TOWARDS IDENTIFYING THE ADRP GENE IN A LARGE SOUTH AFRICAN FAMILY WITH RETINITIS PIGMENTOSA

By

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"Sight is our most precious sense, and any diminution of it is regrettable. Our eyes alone save us from perpetual darkness, and they fill our lives with all the glory of creation. They enable us to scan the vast sweep of the universe a million light years away, or examine the minute details of a flower held in the hand."

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ABSTRACT

Inherited retinal degenerative disorders are a common cause of severe visual dysfunction. Retinitis pigmentosa (RP) comprises of a group of retinopathies characterised by a progressive loss of photoreceptor and retinal pigment epithelium function. Over the past decade numerous RP-causing genes and loci have been identified.

The study of a South African (SA) family with autosomal dominant RP (ADRP) forms the subject of this dissertation. The initial and most ascertainable clinical manifestation of the disorder is night-blindness and is usually evident before 10 years of age. Disease severity correlates with progressive and diffuse retinal pigmentation in affected family members.

Following recruitment and clinical investigation of members of the large family, genetic linkage analysis facilitated the localisation of the disease phenotype to a 23 cM interval on chromosome 17p13 (RP13), the seventh locus identified for ADRP. Recruitment of additional members of the family, with a series of subsequent multipoint linkage analyses and haplotype construction, facilitated the fine mapping of RP13 to a 3 cM region between microsatellite markers *D17S1529* and *D17S831* on chromosome 17p13.3. An additional two RP13-linked families (from the UK and USA) mapped to this locus in subsequent independent studies. Although the families share a common ancestry, the UK family does not share the same disease-associated haplotype (the family from the USA was not available for testing).

Five genes which were considered to be functional candidates and which map to chromosome 17p, were screened for mutations in members of the RP13-linked SA family. The genes investigated were: recoverin (*RCV1*) retinal guanylate cyclase (*RETGC-1*), phosphatidylinositol transfer protein (*PITPN*), pigment epithelium-derived factor (*PEDF*) and beta arrestin 2 (*ARRB2*). These candidates were chosen on the basis of map position on chromosome 17p, expression in the retina and/or involvement in the rod phototransduction cascade. Recombination events between the ADRP locus and a microsatellite polymorphism in *RCV1* provided evidence for the exclusion of this gene. Direct sequence analysis of the coding regions of *RETGC-1* and *ARRB2* in affected family members did not reveal any pathogenic mutations. Furthermore, *RETGC-*

1 was excluded based on reported findings which implicated the gene in the pathogenesis of Leber's congenital amaurosis (LCA), a condition which mapped centromeric to the RP13 interval (Perrault et al., 1996). *ARRB2* was placed centromeric to the RP13 interval based on radiation hybrid mapping studies as shown at the GeneMap98 internet site. Direct DNA sequencing of the coding regions of *PITPN* and *PEDF* including the intron-exon boundaries revealed no potential disease-causing alterations. Genetic mapping strategies did however, place these genes into the RP13 critical interval. These findings were supported by additional data which suggests that *PITPN* and *PEDF* reside on a physical clone contig within the RP13 critical region. The results of several mutation detection techniques, including direct sequencing suggested that *PITPN* and *PEDF* are unlikely to be responsible for the ADRP phenotype in the RP13-linked SA family.

In a further effort to identify the RP13 gene an attempt was made to construct a physical map of the deduced 3cM critical region. To this end a megaclone contig comprising seven yeast artificial chromosomes (YACs) and three P1 artificial chromosomes (PACs) using sequence-tagged-site (STS) content mapping was constructed. Furthermore, a partial physical sequence contig comprising eight sequence files were assembled. The megaclone and electronic sequence contigs provide the valuable tools necessary for the purpose of isolating new genes within the RP13 genetic interval. In this regard, the sequence files were interrogated for transcription units within the interval. Sequence comparisons were performed between these units and the eye subset of the STACK EST database. Using this approach the known retinal genes *PEDF* and *PITPN* were identified, together an additional retinally expressed gene, *PRP8*, and other novel transcripts.

The findings presented in this dissertation will contribute to the increasing 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 RP13 locus on chromosome 17p13. The short term application of the molecular findings is the potential for pre-symptomatic testing of at-risk members from the family. In the long term it is possible that the findings presented will form the foundation for understanding the genetic and biological causation of disease in the large SA and other RP13-linked families; which may lead to the development of a treatment.

ACKNOWLEDGEMENTS

With the authorisation and constant encouragement of my **supervisors,** Prof. Jacquie Greenberg, Prof. Rajkumar Ramesar and Prof. Peter Beighton I undertook to complete this dissertation to furnish reliable information on the RP13 disease locus. It is with my sincere appreciation to them that I submit this dissertation for review.

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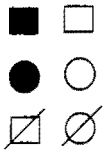
SYMBOLS AND ABBREVIATIONS

°C	degrees Celsius
[α^{32}P]dCTP	radio-isotope of phosphorus
μg	microgram
μM	micromolar
μl	microlitre
%	percentage
θ	theta
A	adenine
AD	autosomal dominant
ADRP	autosomal dominant retinitis pigmentosa
AR	autosomal recessive
ARRB2	beta arrestin 2
ARRP	autosomal recessive retinitis pigmentosa
BACs	bacterial artificial chromosomes
BLAST	basic local alignment search tool
bp	base pair (s)
C	cytosine
Ca^{2+}	calcium ions
[CA]n	CA dinucleotide
cDNA	complementary deoxyribonucleic acid
cen	centromere
CEPH	Centre d'Etudes du Polymorphisme Humain
cGMP	cyclic guanosine 3',5'-monophosphate
cM	centiMorgan
dCTP	α-deoxycytosine-triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
e.g.	for example
ERG	electroretinography/ electroretinogram
EST	expressed sequence tag
et al.	and others
FISH	fluorescent in-situ hybridisation
G or g	guanine

GC	guanylate cyclase
GDP	guanosine diphosphate
GTP	guanosine triphosphate
HCl	hydrochloric acid
HGMP	human genome mapping project
HGP	human genome project
hr/ s	hour (s)
ICI	Imperial Chemical Industries
ICRF	Imperial Cancer Research Fund
IPM	interphotoreceptor matrix
K^+	potassium ions
kb	kilobase (s)
KCl	potassium chloride
kD	kilodaltons
l	litre (s)
LB	Luria-Bertani broth
lod	log of the odds
M	molar
Mb	megabase (s)
MDE	gel matrix
$MgCl_2$	magnesium chloride
min/ m	minute (s)
ml	millilitre
mM	millimolar
mRNA	messenger ribonucleic acid
Na^+	sodium ions
NaCl	sodium chloride
NaOH	sodium hydroxide
NCBI	national center for biotechnology information
ng	nanogram
NRL	neural retina-specific leucine zipper gene
O/N	overnight
PAC	P1-derived artificial chromosome
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PDE	cGMP-phosphodiesterase

PDEA	alpha subunit of PDE
PDEB	beta subunit of PDE
PDEG	gamma subunit of PDE
PEDF	pigment epithelium-derived factor
pH	log H^+ ions
PITPN	phosphatidyl inositol transfer protein
pmol	picomoles
RCV1	recoverin
RD	retinal degeneration
RETGC-1	retinal guanylate cyclase
RFLP	restriction fragment length polymorphism
RH	radiation hybrid
RHO	rhodopsin
RP	retinitis pigmentosa
RPE	retinal pigment epithelium
ROM1	rod outer segment membrane protein 1 gene
SA	South Africa
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
U	units
UCT	University of Cape Town
UK	United Kingdom
URL	universal resource locator
USA	United States of America
UTR	untranslated region
www	world wide web
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 five chapters and the layout is as follows:

- **Chapter One** provides a background to the anatomy of the eye and the biology of vision. It provides a review of hereditary retinal degenerative disorders, focussing on retinitis pigmentosa (RP). A synopsis of the development of RP research in South Africa is provided, and includes an outline of the aims and objectives of the study.

- Chapters Two, Three and Four contains experimental details. Each of these chapters is divided into four sections i.e. Introduction, Materials & Methods, Results and Discussion. **Chapter Two** describes the genetic linkage study undertaken in a large South African family in order to map the gene underlying an autosomal dominant form of RP. **Chapter Three** outlines the investigative approach used to screen the potential and functional candidate genes for RP13. **Chapter Four** deals with the construction of a physical map of the RP13 locus. In addition, this chapter describes the approach used towards the generation of a transcription map for this region.

- While the conclusions drawn for each aspect of this study are discussed at the end of the relevant Chapter, **Chapter Five** provides a final comment on each of these facets and includes comments on future research prospects, based on the results obtained.

CHAPTER 1

INTRODUCTORY BACKGROUND

1.1. REVIEW OF INHERITED RETINAL DEGENERATION

1.1.1. INTRODUCTION

Retinal degeneration (RD) leading to either partial or complete blindness, is the consequence of a very heterogeneous group of conditions. Early family studies revealed that RD could be inherited in dominant, recessive and X-linked modes. Later, molecular analysis of these disorders showed further genetic heterogeneity existed within each group. A subset of the hereditary forms of retinal degeneration, namely, retinitis pigmentosa (RP), in the South African context forms the subject of this thesis.

RP is caused by mutations in several genes, however, because the disorder in several families remains unmapped, it is likely that many additional genes which cause RP, exist. The identification of the entire set of RP-causing genes requires considerable effort. The exercise will nevertheless be valuable as the discoveries may represent a valuable step towards understanding the pathogenic mechanisms underlying RP. Molecular genetic studies will also contribute increasingly to the clinical diagnosis, management and treatment of patients with RP, the ultimate goal being the development of therapies that will preserve sight.

In this introductory chapter the anatomical structures underlying vision, the process of visual transduction and the genetics of RP is reviewed in order to provide a perspective for the investigations trends in this thesis.

1.1.2. THE STRUCTURE OF THE EYE

A description of the general structure of the eye essentially as outlined in Ross and Romrell (1989) is provided as a backdrop to retinal disorders. The eye is made up of three separate concentric layers (figure1.1.):

- an outer layer, the **corneoscleral coat**, which consists of the sclera and cornea;
- a middle layer, the **uvea,** consisting of the choroid, ciliary body, and iris;
- the innermost layer, **the retina**, which consists of an outer pigment epithelium (PE) and an inner neural retina.

The eye is a specialised organ of photoreception, a process that involves the conversion of light energy into nerve action potentials. The photoreceptors consist of two types of nerve cells, the rods and cones. These rod and cone receptors and a system of integrating neurons are located in the inner layer of the eye, the retina. The remaining structures of the eye serve to support the retina. In addition, there are several accessory structures, namely the eyelids, lacrimal gland and conjunctiva, which protect the eye from external damage.

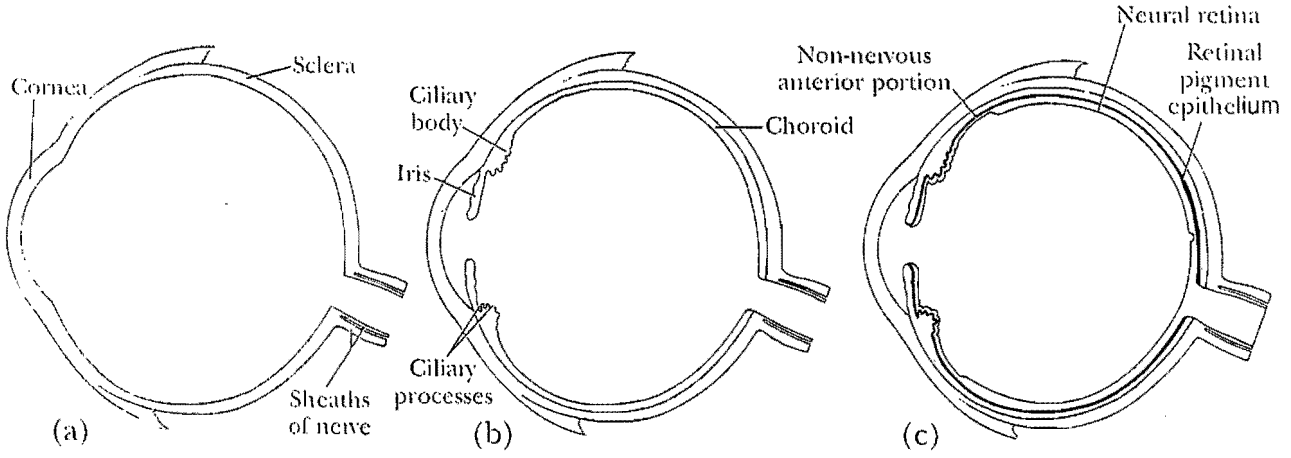


Figure 1.1 Schematic diagram of the layers of the eye.
(a) the corneoscleral coat (b) the uvea (c) the retina

1.1.2.1. The Corneoscleral Coat

The corneoscleral coat forms a tough fibro-elastic support capsule around the eye. The posterior section, the sclera, is opaque and provides an anchor for the extra-ocular muscles. The anterior section, the cornea, is transparent and is the principle refracting medium of the eye. It roughly focuses an image onto the retina.

1.1.2.2. The Uvea

The uveal tract is a vascular layer made up of the choroid, ciliary body and the iris. The choroid lies between the sclera and the retina in the posterior section of the eye. It provides nutritive support for the retina and is heavily pigmented in order to absorb light which enters the eye. Anteriorly, the choroid merges with the ciliary body, which is muscular and controls the shape of the lens. The iris, which extends in front of the lens from the ciliary body, is highly pigmented and adjustable in order to regulate the amount of light reaching the retina.

1.1.2.3. The Retina

A more detailed review of the structure of the retina is provided since this is the primary tissue which is diseased in persons with RP. Nerve fibres from the retina converge to form the optic nerve, which leaves the eye through a part of the sclera known as the lamina cribrosa. The function of the retina is to send appropriate signals to the brain, via the optic nerve, in response to light entering the eye.

Histologically, the retina is divided into several distinct layers and the

distribution of the various cell types of the retina is illustrated in figure 1.2. There are two layers within the retina: (i) the neural retina, and (ii) the retinal pigment epithelium (RPE).

The neural retina consists of two regions that differ in function, based on their response to light (i) a photosensitive and (ii) a non-photosensitive region.

(i)The photosensitive region lines the whole inner surface of the eye, (except where it is pierced by the optic nerve) and is composed of photoreceptor cells. The rod and cone photoreceptors are specialised nerve cells that convert photons into nerve signals (figure 1.3.). Cone cells provide colour vision and are most concentrated at the fovea which is located at the centre of the macula. Rod cells are more sensitive to light than cones, but provide essentially black and white vision.

Rod cells are long slender bipolar cells, which extend beyond the outer limiting membrane (figure 1.3.). Rod cells consist of three parts:

- The outer segment which is closely associated with projections from the adjacent RPE and contains an array of horizontal membranous discs. The discs incorporate the pigment rhodopsin, which is involved in the first step of the visual pathway. These discs are continuously phagocytosed by the cells of the PE at one end of each rod and replaced from the inner segment.

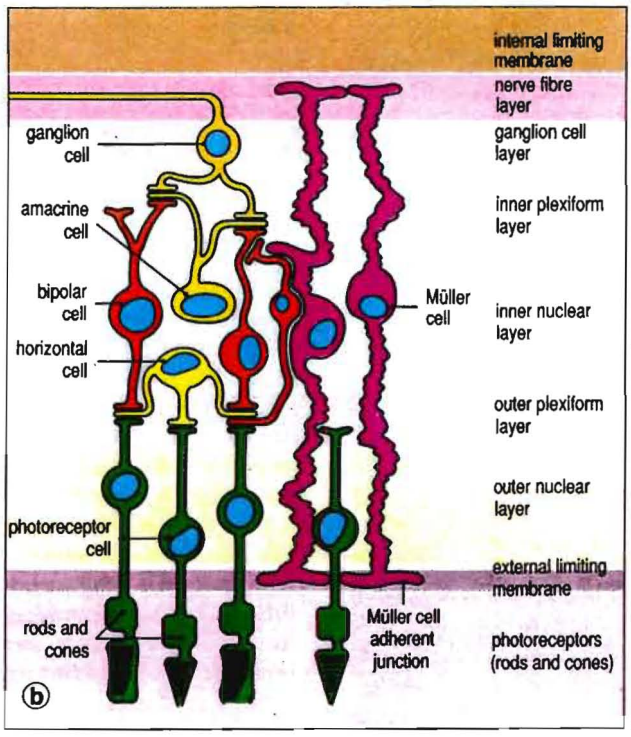


Figure 1.2. Distribution of the retinal cell types.

- The stalk which is a strand of cytoplasm containing nine microtubule doublets and joins the inner and outer segments.
- The inner segment which is associated with a cell actively synthesising proteins. This section contains a prominent golgi apparatus and many mitochondria.

Cone cells are similar in basic structure to the rods but differ in several respects:

- The outer segments of cone cells are long conical structures about two-thirds the length of a rod. The membranous discs are flatter than those in rod cells and are continuous with the plasma membrane so that on one side, the spaces between the discs are continuous with the extracellular matrix (figure 1.3.). The discs are not replaced on a regular basis.
- The inner segments are continuous with the bodies of cone cells.

The outer segments house the photosensitive pigment cells, while inner segments contain components for metabolic processes, which provide energy to facilitate photoreceptor activities.

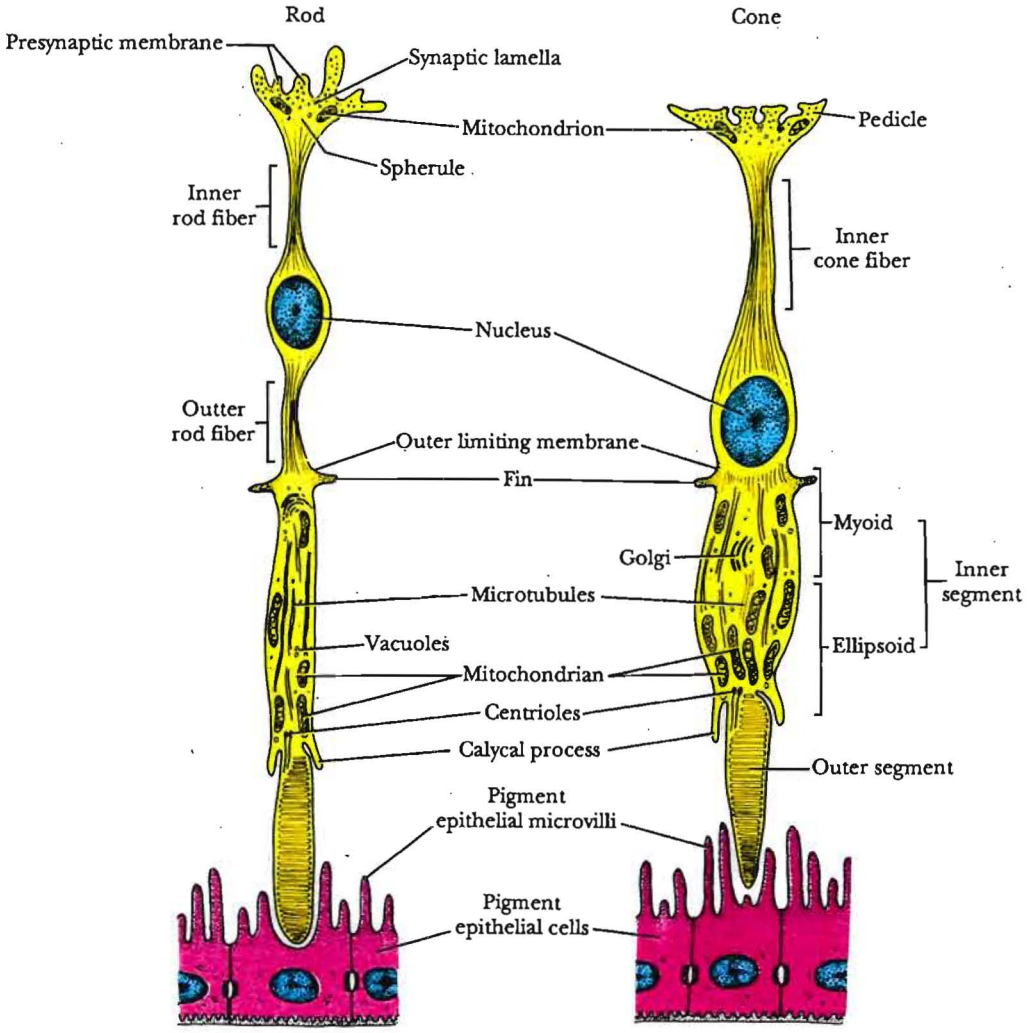


Figure 1.3. Schematic diagram of the ultrastructure of rod and cone cells.

(ii) The non-photosensitive region lines the inner aspect of the ciliary body and the posterior surface of the retina and is composed of the following cell types:

Direct conducting neurones: bipolar cells are the most numerous of the direct conducting neurones and in general they make direct connections between one or more photoreceptors and one or more optic tract neurones. The cell bodies of the optic tract neurones or ganglion cells comprise the ganglion cell layer (figure 1.2.).

Association and other neurons: horizontal cells are neurons involved in processes making connections between adjacent and more distal rods and cones. The amacrine cells are neurons, which form connections with bipolar and optic tract neurones as well as making occasional feedback connections with the photoreceptors.

Supporting cells: An elongated support cell extends between inner and outer limiting membranes (figure 1.2.). These are called Müller cells which have long cytoplasmic processes that embrace and sometimes encircle the retinal neurones, filling all the intervening spaces. The Muller cells provide structural support and may mediate the transfer of metabolites to the retinal neurones.

The region around the optic nerve (figure 1.1.) is devoid of photoreceptors, making it a blind spot in the visual field. In contrast, the macula, located about 2.5mm lateral to the optic nerve, is the area of

greatest visual discrimination as the visual elements are concentrated in this area.

The retinal pigment epithelium (PE) is a single layer of hexagon-shaped cells resting on Bruch's membrane, which is part of the choroid (figure 1.1.). Adjacent cells are connected by a junctional complex and are the site of the "blood-retinal barrier." Complex cytoplasmic processes, typical of cells involved in active transport, project for a short distance between the photoreceptors of the rods and cones. The elongated melanin granules are the most prominent features of the cells. The pigment cells are tallest in the fovea and adjacent area, accounting for the darker colour of this region. The RPE has many important functions, namely:

- absorption of light entering the eye, thereby decreasing light scatter;
- it is a component of the blood-retina boundary, isolating the retinal cells from blood borne substances;
- it is implicated in the photopigment regeneration process, after their response to light.

The RPE also plays a role in the phagocytosis and disposal of the membranous discs of the photoreceptor cells (maintenance).

1.1.3. THE PHOTOTRANSDUCTION PATHWAY

The photoreceptor outer segment is the major location for light transduction and it can be the site of extensive pathology. On this basis it is possible that any protein involved either in the structure or the visual process in the outer segment, could be a candidate for inherited retinopathies. Several research groups have investigated this possibility by looking at the components of the visual transduction pathway.

The first step for perception of light is the enzymatic amplification of the light signal and occurs in the rod outer segment. In this segment, opsin combines with the chromophore 11-cis-retinal to form rhodopsin (RHO), a 40-kDa transmembrane protein (Stryer, 1988). Photoisomerisation or light activation of the 11-cis-retinal chromophore is both efficient and rapid, and is the first step in the visual transduction cascade (Yau, 1994). The subsequent transmission of the signal is illustrated in figure 1.4. The photoisomerisation of RHO produces an intermediate compound called metarhodopsin II (R^*), which is conformationally activated.

The second step in visual excitation is the activation of transducin by R^*. Transducin (T) is a peripherally expressed membrane protein with three subunits: Tα (39kDa), Tβ (36kD), and Tϑ (8kDa). The Tα subunit contains a binding site for guanosine triphosphate (GTP) and guanosine diphosphate (GDP) as well as a catalytic site for the hydrolysis of bound GTP. The Tβ and Tϑ subunits form a Tβϑ subunit. Tα and Tβϑ are

associated when GDP is bound (T-GDP, the inactive dark state), and separate when GTP is bound (T-GTP, the light-activated state). The GTPase activity of transducin is essential for bringing the system back to the dark state. Following illumination, T-GDP encounters R^* in the plane of the disc membrane. R^* induces the release of GDP from transducin and allows GTP to enter.

R^*-T-GTP then dissociates into Tα-GTP, Tβδ and R^*. Tα-GTP carries the excitation signal to cGMP phosphodiesterase (PDE) and R^* is free to catalyse another round of GTP-GDP exchange. Hundreds of transducin molecules are activated by a single R^* and this is the primary stage of amplification in vision. The transducin cycle (figure 1.5.) flows unidirectionally where both the formation of Tα-GTP and its hydrolysis are essentially irreversible.

Tα-GTP activates PDE by carrying away its inhibitory subunits. PDE consists of three polypeptide chains: α (88kDa), β (85kD), and δ (9kDa), and is a peripherally expressed membrane protein with a subunit structure of αβδ2. Tα-GTP first interacts with the αβδ2 holoenzyme and removes one of its δ subunits to form a partially active αβδ complex. The remaining δ subunit is removed by another Tα-GTP to form a fully active αβ.

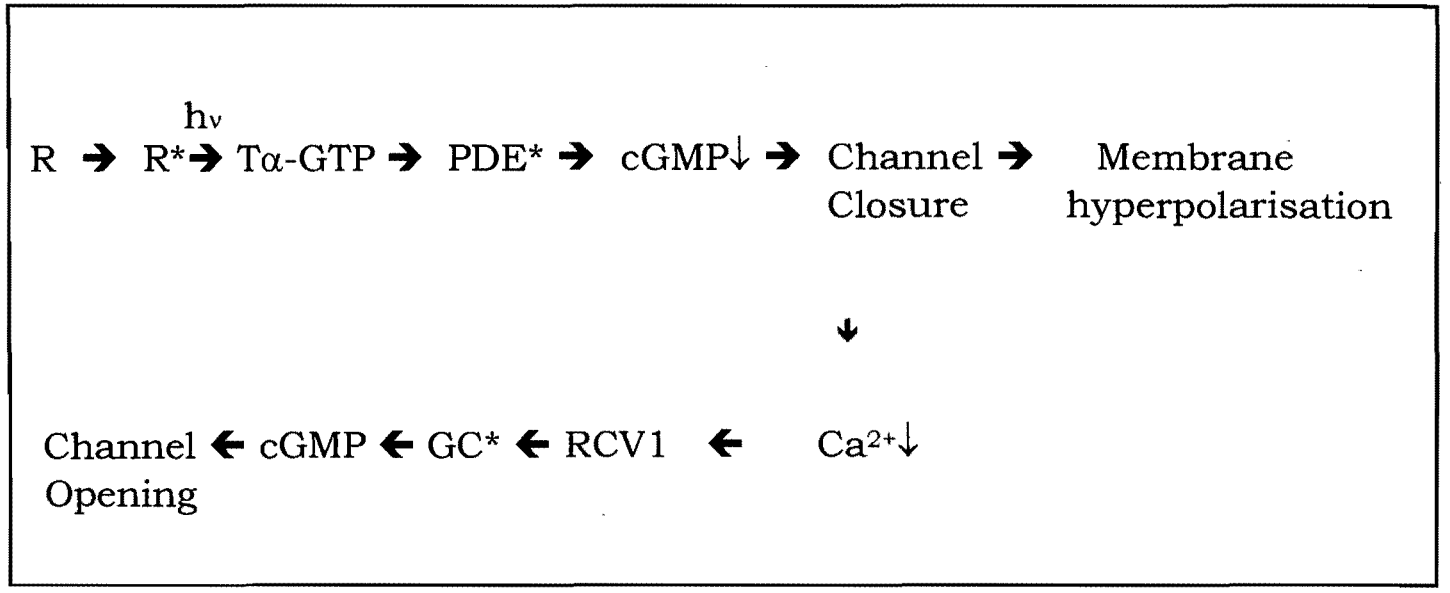


Figure 1.4. The visual excitation and recovery pathway (Stryer, 1991).
Photoactivation of rhodopsin (R) triggers a cascade leading to closure of membrane channels, which generates a nerve signal. Channel closure also causes a drop in the cytosolic calcium level, which leads to the reopening of channels. T-transducin, GTP-guanosine triphosphate, PDE-cGMP-phosphodiesterase, cGMP-cyclic guanosine 3', 5'-monophosphate, Ca^{2+}-calcium ions, RCV1-recoverin.

Fully activated PDE hydrolyses cGMP (bound to the rod photoreceptor cGMP-gated channel protein) which leads directly to channel closure. Channel closure is a result of the lowering of cytoplasmic cGMP concentration. Closure of the channel causes hyperpolarisation of the rod cell plasma membrane, which initiates the visual transduction signal.

The channel opens and closes within milliseconds in response to changes in the cGMP level. The plasma membrane of the rod outer segment is in essence a cGMP electrode as it is gated solely by cGMP. Most of the light-induced hyperpolarisation, the excitation signal, comes from blockage of Na^+ influx.

Channel reopening depends on the restoration of the cGMP level, which requires activation of guanylate cyclase (GC) and inhibition of PDE. Deactivation of PDE* requires hydrolysis of Tα-GTP to Tα-GDP (figure 1.4.). For channel opening, photoexcited rhodopsin (R*) must also be deactivated. This shut-off is achieved by the phosphorylation of multiple serines and threonines in the carboxyl terminal region of R* by rhodopsin kinase, a cytosolic protein. Another cytosolic protein, arrestin, then caps multiple phosphorylated R* to prevent it from interacting with transducin. Rhodopsin is regenerated minutes later by the insertion of 11 cis-retinal, the release of arrestin, and the removal of

the COOH-terminal phosphates by protein phosphatase 2A (Fowles et al., 1989). The restoration of the dark state also requires the activation of GC. It is thought that recoverin is also involved in the recovery from light to dark state.

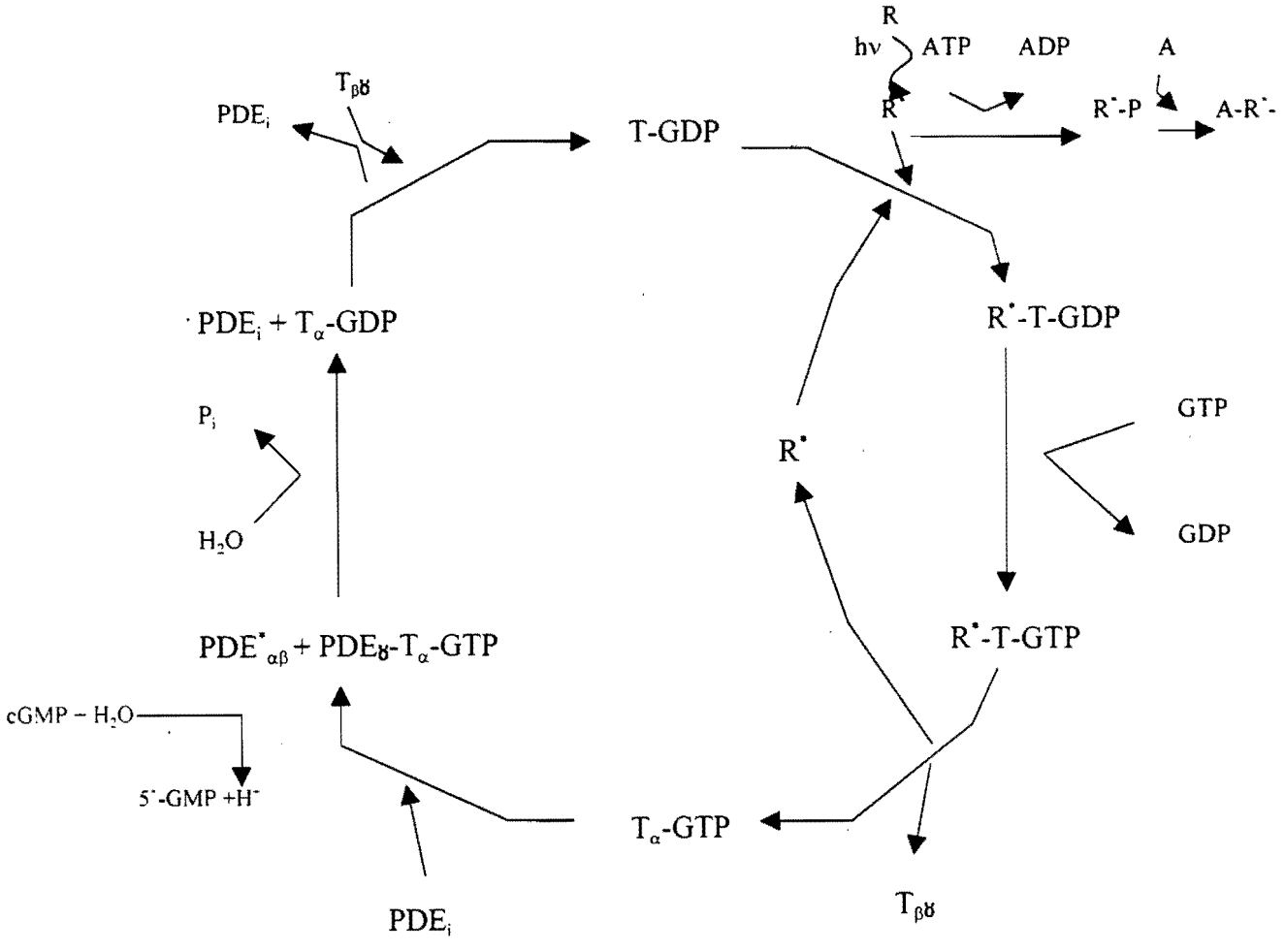


Figure 1.5. Light-activated transducin cycle.
R - Rhodopsin; R* - photoexcited rhodopsin; T – transducin; PDE_i – inhibited phosphodiesterase; PDE* - activated phosphodiesterase; A – arrestin.

In the dark, the entry of Ca^{2+} through the cGMP-gated channel is matched by its efflux through an exchanger that is driven by the influx of three Na^{+} and the efflux of one K^{+}. Following illumination, the influx of Ca^{2+} through the cGMP-gated channel ceases, but its export by the exchanger continues until the Ca^{2+} intracellular concentration drops markedly. A lowering of Ca_i stimulates recoverin, which in turn activates GC (Shastry, 1997), resulting in a rise in cGMP production which reopens the cGMP-gated channel. This process together with the phosphorylation of photoactivated rhodopsin by rhodopsin kinase converts the rod cells to the ground state.

This pathway takes place in both rods and cones. Many of the proteins involved, however, exist in either rod-specific or cone-specific versions.

1.1.4. MOLECULAR GENETICS OF HUMAN INHERITED RETINAL DEGENERATION

Inherited disorders that cause the retina to degenerate lead to either partial or total blindness. Much progress has been made in the identification of the genetic defects underlying common inherited retinal conditions such as RP, macular degeneration and other rare forms of retinal disease, which manifest primarily as degeneration of the photoreceptor cells.

Photoreceptors may degenerate due to inherent structural or

biochemical defects in the cell. If the defect affects rods, visual field loss progresses from the periphery towards the centre of the retina. Conversely, defects that affect cones have the opposite effect, destroying the central region of the retina (the macula) where cones are most abundant (Zhang et al., 1995).

It appears that many factors can initiate RD, and it is possible that several different processes are involved. The process leading from genetic defect to RD is not well understood. One current theory is that regardless of the fundamental genetic defect, apoptosis, a form of programmed cell death, is ultimately responsible for RD (Chong et al., 1999).

RP, the group of disorders which forms the subject of this thesis will be discussed in this section.

1.1.4.1. Retinitis Pigmentosa (RP)

One group of the degenerative retinopathies, RP, has attracted special attention in this decade. The nomenclature encompasses many conditions, involving progressive photoreceptor and retinal pigment epithelial degeneration in the retina (Bunker et al., 1984). This group of disorders which occur in all population groups is the most common cause of genetic blindness (Inglehearn, 1998) and affects approximately 1 in 4000 people (Bunker et al., 1984). RP is untreatable.

The diagnostic criteria for RP are bilateral involvement, loss of peripheral vision and abnormal rod responses in the electroretinogram (ERG). These criteria reflect rod dysfunction and progressive loss of both rod and cone photoreceptor function. The corresponding clinical symptoms are:

- night blindness (nyctalopia);
- progressive concentric reduction of the visual fields;
- usually later, signs of macular involvement like loss of reading acuity, abnormal colour vision and sensitivity to glare, and
- in the final stages of the disease, most patients experience blindness.

Clinical variability is a feature in RP, even within the same family (Berson, 1996). In some instances the disorder manifests early, within the first decade of life, followed by a rapid progression to blindness. Conversely, symptoms may not manifest until the fifth decade or beyond, with a more gradual progression of the disorder. Other ocular findings include atrophic changes of the RPE followed by the appearance of melanin-containing structures in the vascular layer and around the Müller cells resulting in the typical bone-spicule-like pigmentation, waxy pallor of the optic discs and attenuated retinal vessels. Myopia is frequent, with subcapsular posterior cataract and destruction of the vitreous usually occurs in patients over 40 years (Gal et al., 1997).

The genetics of inherited forms of RP is complex, with families showing

either X-linked (10-15%) (XLRP), autosomal dominant (15-20%) (ADRP), autosomal recessive (20-25%) (ARRP), maternal (mitochondrial), or digenic inheritance patterns. Besides the genetically classifiable cases, however, many families which have been reported cannot be ascribed to any genetic form (Phelan and Bok, 2000). Such cases are labelled "simplex" and this cohort may account for at least 40% of cases (Jay and Jay, 1982). Individuals in the simplex group have no family history of the condition. The condition may also appear as part of a syndrome where RP presents with additional features (Chong et al., 1999).

The molecular defects underlying several forms of genetic blindness have already been identified. The majority of the genetic categories of RP mentioned above are caused by defects in various genes. It has been shown that mutations reside not only in genes whose transcription products are directly involved in the visual pathway, but may also occur in other retinally-expressed genes whose products have an indirect effect on the pathway.

Autosomal dominant retinitis pigmentosa (ADRP): The published incidence of the different genetic forms of RP in the USA and UK suggests a higher prevalence of the dominant form of the disorder compared to the others (Krumpaszky and Klauss, 1996). The evidently higher prevalence of ADRP has resulted in a greater effort being invested in its investigation internationally.

Clinically, ADRP is classified into two main groups based on differences in the relative loss of rod and cone function. Type 1 ADRP refers to an early and diffuse loss of rod function followed by a progressive loss of cone function (Massof and Finkelstein, 1981). In type 2 ADRP, the condition progresses in a more regional fashion, with a concurrent loss of both rod and cone function. In comparison with type 1, patients with type 2 ADRP usually have a later onset of night blindness and the visual fields are better preserved; individuals with the latter type, therefore, usually have a more favourable visual prognosis. ADRP types 1 and 2, described by Massof and Finkelstein (1981) correspond well to the D-type and the R-type, respectively, described by Lyness et al. (1985).

Considerable progress has been made in the understanding of the molecular pathology of ADRP resulting in 12 chromosomal loci being mapped to date (Clarke et al., 2000). These ADRP loci reside on chromosomes 3q (McWilliam et al., 1989), 6p (Farrar et al., 1991), 8q (Blanton et al., 1991), 7p (Ingelhearn et al., 1993), 7q (Jordan et al., 1993), 19q13.4 (Al-Maghtheh et al., 1994), 19q13.3 (Sohocki et al., 1998), 17p13.3 (Greenberg et al., 1994b), 17p13.1 (Perrault et al., 1998), 17q (Bardien et al., 1995), 1q (Xu et al., 1996) and 14q (Bessant et al., 1999).

Disease-causing mutations have been identified in genes at six of these ADRP loci:

- Rhodopsin (*RHO*), on chromosome 3q, encodes the visual pigment that initiates the phototransduction cascade. This was the first gene to be implicated in ADRP (Dryja et al., 1990). Since the first report of a mutation in the *RHO* gene, a variety of other mutations have been described in this gene in families manifesting ADRP (Al-Maghtheh et al., 1993). Many of the gene alterations are missense mutations and occur only in individual families (Inglehearn, 1998). *RHO* gene mutations account for approximately 20% of ADRP. Interestingly, some mutations in the *RHO* gene are believed to cause ARRP, simplex and congenital stationary night blindness (Phelan and Bok, 2000).
- *Peripherin/ RDS*, on chromosome 6p, the second gene implicated in ADRP (Dryja and Li, 1995), encodes a membrane protein located in the rim of outer segment discs (Molday et al., 1987). The postulated function of the protein is the creation of a conformational bend at the periphery of disc membranes, or to anchor the discs to the cytoskeleton of photoreceptor cells (Arikawa et al., 1992). In addition, the protein is essential for the biogenesis of photoreceptor outer segments. *Peripherin/ RDS* mutations segregate in families with the AD and the AR form of RP (Inglehearn, 1998). While the majority of *peripherin/ RDS* mutations are believed to cause RP, some are associated with macular dystrophy (Wells et al., 1993), pattern

macular dystrophy (Weleber et al., 1993), vitelliform macular dystrophy (Wells et al., 1993), butterfly macular dystrophy (Nichols et al., 1993), or fundus flavimaculatus (Weleber et al., 1993). Since mutations in the same gene can produce such different phenotypes, it is thought that other gene products or environmental factors could be associated with these disorders (Phelan and Bok, 2000).

- *RETGC-1*, on chromosome 17p, was implicated in the phenotype of AD cone-rod dystrophy patients (Perrault et al., 1998). The cone-rod dystrophy group of disorders is a common variant of RP which shows the simultaneous involvement of cone and rod photoreceptor degeneration (Phelan and Bok, 2000). RETGC-1 is one of two isoforms which dimerise to form the functional RETGC. The active enzyme is required during the phototransduction cascade to return the levels of cGMP to normal.
- The cone-rod homeobox gene (*CRX*), on chromosome 19q, encodes a photoreceptor-specific transcription factor. The gene was shown to underlie a late-onset dominant form of RP (Sohocki et al., 1998). The CRX protein regulates the expression of *RHO* and other outer segment proteins genes including rod transducin and arrestin (Clarke et al., 2000).
- The neural retina-specific leucine zipper gene (*NRL*), on chromosome 14q (Yang-Feng and Swaroop, 1992), shown to underlie ADRP (Bessant et al., 1999), encodes a basic motif-leucine zipper protein implicated in regulating the *RHO* gene. Expression studies indicate

that the mutant NRL protein up-regulates the *RHO* gene promoter.

- The *RP1* gene, on chromosome 8q, was recently shown to underlie the ADRP phenotype (Sullivan et al., 1999). Several disease-causing alterations in *RP1* have been elucidated in families previously mapped to this locus. *RP1* gene-expression is retina-specific and the product is believed to be a protein kinase involved in the development and/or maintenance of the neural retina (Sullivan et al., 1999).

For the remaining six loci, the disease-causing genes have not yet been identified. Based on the observation that the disorder in several ADRP families has been excluded from the above loci (by linkage analysis) suggests that further ADRP loci remain unmapped.

Autosomal recessive retinitis pigmentosa (ARRP): The clinical manifestations of RP are most severe in persons with the autosomal recessive form of the condition (Jay and Jay, 1982). ARRP accounts for approximately 20% of all cases of RP. However, simplex forms of RP, which are presumed to be recessive in most cases, account for a further 50% (Jay, 1982). ARRP is characterised by allelic and non-allelic heterogeneity, and several loci have been identified for this form of the disorder, on chromosomes 1p31 (Gu et al., 1997), 1p21 (Cremers et al., 1998), 1q31 (van Soest et al., 1996), 2p11 (Gu et al., 1999), 2q31 (Bayes et al., 1998), 2q37 (Nakazawa et al., 1998), 3q21 (Rosenfeld et al., 1992), 4p16 (Valverde et al., 1996), 4p14 (Dryja et al., 1995), 5q31

(Huang et al., 1995), 6p21 (Knowles et al., 1994), 6q15 (Ruiz et al., 1998), 15q26 (Maw et al., 1997), and 16p12 (Finckh et al., 1998).

In some instances, specific genes and causative mutations have been identified in forms of ARRP:

- *RHO*, on chromosome 3q (Rosenfeld et al., 1992);
- α and β *PDE*, on chromosomes 5q and 4p, respectively (Huang et al., 1995, McLaughlin et al., 1995, Danciger et al., 1995 and Valverde et al., 1996);
- the α subunit of cGMP-gated channel protein (*CNGC*), on chromosome 4p14 (Dryja et al., 1995);
- *RPE65*, on chromosome 1p31 (Gu et al., 1997);
- *RLBP1*, on chromosome 15q (Maw et al., 1997);
- *ABCR*, on chromosome 1p21 (Martinez-Mir et al., 1998);
- *TULP1*, on chromosome 6p (Banerjee et al., 1998 and Hagstrom et al., 1998);
- *SAG*, on chromosome 2q37 (Nakazawa et al., 1998); and
- *CRB1*, on chromosome 1q31 (den Hollander et al., 1999).

Interestingly, both *RPE65* and *RLBP1* are expressed primarily in the RPE, and not the neural retina (Inglehearn, 1998). Each encodes proteins involved with the transport of retinoids from the RPE to the neural retina. It is believed that RD caused by mutations in these genes is a result of the inefficient transfer of fat-soluble retinoids to the neural retina.

X-linked retinitis pigmentosa (XLRP): Persons exhibiting the XLRP have earlier manifestations compared to individuals with ARRP or ADRP. Males are consistently severely affected and never pass the disorder on to their male off-spring. Molecular investigations have revealed genetic heterogeneity in this form of the condition, where at least six disease loci have been identified (Clarke et al., 2000):
RP2 (Xp11.3) between markers *DXS7* and *DXS255* (Teague et al., 1994), RP3 (Xp21.1) which is closely linked and distal to *DXS7* (Chen et al., 1989), RP6 (Xp21.3) (Ott et al., 1990, Aldred et al., 1994), RP15, RP23 (both at Xp22) and RP24 (Xq26) (Clarke et al., 2000).

Two genes have been cloned, characterised and shown to underlie the XLRP disease phenotype:

- mutations in the RP guanosine triphosphate (GTP)ase regulator gene (*RPGR*) (at the RP3 locus) were found in approximately 20% of XLRP patients (Buraczynska et al., 1997), and
- mutations in the RP2 gene (*RP2*) are thought to account for 20-30% of XLRP cases in European populations (Clarke et al., 2000).

A range of other X-linked retinal phenotypes all map to loci overlapping the XLRP interval. These include cone dystrophy (Meire et al., 1994), Norrie's disease (Berger et al., 1992), congential stationary night blindness (CSNB) (Hardcastle et al., 1997) and Aland Island eye disease (Glass et al., 1993). These may or may not be allelic.

Digenic retinitis pigmentosa: The phenotype segregating in most RP families is the result of defects in single genes (monogenic). In 1994, however, three families with RP were identified, each with the same missense mutation in *peripherin/RDS* (on 6p) (Kajiwara et al., 1994). Each of the three families had a second mutation in *ROM1* (a gene on 11q), thereby demonstrating the phenomenon of digenic inheritance. Parents of affected individuals were carriers for either the *ROM1* or the *peripherin/RDS* mutations but did not manifest the condition.

Peripherin/RDS and *ROM1* encode the polypeptide subunits of an oligomeric transmembrane protein complex present at photoreceptor outer segment disc rims (Phelan and Bok, 2000). Goldberg and Molday (1996) reported that, when defective, the protein subunits of the complex do not assemble correctly.

Syndromic retinitis pigmentosa: RP is a component of a number of genetic diseases. Usher syndrome (USH) is comparatively common, comprising RP and genetic deafness. USH is an AR heterogeneous condition (Clarke et al., 2000). Three phenotypic variants of USH are defined according to the severity of hearing loss and the degree of vestibular involvement (Smith et al., 1994). Type 1 USH (USH1) is characterised with profound hearing loss with vestibular dysfunction and progressive photoreceptor degeneration. USH1 accounts for 60%-

90% of all USH cases (Kaplan et al., 1992). USH2 is the most common form of the disease. Patients with USH2 present with RP, moderate hearing loss and normal vestibular involvement. The condition is not congenital. In USH3, the rarest form, the RP and hearing loss is progressive and the condition manifests in adulthood (Smith et al., 1994).

The clinical heterogeneity of USH is reflected by the assignment of this group of disorders to at least nine loci on chromosomes 1q (Kimberling et al., 1990), 3p (Hmani et al., 1999), 3q (Sankila et al., 1995), 5q (Pieke Dahl et al., 2000), 14q (Kaplan et al., 1992), 11q (Kimberling et al., 1992), 11p (Smith et al., 1992), 10q (Wayne et al., 1996) and 21q (Chaib et al., 1997).

Two USH genes have been characterised (Kimberling et al., 2000):

- The myosin VIIA gene (*MYO7A*) has been shown to be causative of the USH1 form of the disorder at the 11q locus (Weil et al., 1995). Reported findings suggest that myosin is required for the transport of opsin to the outer segment of photoreceptor cells (Liu et al., 1998), and
- *USH2A*, on chromosome 1q41 (Liu et al., 1999). The USH2A protein sequence suggests that it may represent a novel class of interphotoreceptor cell matrix (IPM) proteins or a cell adhesion molecule (Liu et al., 1999).

Bardet-Biedl syndrome is a recessive condition which involves RP, polydactyly, obesity, hypogenitalism, mental retardation and renal anomalies. Four loci have been associated with the condition through linkage analysis, on chromosomes 11q (Leppert et al., 1994), 16q (Kwitek-Black et al., 1993), 3p (Sheffield et al., 1994)and 15q (Carmi et al., 1995).

Refsum disease is characterised by RP, peripheral neuropathy, cerebellar ataxia and elevated cerebrospinal fluid protein levels (Jansen et al., 1997). Defects in the phytanoyl-CoA hydrolase gene on chromosome 10p has been shown to underlie this condition (Jansen et al., 1997).

Congenital stationary night blindness (CSNB): Individuals with CSNB have normal day vision, mediated by the cone cells of the retina. They experience night blindness due to the malfunctioning of the rod photoreceptors in dim light. The disorder is non-progressive and genetically heterogeneous (Hardcastle et al., 1997). It is believed that the genetic colocalisation of CSNB and RP for at least four loci suggests that CSNB is a mild, non-progressive form of RP.

Distinct genetic loci have been reported for the X-linked form of CSNB at Xp21.1, one which is allelic to the RP3 gene RPGR (Apfelstedt-Sylla et al., 1996), and another which is possibly allelic with the RP2 gene

(Aldred et al., 1992). Furthermore, there is yet another locus for X-linked CSNB (CSNB4) between the RP2 and RP3 loci on proximal Xp (Hardcastle et al., 1997). A recessively inherited form of CSNB, Oguchi disease, results from mutations in *SAG* (Fuchs et al., 1995) and rhodopsin kinase (Yamamoto et al., 1997). Both these genes encode proteins which function to restore the resting phase in rods after response to light. CSNB is also transmitted in an autosomal dominant fashion. Dominant CSNB is associated with mutations in the α subunit of rod transducin, *GNAT1* (Dryja et al., 1996), *PDE*β (Gal et al., 1994) and *RHO* (Gal et al., 1997).

Macular Degeneration: The phenotype of macular degeneration encompasses a heterogeneous group of disorders characterised by a progressive loss of central vision due to the degeneration of the macula and the underlying pigment epithelium (Zhang et al., 1995). Much progress has been made in the elucidation of the macular dystrophies, with the genes responsible for dominant and recessive forms of the condition being mapped to specific chromosomes. Some of these are: 11q13 - Best macular dystrophy, an autosomal dominantly inherited disorder where patients have bilateral macular lesions at a young age (Stone et al., 1992). Funduscopically, features include a yellow appearance of the macula. 1p21-p13 – Stargardt macular dystrophy, characterised by bilateral atrophic changes in the macula, degeneration of the RPE, and the presence of yellow-white flecks in the posterior pole.

A recessive form of Stargardt disease maps to chromosome 1p (Kaplan et al., 1993). 6q14-q16.2 – Autosomal dominant Stargardt dystrophy (Stone et al., 1994). 13q34 - Autosomal dominant Stargardt dystrophy (Zhang et al., 1994).

Specific genes and genetic alterations have been found to underlie some forms of macular degeneration:

- *Peripherin/RDS*, on chromosome 6p, has been implicated in some macular dystrophies (Shastry, 1994).
- retina-specific ATP-binding cassette transporter (*ABCR*) gene, on chromosome 1q, has been shown to be causative of dominant age-related macular dystrophy (AMD) and recessive Stargardt dystrophy (Allikmets et al., 1997a; b). AMD is the leading cause of blindness in the elderly. The condition is characterised by progressive degeneration of the retina, the RPE and the choriocapillaris (the blood supply to the outer retina).
- A 7-kb heteroplasmic deletion of the mitochondrial genome was found in a patient with diabetes, deafness, cataract, and maculopathy (Souied et al., 1998). This was the first report of a mitochondrial mutation associated with macular degeneration.

1.2. RETINITIS PIGMENTOSA IN SOUTH AFRICA

SA RP families have been documented in the Department of Human Genetics at the University of Cape Town for the past 27 years. A nation-wide survey of individuals with RP was conducted in 1985 (Oswald et al., 1985). The figures reported at that stage were recognised as being incomplete, however, the aim of the study was to provide a perspective on the genetics of RP in SA. Sixty three families comprising one hundred and thirty affected individuals participated in the survey. The results showed that the proportions of the different genetic categories of RP were generally in accordance with those found in international studies.

The situation in SA was reviewed again in 1993 (Greenberg et al., 1993). This review took place three years after the establishment of a DNA banking centre in the molecular laboratory of the Department of Human Genetics, in 1990. By 1994, blood specimens for DNA extraction and archiving had been received from 153 affected individuals, from 75 families. At the time of the review, none of the families had been mapped to any of the candidate loci screened. In the first report by Oswald et al. (1985) and in the later report by Greenberg et al. (1994a), the apparent small proportion of XLRP families in SA was noted. Oswald also proposed a possible "reverse" founder effect. And in 1994 it was postulated that each of the SA RP families is likely to have a different mutation at the molecular level.

The localisation of the first RP gene locus to the long arm of chromosome 3 in 1990, prompted molecular genetic studies aimed at identifying disease-associated loci in SA RP families (Greenberg et al., 1992).

1.2.1. CURRENT STATISTICS

DNA samples from 1501 affected and unaffected individuals from 383 families with RP had been archived in the molecular laboratory at the department of Human Genetics at UCT (as at June 1999). The genetic categorisation of 383 families is presented in table 1.1. The proportions of the different genetic forms of RP archived continue to be in accordance with those published internationally. Families have been categorised where pedigree data indicated typical Mendelian patterns of inheritance. The term simplex is used to define a single affected individual with no family history or consanguinity evident in the pedigree. Families with more than one affected individual, but with insufficient information to indicate any specific Mendelian pattern of inheritance were termed indeterminate.

Table 1.1. Genetic categorisation of 383 RP families

Inheritance Pattern	No. of Families	No. of Affected individuals	Total no. of individuals
Autosomal dominant	68	211	591
Autosomal recessive	45	69	173
X-Linked recessive	17	26	69
Simplex	31	33	128
Indeterminate	61	77	91
Macular dystrophy	40	41	71
Stargardts disease	68	96	230
Lebers congenital amaurosis	3	3	3
Other	11	13	25
Syndromic:	39	51	120
Totals	383	620	1501

1.3. AIMS AND OBJECTIVES

The present study was initiated with the aim of elucidating the molecular genetic basis of the RP phenotype segregating in a large SA family of British origin. The 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 two.

The identification of the gene underlying the phenotype in the family is significant, as it will facilitate pre-symptomatic testing and informed genetic counselling for at-risk members from this large family. The same advantage will be applicable to any other ADRP families linked to this gene.

Findings from a study such as this could lead to an improved understanding of the pathogenic pathways which underlie the ADRP phenotype. This knowledge will in turn improve our understanding of the visual process. In addition, information gleaned from the function of the disease-causing gene is necessary for the development of appropriate therapeutic measures for the affected family members. Furthermore, it is believed that therapeutic modalities will be gene- or mutation-specific, therefore, defining the set of ADRP genes in the SA context is a vital step in the process.

CHAPTER 2
LOCALISATION OF THE RP13 GENE

2.1. INTRODUCTION

In the study of retinal disease many genes involved in the visual phototransduction pathway have either been mapped or cloned. These efforts have facilitated the investigation of the possible role of these genes in various forms of retinal degenerative disorders, including ADRP. The investigation of the reported loci in a large number of families with ADRP confirmed the high level of heterogeneity and the existence of other loci (Inglehearn, 1998). To date several genes have been implicated in the ADRP form of RD. The purpose of the present study was to find the location of the genetic defect in a large SA family (ADRP-SA1) in which retinitis pigmentosa segregates as an autosomal dominant condition.

At the outset of this investigation, five loci had already been reported to be associated with ADRP. The disorder was first shown to be linked to a marker, *C17*, close to the *RHO* gene on chromosome 3q21-q24 (McWilliam et al., 1989). *Peripherin-RDS*, on chromosome 6p21.1-cen, was the other gene shown to be associated with ADRP (Farrar et al., 1991). The remaining three disease-associated loci represented anonymous chromosomal regions. These were, the pericentric region of chromosome 8 (Blanton et al., 1991), the short arm of chromosome 7

(7p) (Ingelhearn et al., 1993), and the long arm (q) of the same chromosome (Jordan et al., 1993). These five confirmed ADRP loci were the obvious candidates with which to commence the search for the gene underlying the ADRP-SA1 disease phenotype. Additional functionally and structurally significant candidate genes, not previously associated with RD, were investigated for linkage to ADRP-SA1. These included:

- *cGMP-PDE* β, on chromosome 4p (Weber et al., 1991);
- the interstitial retinal binding protein gene (*IRBP*), on chromosome 10p (Liou et al., 1987);
- the retinoid binding protein gene (*RBP*), on chromosome 10q (Rocchi et al., 1989);
- the rod outer membrane 1 gene (*ROM1*), on chromosome 11q, which at the time was thought to be involved in ADRP (Bascom et al., 1992a; b);
- *cGMP-PDE* α, on chromosome 5q (Pittler et al., 1990);
- the rod α-transducin gene (*GNAT1*), on chromosome 3p (Ngo et al., 1993);
- the S-antigen gene (*SAG*), on chromosome 2q (Ngo et al., 1990); and
- the recoverin gene (*RCV1*) on chromosome 17p (Wiechmann et al., 1994a).

The positional candidate approach was the strategy of choice used to test the various loci in the ADRP-SA1 family. This approach involved the analysis of polymorphic DNA markers tightly linked to the genes of interest in affected and unaffected members of ADRP-SA1. The resulting

genotypes were then tested for linkage in the family. The principle of linkage is a statistical estimate of the tendency of genes or DNA sequences at specific loci to be inherited together as a result of their physical proximity on a single chromosome (Terwilliger and Ott, 1994).

2.2. PATIENTS, MATERIALS AND METHODS

2.2.1. RECRUITMENT OF PATIENTS AND ACCUMULATION OF BIOLOGICAL SAMPLES

A retinal degeneration (RD) research centre was established in the Department of Human Genetics in Cape Town, with the goal of elucidating the molecular basis of this group of disorders in South Africa. The centre archives patient data and biological material from individuals and families with all forms of inherited RD (Greenberg et al., 1994a, Greenberg and Peters, 1995).

Patients with RD are recruited through ophthalmologists and the support group, Retinal Preservation Foundation of South Africa. Before blood samples are taken, patients are counselled and patient data and DNA consent forms are completed in the presence of the patient (Appendix C, section C.1). The patient signs the form once he/she has a clear understanding of its content. The form is distributed to Ophthalmologists and genetic centres regionally and nationally, schools for the blind, patient support groups for the visually impaired and the RP Foundation of SA. In every instance, patients are assured of the confidentiality of the information. The forms together with the blood

samples are sent to the laboratory. Specimens are coded and the clinical and personal data captured on computer. The patient data and DNA consent forms were filed together with any additional information on the family.

Subjetcs, who were generally affected with RD, or first degree relatives of such individuals were evaluated ophthalmologically at the Department of Ophthalmology, Groote Schuur Hospital, or by ophthalmologists in other centres. Clinical signs and electrophysiological information is documented according to a clinical data form (Appendix C, section C.2) designed for the consistent collection of accurate clinical data.

Analysis of the genealogical data compiled from each of the families archived, revealed a large autosomal dominant RP kindred (Oswald et al., 1985, Greenberg et al., 1993, Greenberg et al., 1994a). The family was designated, ADRP-SA1.

2.2.1.1. The ADRP-SA1 Family

ADRP-SA1 (figure 2.1.) was first identified in 1985 (Oswald et al., 1985) through the proband (individual IV-17) and her two affected sons. Individual IV-17 was eventually instrumental in tracing 60 South African relatives, 24 of whom manifest RP. A genealogical study revealed that the progenitor emigrated from England in the early twentieth century, with many descendants still resident in South Africa. The progenitor, who is the most likely carrier of the defective gene, was the

only affected individual of nine siblings and possibly represents a new mutation.

The disorder in the ADRP-SA1 pedigree showed a clear autosomal dominant inheritance pattern (figure 2.1.). Both males and females were affected and the disorder could be traced through six successive generations.

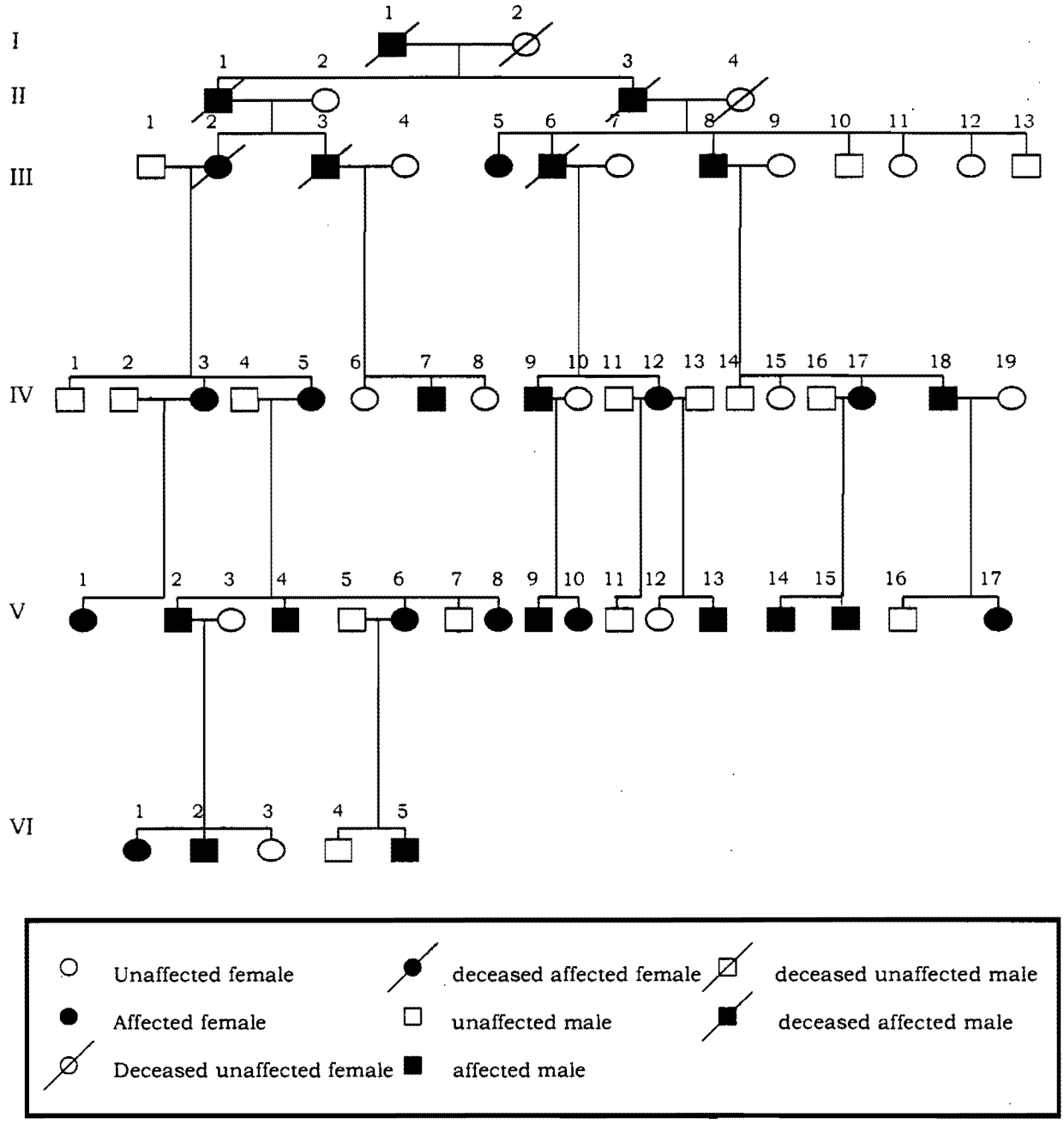


Figure 2.1. Pedigree of the ADRP-SA1 family showing a clear autosomal dominant pattern of inheritance in six successive generations.

2.2.1.2. Clinical Presentation

In the ADRP-SA1 family the onset of night-blindness was consistently around 4 years of age with a diffuse pattern of retinal involvement. These clinical signs are indicative of type I ADRP as described by Massof and Finkelstein (1981). This diagnosis was confirmed by various ophthalmologists who investigated the affected family members. In every recorded instance, affected family members experienced night-blindness followed by characteristic tunnel vision. Affected individuals exhibited abnormal ERGs of both the rods and cones, however, the rods were predominantly involved. Generally, the diffuse pattern of pigmentation (type I ADRP) was observed in the mid-equatorial region of the retina before 10 years of age. The RP phenotype was consistent within the family and appeared to be fully penetrant with no evidence of skipped generations. Bilateral cataracts were commonly observed in affected individuals. Minimal clinical details were available regarding the retinal defects in the family.

A detailed ophthalmological report on affected individual **VI-1** (figure 2.1.) revealed that at 17 years of age she complained of reduced vision in both eyes. On examination, her visual acuity was 6/7.5 and 6/9, without correction. She had sight astigmatism and, with a correction, the visual acuity could be improved to 6/6. Intra-ocular pressures were normal. After dilation very little pigment was observed in the retina. She also had a posterior lenticonus, again of little significance. On subsequent examination, at 20 years of age, the visual acuity in her right eye was 6/7.5, with correction, and that in the left eye was 6/9

without, and 6/7.5 with correction. Colour vision, stereo vision and intra-ocular pressures were normal. A small central subcapsular cataract was present in both eyes. A partially collapsed vitreous and signs of severe vitreous floaters were present in both eyes. The retinal signs were still typical of RP and the electroretinogram (ERG) showed abnormalities of both rod and cone cells, with greater involvement of the rods.

2.2.2. LINKAGE ANALYSES WITH CANDIDATE LOCI

Several candidate genes and loci were investigated towards localising the disease gene in ADRP-SA1. The study was undertaken using linkage analysis with markers that were selected because they were either intragenic or tightly linked to the genes investigated.

The object of linkage analysis is the estimation of the recombination fraction (θ) between two genetic traits and to test whether θ is less than 0.5. The recombination fraction, is therefore the rate of recombination between loci, which is taken as a measure of the genetic distance between genetic loci. The standard tools for linkage analysis are generally genetic markers, which have undergone major developments over the past century (Strachan and Read, 1996). During recent years the markers of choice have been microsatellites. Microsatellites are small arrays of tandem repeats interspersed throughout the genome of which the bulk are (CA)n repeats (Strachan and Read, 1996). These arrays of CA repeats are very common in the genome and highly polymorphic, and therefore have been chosen as the markers of choice

during this study. However, as tri- and tetranucleotide repeats produce clearer results (genotypes), these replaced dinucleotide repeats as the markers of choice over the extended period of this study.

2.2.2.1. DNA ISOLATION

Genetic linkage studies were undertaken using genomic DNA which was extracted from whole blood, obtained from subjects, using the Genomix™ blood scale-up kit [*Talent*, Italy] (Appendix A, section A.1). Peripheral blood (10ml) was obtained by venipuncture from 46 family members.

2.2.2.2. Candidate Loci And Polymorphic Markers

The five known ADRP-causing genes/loci on chromosomes 3q (McWilliam et al., 1989), 6p (Farrar et al., 1991), 8q (Blanton et al., 1991), 7p (Ingelhearn et al., 1993), and 7q (Jordan et al., 1993), were chosen as the candidate loci to commence the investigation for linkage in ADRP-SA1. The study was subsequently extended to eight other functional candidate genes including *cGMP-PDE* β (4p) (Weber et al., 1991), *IRBP* (10p) (Liou et al., 1987), *RBP* (10q) (Rocchi et al., 1989), *ROM1* (11q) (Bascom et al., 1992a), *cGMP-PDE* α (5q) (Pittler et al., 1990), *GNAT1* (3p) (Ngo et al., 1993), *SAG* (2q) (Ngo et al., 1990) and *RCV1* (17p) (Wiechmann et al., 1994a).

The DNA markers used are listed in tables 2.1. and 2.2. The PCR markers *D4S227* and *ID53/54* were the generous gift of Dr Bernard Weber (Germany) and *D8S165* and *D8S108* of received from Dr Steven Daiger (USA). The remaining microsatellite markers were purchased

Table 2.1. List of the microsatellite markers used to test each candidate locus for linkage.

Candidate Genes/Loci	Markers
RHO	*D3S621* *RHO*
Peripherin/RDS	*RDS* *D6S105* *D6S89*
8q Locus	*D8S165* *D8S87*
7p Locus	*D7S460* *D7S435*
7q Locus	*D7S480*
PDE-β	*D4S227* *ID53/54*
IRBP	*D10S193* *D10S225*
RBP	*D10S220* *D10S199*
ROM1	*D11S527* *D11S905* *D11S935* *PYGM* *INT2*
PDE-α	*D5S393* *D5S403*
GNAT1	*D3S1210* *D3S1217* *D3S643* *HRG*
RCV1	*D17S849* *D17S938* *D17S796*

Table 2.2. List of supplementary RFLP markers used to test candidate loci for linkage.

Candidate Genes	Marker	Restriction Enzymes	Probe
RHO	*D3S21*	*MspI*	L182
	D3S14	*MspI*	R208
	D3S19	*MspI*	U1
SAG	*D2S50*	*MspI*	CYNA4
	PLAP	*RsaI*	ALPI
	SAG	*RsaI*	Pekz105

from Research Genetics (Huntsville, USA) (Appendix A, section A.3). The RFLP genomic probes on chromosome 3 were inserts in the vector, Charon 4A and were propagated in bacteriophage lambda (Sambrook et al., 1989). The genomic probes, L182, R208 and U1 were polymorphic for the restriction enzyme *MspI* (Donnis-Keller et al., 1987) and purchased from the American Type Culture Collection (ATCC Baltimore, USA). The chromosome 2 RFLP probes were obtained from the Waardenberg Consortium (Farrar et al., 1992). The Southern blot technique was carried out using conventional methods (Sambrook et al., 1989).

2.2.2.3. Polymerase Chain Reaction

Microsatellite markers were processed by amplification using the polymerase chain reaction (PCR). PCRs contained 0.2µg of genomic DNA, as template; 50mM KCl, 10mM Tris-HCl pH8.3, 1.5mM $MgCl_2$, 200µM of dATP, dGTP, dTTP and dCTP; 1µCi of α-[^{32}P]dCTP; 10-20 pmol of each primer and 1 unit of *Taq* polymerase [*GibcoBRL*, UK], in a final volume of 25µl. The samples were generally amplified through 30 cycles in an Omnigene [*Hybaid*, UK] thermal cycler denaturing at 95 °C, annealing temperatures of 55°C to 60°C, and extension at 72 °C. PCR products were checked for size against a standard 1kb molecular weight marker [*Gibco*, UK] on 2% agarose [Sigma, USA] gels. Twenty µl of formamide loading buffer (Appendix B, section B.3.) was added to each reaction after PCR. Samples were denatured for 3 min at 95°C and chilled on ice for 10 min, followed by electrophoresis through 6% denaturing polyacrylamide gels (Appendix B, section B.4). The gels were dried onto 3MM paper [*Schleicher and Schuell*, Germany] on a slab gel drier [*Hoefer Scientific Instruments*, USA] for 1-2 hrs at 80°C and exposed to X-ray film [*Agfa*, Germany] for 16-32 hrs at –70°C.

2.2.2.4. Calculation Of Lod Scores

In order to establish the likelihood that the ADRP-SA1 disease-causing gene locus was linked to the markers tested, two-point analyses were performed during the initial test of candidate loci, and multipoint analyses once an indication of linkage was observed. The latter was used to confirm the order of markers on the genetic map and to fine

map the disease gene with respect to these markers. Analyses were performed using the LINKAGE software package Ver.5.03 (Lathrop and Lalouel, 1984). The Genethon map order of microsatellite markers for each chromosome was used (Gyapay et al., 1994). In order to facilitate processing by the software, the number of alleles for each marker was reduced to five, ensuring no loss of information in the family. The RP gene was assumed to be completely penetrant and lod scores between the markers and the ADRP locus were calculated using recombination fractions from 0.00 to 0.25. A gene frequency of 0.001 was assumed for the disease locus. For multipoint analyses, allele frequencies for each of the markers were calculated from a number of unaffected unrelated control individuals from the population of origin.

2.2.3. HAPLOTYPE CONSTRUCTION

The alleles of markers situated close together on a small genomic segment tend to be inherited as a set or haplotype through generations within a pedigree. Haplotype analysis is powerful in mapping genes because it facilitates the identification of recombinant events within the genomic segment. These events enable the researcher to pin-point the exact region within the segment which harbours the gene of interest. A set of eight markers on chromosome 17p which span the RP13 critical interval was used to construct haplotypes for the ADRP-SA1 family.

2.3. RESULTS

2.3.1. LINKAGE ANALYSES WITH CANDIDATE LOCI

2.3.1.1. Exclusion of 11 Candidate Loci

A range of 34 markers was used to investigate 11 distinct loci as candidates underlying the RP phenotype in the ADRP-SA1 family. Results from a series of two-point linkage analyses between the disease phenotype and test markers. are presented in table 2.3. Significantly negative lod scores provided evidence for exclusion of the candidate genes on chromosomes 2q (*SAG*), 3p (*GNAT1*), 4p (*cGMP-PDE* β), 5q (*cGMP-PDE* α), 10p and q (*IRBP and RBP*), and 11q (*ROMI*), and the anonymously linked loci on, 7p, 7q and 8q. The genotypes produced for the two markers used to test the *RHO* and *peripherin/RDS* genes for linkage were uninformative, resulting in low positive lod scores in each instance. Observed meiotic recombinants were, however, detected between the disease phenotype and the intragenic markers (*RHO* and *RDS*) used at each locus (Greenberg et al., 1993), thereby excluding these 2 genes.

These results provided evidence for exclusion of the five reported primary ADRP candidate loci and a further six functional candidate genes in the ADRP phenotype that was investigated in this study.

Table 2.3. Pairwise lod scores (z) showing exclusion of linkage between ADRP and selected marker at 11 candidate loci.

Marker	Recombination Fraction (θ)					
	0.0	0.05	0.10	0.15	0.20	0.25
D3S621	-99	-4.96	-2.98	-1.92	-1.25	-0.77
R208	-99	-5.90	-3.70	-2.49	-1.69	-1.13
U1	-99	-4.93	-3.11	-2.1	-1.43	-0.95
L182	-99	-0.34	-0.10	0.01	0.06	0.09
RHO	-99	0.98	1.02	0.94	0.80	0.65
RDS	0.67	0.57	0.45	0.34	0.24	0.16
D6S105	-99	-2.88	-1.61	-0.97	-0.59	-0.36
D6S89	-99	-1.05	-0.37	-0.08	0.06	0.12
D8S165	-99	-6.17	-3.74	-2.44	-1.59	-1.03
D8S87	-99	-2.39	-1.40	-0.89	-0.59	-0.40
D8S108	-99	-3.22	-2.05	-1.32	-0.79	-0.42
D7S460	-99	-7.56	-4.94	-3.47	-2.49	-1.77
D7S435	-99	-1.12	-0.36	-0.03	0.11	0.16
D7S480	-99	-7.22	-4.48	-2.99	-2.02	-1.35
D4S227	-99	-3.52	-2.18	-1.46	-1.00	-0.68
ID53/54	-99	-1.24	-0.76	-0.49	-0.32	-0.19
D10S193	-99	-2.57	-1.34	-0.74	-0.39	-0.18
D10S199	-99	-1.76	-1.00	-0.62	-0.39	-0.24
D10S220	-99	-1.80	-1.00	-0.58	-0.33	-0.16
D10S225	-99	-4.21	-2.59	-1.73	-1.18	-0.79
D11S527	-99	-5.93	-3.69	-2.48	-1.68	-1.13
D11S905	-99	-7.84	-4.84	-3.22	-2.17	-1.44
D11S935	-99	-5.73	-3.38	-2.15	-1.39	-0.88
PYGM	-99	-1.45	-0.74	-0.41	-0.22	-0.10
INT2	-99	-1.37	-0.88	-0.63	-0.48	-0.37
D5S393	-99	-3.42	-2.29	-1.66	-1.23	-0.90
D5S403	-99	-4.11	-2.65	-1.84	-1.30	-0.91
D3S1210	-99	-3.48	-2.13	-1.39	-0.92	-0.60
D3S1217	-99	-5.12	-3.23	-2.20	-1.53	-1.06
HRG	-99	-4.03	-2.34	-1.44	-0.87	-0.50
D3S643	-99	-5.02	-2.97	-1.92	-1.29	-0.88
D2S50	-99	-3.58	-2.27	-1.58	-1.14	-0.83
ALP1	-99	-2.54	-1.65	-1.16	-0.83	-0.59
PekZ105	-99	-0.46	-0.26	-0.17	-0.11	-0.07

2.3.1.2. Linkage To Chromosome 17p

Evidence for linkage was first noted on visual examination of the genotypes produced with the set of markers (*D17S849*, *D17S938* and *D17S796*) used to test the candidate gene, recoverin (*RCV1*), on chromosome 17p for linkage to the phenotype in ADRP-SA1 (figure 2.2). Significantly positive two-point lod scores of 3.60, 5.43 and 4.82 were subsequently obtained for markers *D17S849*, *D17S938* and *D17S796*, respectively (all at $\theta = 0.10$). In order to further investigate these preliminary findings, the DNA from subjects in the family was genotyped with six additional markers *(D17S926, D17S960, D17S578, D17D786, D17S804* and *TP53*) spanning a genetic distance of 23cM within the 17p13 chromosomal region. Two-point lod scores between the phenotype and the initial three markers used to test the locus and each of the six additional markers are shown in table 2.4. The gene underlying the phenotype in ADRP-SA1 was most closely linked to *D17S938*, which produced the highest lod score at lowest θ value (lod score of 5.43, at $\theta = 0.10$), between markers *D17S849* and *D17S796* (Greenberg et al., 1994b). The locus was subsequently designated RP13 (MIM 600059).

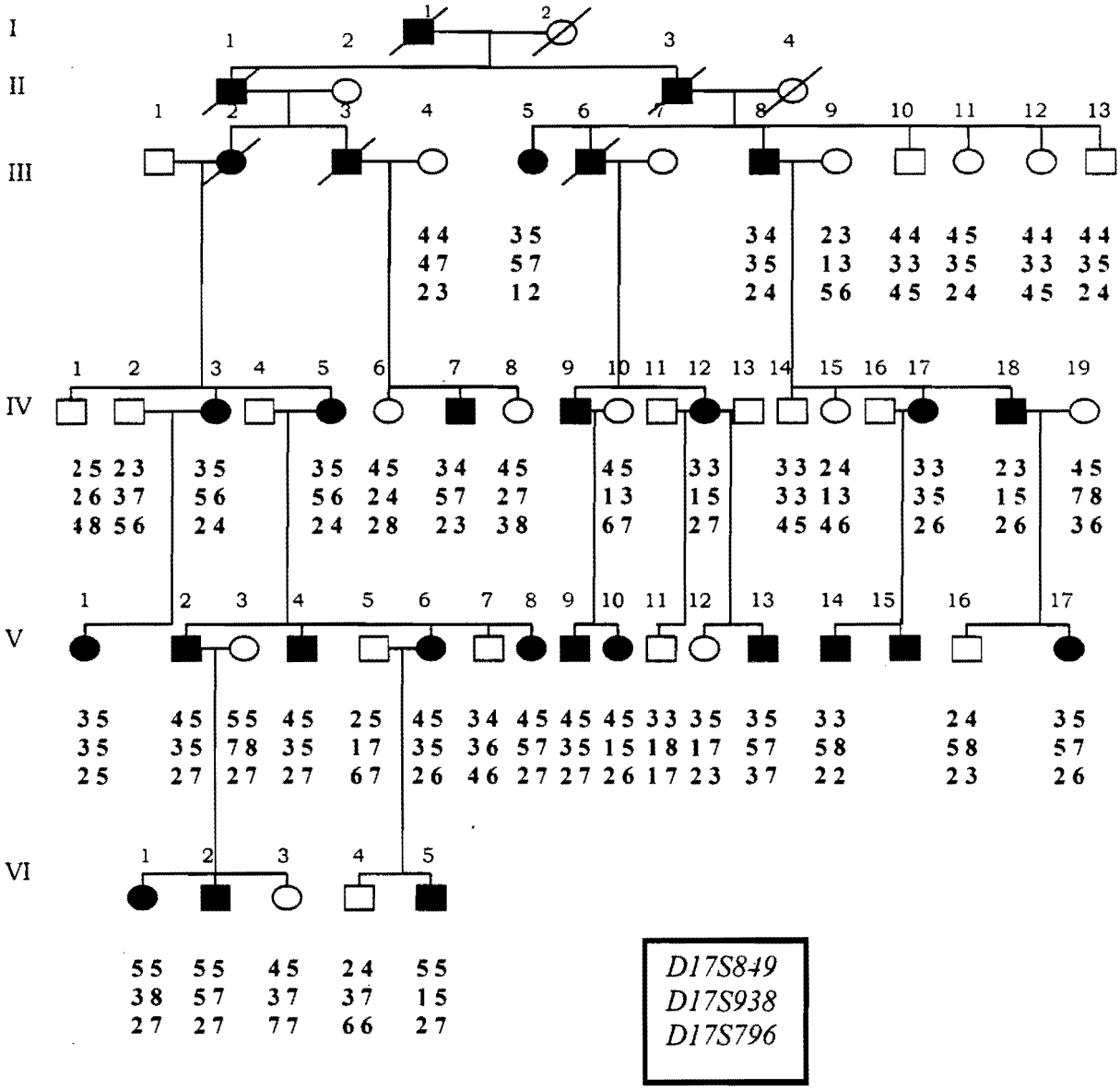


Figure 2.2. Representation of the segregation of the genotypes for markers *D17S849* (five alleles), *D17S938* (eight alleles) and *D17S796* (eight alleles).

Table 2.4. Pair-wise lod scores between ADRP and nine polymorphic DNA markers on chromosome 17p.

Recombination Fraction (θ)

Marker	0.00	0.05	0.10	0.20	0.30	0.40
D17S926	-∞	0.07	1.09	**1.51**	1.15	0.45
D17S849	-∞	3.35	**3.60**	3.14	2.17	0.97
D17S938	-∞	5.07	**5.43**	4.82	3.49	1.78
D17S796	-∞	4.72	**4.82**	4.01	2.69	1.19
D17S960	-∞	3.09	**3.16**	2.62	1.76	0.77
D17S578	-∞	2.83	**3.29**	2.93	1.97	0.83
D17S786	-∞	3.26	**3.55**	3.14	2.24	1.11
D17S804	-∞	-0.47	0.14	**0.47**	0.41	0.22
TP53	-∞	3.34	**3.55**	2.95	1.89	0.74

The bold numbers indicate the highest lod score at the corresponding (θ) value obtained for each marker

2.3.1.3. Fine Mapping The RP13 Locus

Further genetic mapping studies were carried out to establish the smallest possible interval harbouring the RP13 locus and to facilitate the identification of the disease-causing gene.

To establish which interval between the nine markers should be exploited to fine map the RP13 locus, a series of three-point analyses were undertaken using two markers (from the initial nine) and the disease phenotype in subjects from the ADRP-SA1 family. The highest lod score at the lowest θ was obtained in the interval between *D17S849*

and *D17S938*. DNA from family members was genotyped with five markers (*D17S1529, D17S1528, D17S831, D17S829* and *D17S1353*) within the 15cM interval between *D17S849* and *D17S938* (figure 2.3.). Two point lod scores obtained between the disease locus and each of these markers are shown in table 2.5. Marker *D17S1528* showed no recombination events and produced a lod score of 7.19 ($\theta = 0.00$). Markers *D17S1529* and *D17S831*, located on either side of *D17S1528* also showed significantly positive lod scores of 5.64 and 6.66 respectively, at $\theta = 0.05$.

In order to confirm the order of markers on the genetic map of chromosome 17p, another set of multipoint analyses was performed, using 10 markers, starting at the telomeric marker, *D17S849*, and ending at the centromeric marker, *D17S804*. The multipoint analysis results illustrated in figure 2.4., show that a peak lod score of 8.4 was obtained between *D17S1529* and *D17S1528*, indicating that this was the most likely interval to contain the disease gene. The 1-unit-of-lod-score support interval (an estimation of a two-point recombination fraction) only encompasses the region between *D17S1529* and *D17S831*, with both these markers being excluded.

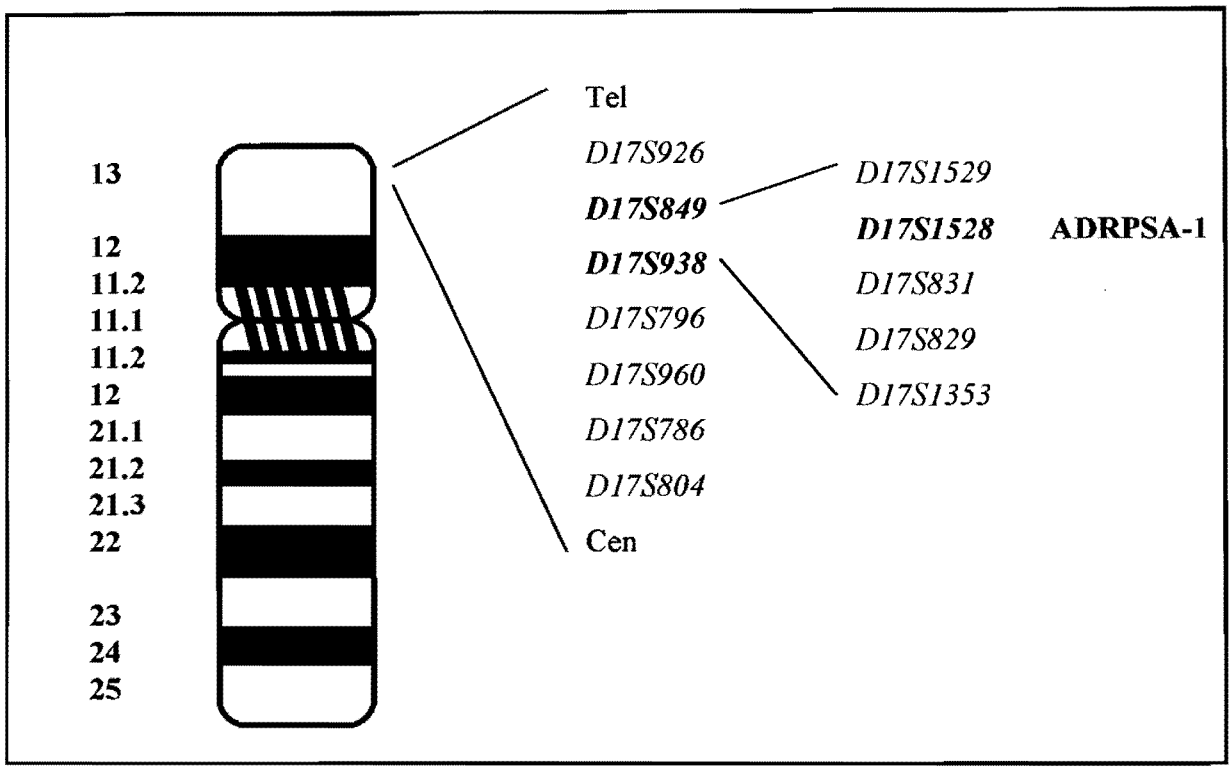


Figure 2.3. Ideogram of human chromosome 17, with an expanded genetic map of the region to which the ADRP-SA1 phenotype localised.

Table 2.5. Pair-wise lod scores between microsatellite markers and the ADRP-SA1 disease phenotype.

Recombination fraction (θ)

Marker	0	0.05	0.1	0.2	0.3	0.4
D17S849	-∞	3.35	**3.60**	3.14	2.17	0.97
D17S1529	2.12	**5.64**	5.21	3.95	2.43	0.88
D17S1528	**7.19**	6.70	6.10	4.71	3.12	1.43
D17S831	2.97	**6.66**	6.22	4.93	3.35	1.57
D17S829	-9.15	**0.92**	0.92	0.64	0.27	0.00
D17S1353	-5.13	2.40	**2.72**	2.39	1.63	0.73
D17S938	-∞	5.07	**5.43**	4.82	3.49	1.78

The bold numbers indicate the highest lod score at the corresponding (θ) value obtained for each marker.

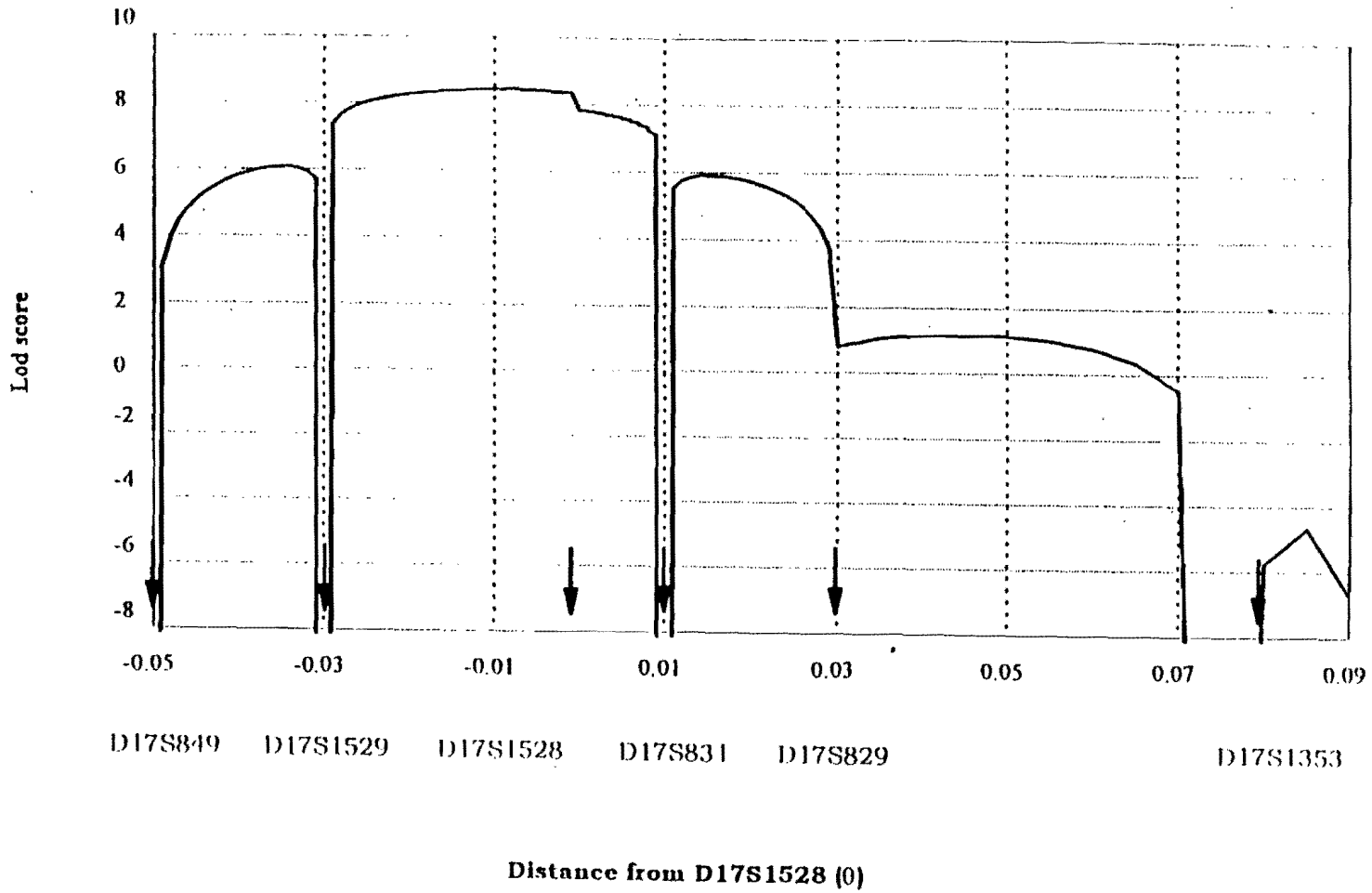


Figure 2.4. Results of multipoint analyses of the ADRP phenotype against two markers in the family.

2.3.2. HAPLOTYPE ANALYSIS

Towards confirming and further narrowing the RP13 genetic interval, haplotypes comprising 8 markers ranging from *D17S926* to *D17S938* were constructed. The support interval as defined by genetic mapping studies was confirmed by observed meiotic recombinants on haplotype analysis (figure 2.5.). It would appear that affected individual III-2 is recombinant for the markers *D17S926* through to *D17S1529*. The recombinant chromosome was transmitted to all the affected individuals in her branch of the pedigree through three generations, except to individual VI-1. It is evident that in individual VI-1 the paternally inherited chromosome had recombined between markers *D17S1528* and *D17S831*. Individual VI-1 is unequivocally affected with ADRP. She is currently aged 26 years and fundal examination revealed typical features of RP (Ophthalmologist's report in section 2.2.1.2., page 41). The recombination events (in individuals III-2 and VI-1) provide conclusive evidence that the RP13 gene maps proximal to *D17S1529* and distal to *D17S831*.

The two-point, multipoint linkage and haplotype data presented support the fine localisation of RP13 from a 15cM to a 3cM interval between markers *D17S1529* and *D17S831* on chromosome 17p13.3 (Goliath et al., 1995).

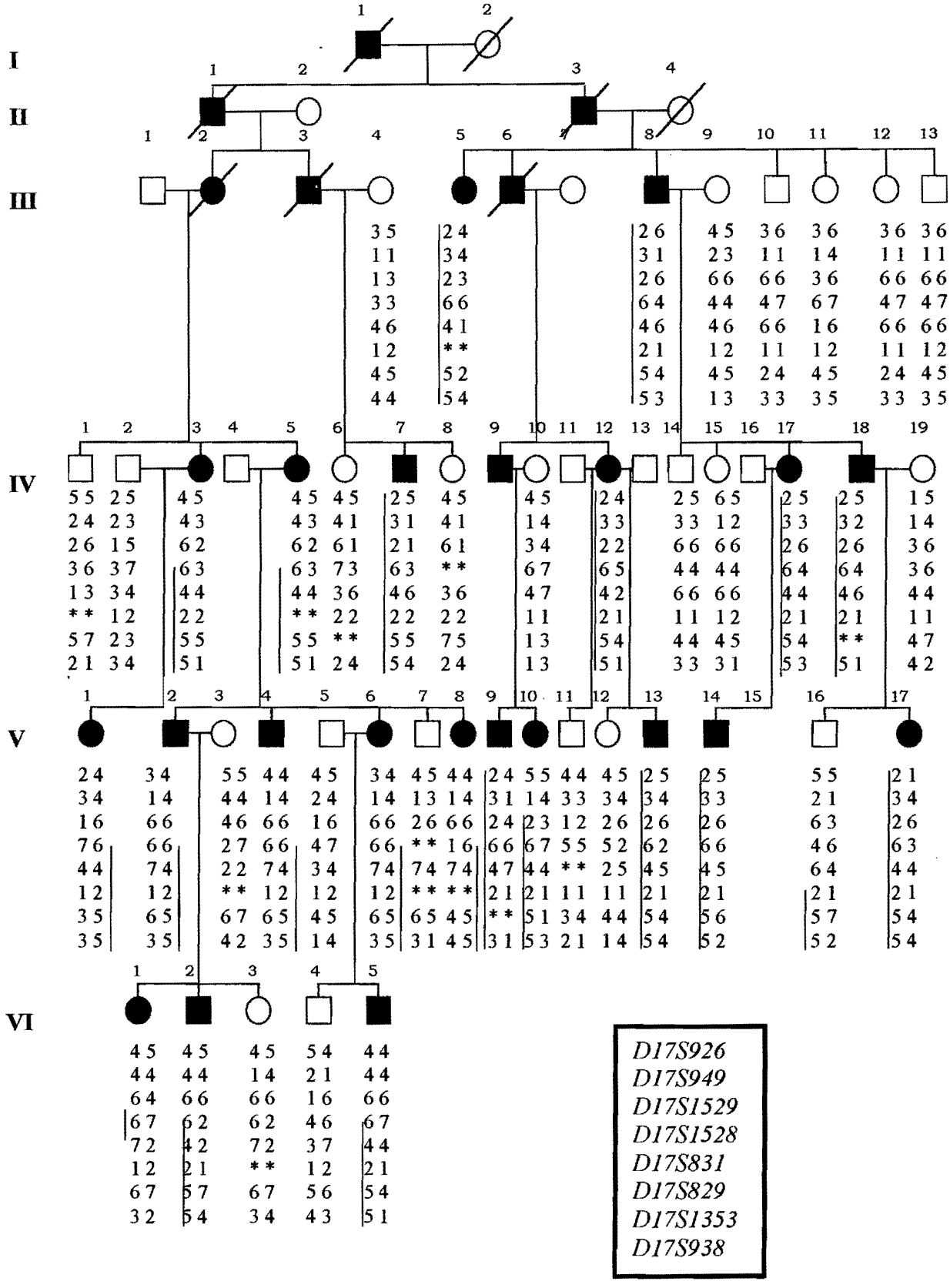


Figure 2.5. ADRP-SA1 pedigree showing the segregation of the affected haplotype in red and critical recombination events (* = undetermined genotypes).

2.4. DISCUSSION

This study reports the identification of the ADRP locus (RP13) which occurs on chromosome 17p in a large SA family (ADRP-SA1), during a positional candidate-based linkage approach (Greenberg et al., 1994b). The subsequent fine mapping of the disease locus placed it on the 17p13.3 chromosomal band, within a 3cM interval between markers *D17S1529* and *D17S1528* (Goliath et al., 1995).

The positional candidate approach used in the search for the ADRP-SA1 disease-causing gene consisted of two phases. The first phase involved screening the five extantly known ADRP loci for linkage to the ADRP-SA1 RP phenotype. The disease locus was excluded from each of the five disease loci: The *RHO* and *RDS* intragenic microsatellite markers at the *RHO* and *peripherin/RDS* genes, respectively, proved to be mostly uninformative, hence the low positive lod scores obtained (table 2.3.). These genes were, however, excluded due to the recombination events observed between the disease locus and alleles of the respective intragenic markers used. The ADRP loci on chromosomes 8q, 7p and 7q were excluded based on linkage analyses which produced lod scores of less than -0.20, at a recombination fraction of 0.05, the generally accepted value for significant exclusion (Lester et al., 1990). Exclusion of ADRP-SA1 from the known loci was not surprising as genetic heterogeneity has been observed in several ophthalmic disorders, most notably RP.

Exclusion of the five known disease loci directed the mapping efforts into the second phase of the positional candidate gene screen. This phase involved screening markers associated with retinally-expressed genes not previously reported to be associated with ADRP. Candidate genes *cGMP-PDE* α and β, *GNAT1*, *SAG* and *RCV1* on chromosomes 5q, 4p, 3p, 2q, and 17p, respectively, were chosen because of the established function of each gene product in the visual phototransduction cascade (chapter1, section 1.1.3., page 11). *IRBP* and *RBP* on chromosome 10 were chosen because of the important transport functions of each gene product in photoreceptor cells. IRBP binds hydrophobic ligands of both retinoids and analogs in the interphotoreceptor space preventing the degradation of these molecules (Nickerson et al., 1998). RBP is involved with the transport of vitamin A to target cells (Naylor et al., 1999). ROM-1 is a transmembrane protein present at photoreceptor outer segment disc rims (Goldberg and Molday, 1996).

It was during the second phase of the study that evidence for linkage was observed with markers flanking the *RCV1* gene locus (*D17S849*, *D17S938* and *D17S796*). This result was confirmed by genotyping DNA from family members with a range of markers spanning a genetic distance of 23 cM. The data presented strongly suggested that the causative gene for ADRP in ADRP-SA1, maps to the short arm of chromosome 17 (RP13) (Greenberg et al., 1994b). Based on further genetic fine mapping studies, the interval was eventually reduced to a 3cM interval between markers *D17S1529* and *D17S831* (Goliath et al.,

1995). Haplotype analysis using eight markers spanning the chromosomal region confirmed this support interval. The haplotypes revealed meiotic recombinants in two affected individuals with markers (*D17S1529* and *D17S831*) on either side of the critical interval.

RP13 represented the seventh locus for ADRP assigned by linkage analysis. It is not yet known what proportion of patients with RP have mutations in the corresponding gene on chromosome 17p. Literature reviews suggest that mutations of *RHO* and *peripherin/RDS* account for approximately 25% and 5% of all ADRP cases, respectively (Inglehearn, 1998). The recently identified *RP1* gene appears to account for at least 7% of ADRP (Bowne et al., 1999). In contrast, the majority of the remaining seven ADRP loci have each been assigned in a single family or have been detected in just a few families (Greenberg et al., 1994b, Ingelhearn et al., 1993, Jordan et al., 1993, Bardien et al., 1995, Al-Maghtheh et al., 1994, Blanton et al., 1991 and Xu et al., 1996). The RP13 locus has been excluded in at least three large SA ADRP families in which the disease locus has been excluded from all known loci (unpublished results). This observation is suggestive of the existence of at least one other ADRP locus.

Amongst the three anonymous ADRP loci tested for linkage (7p, 7q and 8q) in the ADRP-SA1 family, only the gene on chromosome 8q (*RP1*) has since been identified (Bowne et al., 1999). Several pathogenic alterations in *RP1* were elucidated in families previously mapped to this locus. Prelimary findings suggest that *RP1* gene-expression is retina-specific

and the product may be a protein kinase involved in the development or maintenance of the neural retina (Sullivan et al., 1999). An additional three ADRP loci have been mapped to chromosomes 19q (Al-Maghtheh et al., 1994), 17q (Bardien et al., 1995) and 1q (Xu et al., 1996). Furthermore, ADRP-associated defects have been identified in an additional three genes (*NRL, CRX* and *RETGC-1*) (Bessant et al., 1999, Sohocki et al., 1998, Perrault et al., 1998). The previously described retina-specific gene, *NRL* (on chromosome 14q) (Yang-Feng et al., 1992) encodes a transcription factor which has been shown to regulate the expression of the photopigment, rhodopsin, *in vivo* (Kumar et al., 1996). The gene, *CRX* (on chromosome 19q) encodes another transcription factor, which regulates rhodopsin (Sohocki et al., 1998). The *RETGC-1* gene (on chromosome 17p) product is required during the phototransduction cascade to return the levels of cGMP to normal. Taken together, to date these findings bring the total number of ADRP-causing genes/loci to 12.

Of the eight additional non-disease associated candidate genes investigated in this study, six have since been implicated in various forms of retinal disease. Defects in *SAG*, on chromosome 2q have been identified to be causative of Oguchi disease, a rare autosomal recessive form of congenital stationary night blindness (Fuchs et al., 1995). The *GNAT1* gene, on chromosome 3p was implicated in an autosomal dominant form of congenital stationary night blindness termed Nougaret disease (Dryja et al., 1996). The *cGMP-PDE* β gene on 4p underlies ARRP (Valverde et al., 1996) as well as autosomal dominant

congenital stationary night blindness (Gal et al., 1994). *cGMP-PDE* α and *IRBP* on 5q and 10p, respectively, were shown to be causative of ARRP (Huang et al., 1995; Valverde et al., 1998), and *ROM*-1 on chromosome 11q together with *peripherin/RDS* (6p) was shown to underlie the digenic form of RP (Kajiwara et al., 1994).

The localisation of the chromosome 17p ADRP locus (RP13) in this study allowed for the mapping of two additional families to this locus, one from the USA (of British origin) (Kojis et al., 1996) and the other from the UK (Tartellin et al., 1996). Other large SA families, however, have been excluded from the RP13 locus. It was interesting to note that the three RP13-linked families (Greenberg et al., 1994b, Kojis et al., 1996; Tartellin et al., 1996) were each of British origin. The possibility that the separate families were part of a common genealogy. Genealogical data, however, indicated that the immigrant progenitor of the ADRP-SA1 family was the only visually impaired individual of nine siblings with no further family history of disease. Furthermore, haplotype analysis of each of the SA and UK families refuted the possibility that they were related (unpublished data). Two distinct chromosomal haplotypes segregated with the disorder in the two families. These findings suggested that the families were not related. The USA family was not available for testing. The critical genetic intervals as defined by the three studies are illustrated in figure 2.6.

To date, several retinal diseases have been mapped to chromosome 17p. These include:

- Leber's congenital amaurosis (LCA1), is an autosomal recessive condition responsible for severe congenital visual impairment. The disorder maps to a 1cM genetic interval between *D17S938* and *D17S1353* (Camuzat et al., 1995; 1996).
- LCA4, presents with the same clinical picture as LCA1, together with keratoconus. The combined phenotype maps to a distinct locus on chromosome 17p13 (Hameed et al., 2000).
- Autosomal dominant progressive cone degeneration (CORD5) is closely linked to marker *D17S796* in a single large family from the USA (Small et al., 1996). A Swedish family with apparently the same clinical picture mapped to the same chromosome 17p region (Balciuniene et al., 1995).
- Central areolar choroidal dystrophy (CACD) a rare autosomal dominant condition maps close to marker *D17S938* (Lotery et al., 1996).
- Sjogren-Larsson syndrome (SLS) is an autosomal recessive disorder where about 20-30% of patients develop chorioretinitis with macular and perimacular pigmentary degeneration. SLS maps close to marker *D17S805* (Rogers et al., 1995). Three mutations in the fatty aldehyde dehydrogenase gene, which maps to chromosome 17p11.2, were identified in three unrelated SLS patients.
- Cone-rod dystrophy is a severe retinal degenerative condition which maps to an 8cM interval between the markers *D17S796/D17S938* and *D17S954* (Kelsell et al., 1997) (CORD6).

In addition to several retinopathies localising to chromosome 17p, a

number of retina-specific genes map to the same chromosomal region (chapter 3). The phenotypic variation between the disorders that map to chromosome 17p, may be due to allelic heterogeneity as is the case with *peripherin/RDS* where one or more of the retinal dystrophies may result from mutations in the same gene at the 17p13 locus. Furthermore, the phenomenon of epistasis cannot be ruled out. Varied phenotypic expressions could also be the result of alterations in the same gene which in turn cause changes in the amounts and pattern of gene expression. The identification of the disease-causing genes and the elucidation of the molecular pathology of these disorders will clarify these questions.

Localising the RP13 locus relied heavily upon the use of the available genetic marker maps which consist of highly polymorphic microsatellite DNA markers. It has been the goal of researchers internationally to establish a genetic map of the genome where these "landmarks" are spaced approximately 1cM apart. The increased density of markers facilitates and expedites the mapping of disease genes with a high level of confidence. During the past decade the use of these powerful tools have allowed for the mapping of several disease-causing genes. Indeed several of the ADRP genes and/or loci have been localised in this way. The recent surge of interest in single nucleotide polymorphisms (SNPs), an abundant form of genome variation, distinguished from rare variations by a requirement for the least abundant allele to have a frequency of 1% (Brookes, 1999), is now set to benefit a wide range of genetic-related disciplines. The dense distribution of SNPs and their

expected concentration in or near to transcribed sequences (Brookes et al., 2000) is set to revolutionise mapping stategies in the third millenium. For these reasons there is considerable interest in the discovery and characterization of SNPs to enable the analysis of the potential relationships between human genotype and phenotype (Picoult-Newberg et al., 1999). Analysis of human genetic variation around candidate genes can shed light on the problem of the genetic basis of complex disorders. The complete elucidation of SNPs in candidate genes will enable further association studies on panels of affected and unaffected individuals.

The mapping of the RP13 locus by linkage analyses has contributed to the world-wide endeavour in the development of a gene map of the genome. As this research initiative progresses, it becomes an increasingly powerful tool in assigning disease loci to chromosomes or smaller sub-regions, as described in this chapter. When specific genes are presumed candidates of a disorder, the tool of linkage provides a rapid, standard approach to investigate the locus. In this way linkage provides a valuable diagnostic tool as well as a handle for unravelling the molecular basis of a disease.

Although ADRP is rare, linkage to chromosome 17p in ADRP-SA1 is important not only in relation to the disease, but also as it highlights genes (that map to the same chromosomal region) which may be implicated in the pathology of the disorder.

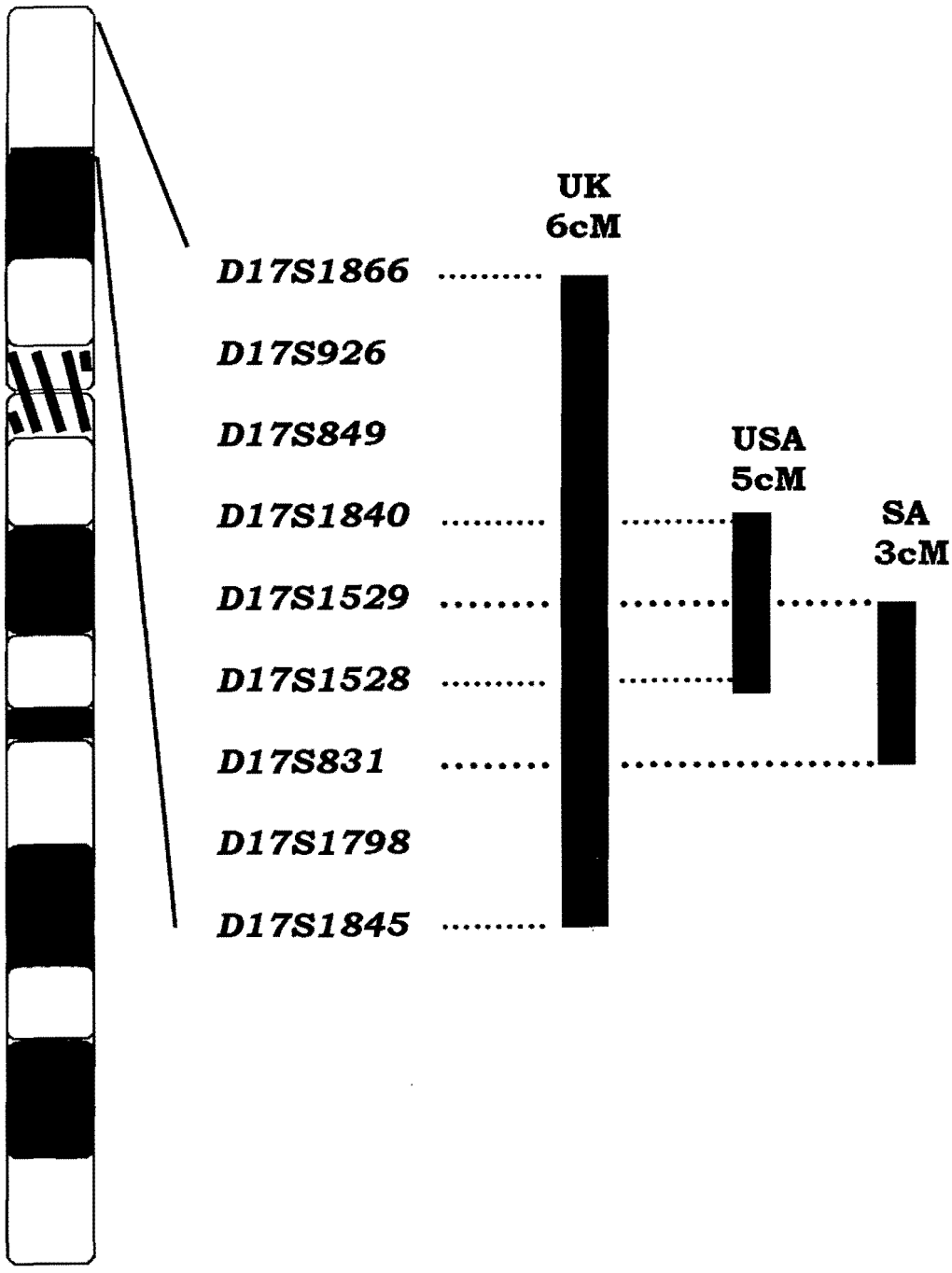


Figure 2.6. RP13 critical intervals as defined by the three groups.

CHAPTER 3
INVESTIGATION OF CANDIDATE GENES AT THE RP13 LOCUS

3.1. INTRODUCTION

Once the neighbourhood of a gene of interest has been identified, several strategies can be used to find the gene itself. Because of a potentially large number of genes in a specific interval, it is reasonable to establish a priority scale in further investigations. One way of ranking candidate genes in order of priority involves the expression of that gene in the tissue of interest, e.g., a gene responsible for kidney disease is likely to be expressed in the kidney and probably less likely in other tissues or organs. This strategy will limit the search to one or a few genes. A gene that is potentially involved has to be compared between unaffected and affected subjects. DNA variation within a candidate gene, between unaffected and affected subjects and its absence in a significant number of a population of matched control individuals, will suggest that the gene of interest has been found.

Since the initial identification of the RP13 locus on the telomeric end of chromosome 17p (Greenberg et al., 1994b), this genomic region has been intensively tested for co-localisation of phenotypically distinct retinal disorders. This gross region has proven to be relatively fruitful in such investigations and has been shown to harbour the genes for LCA

(Camuzat et al., 1995; 1996), CORD5 (Balciumiene et al., 1995). CACD (Lotery et al., 1996), SLS (Rogers et al., 1995) and CORD6 (Kelsell et al., 1997). Furthermore, several retinal genes which are functionally significant map to the same chromosomal region, including recoverin (*RCV1*) (Wiechmann et al., 1994a), retinal guanylate cyclase-1 (*RETGC-1*) (Oliveira et al., 1994), phosphatidylinositol transfer protein (*PITPN*) (Fitzgibbon et al., 1994), β-arrestin 2 (*ARRB2*) (Calabrese et al., 1994) and pigment epithelium-derived factor (*PEDF*) (Tombran-Tink et al., 1994). Although any gene which co-localises with a disease phenotype is a candidate, those which are functionally tissue-specific assume a higher priority for further investigation. Based on map position and function, the *RCV1*, *RETGC-1*, *PITPN*, *ARRB2* and *PEDF* genes were ranked as significant candidates for the disorder in the ADRP-SA1 family. The following is a compilation of the functionally significant candidates, which were considered for further investigation.

Recoverin (RCV1): In moving from darkness into bright light, a state of temporary blindness is experienced by the subject. Photoreceptor cells within the retina subsequently, are able to rapidly adapt to the influx of photons of light into the eye and vision is reinstated. The calcium-binding protein, RCV1, is believed to play a role in this "recovery" process by affecting the termination of the phototransduction pathway (Hurley et al., 1993). RCV1 alters the ability of rhodopsin kinase to phosphorylate activated rhodopsin (chapter 1, page 11).

Human RCV1 is retina-specific and highly conserved between species

(Murakami et al., 1992). The RCV1 gene (*RCV1*) is expressed in the photoreceptors as well as in the bipolar and ganglion cell layers of the retina (Wiechmann and Hammarback, 1993) and has been localised to chromosome 17p12-13 by *in situ* hybridisation (Wiechmann et al., 1994a).

RCV1 is encoded by a 9kb single-copy gene with the entire coding sequence contained within three exons (Murakami et al., 1992). Exons 1, 2 and 3 are 568bp, 113bp and 493bp, respectively. The three calcium-binding regions are all contained within exon 1. This is unusual as other gene sequences that contain calcium-binding domains are usually separated by introns (Xiang et al., 1991).

The function, expression and genetic mapping of *RCV1* rendered it a good candidate for retinal degenerative disorders that mapped to chromosome 17p.

Retinal guanylate cyclase (RETGC): RETGC is a critical component of the visual transduction pathway and is involved in the recovery from the light to dark state in the retina. Light activation leads to a drop in the calcium levels within rod photoreceptor cells. The drop in calcium levels in turn leads to a stimulation of membrane-bound RETGC production via the activation of guanylate cyclase-activating proteins (Dizhoor et al., 1994). Activated RETGC, in turn, catalyses the conversion of guanosine triphosphate to cyclic 3',5'-guanosine monophosphate (cGMP). Two isoforms of RETGC, encoded by separate

genes, *RETGC-1* and *RETGC-2*, exist (Yang et al., 1995). The two isoforms dimerise to form the active cyclase (Yang et al., 1996).

RETGC is a membrane-associated retinal enzyme with a high concentration in the outer segments of photoreceptor cells (Shyjan et al., 1992). The prominent expression of RETGC transcripts in only photoreceptors in itself implies that the enzyme has a very specialised function in these cells.

The gene encoding RETGC-1 maps to chromosome 17p13.1 (Oliveira et al., 1994). The autosomal recessive condition, LCA, which also maps to chromosome 17p, was genetically fine mapped to the interval between markers *D17S1353* and *D17S838* (Perrault et al., 1996). Three mutations, one in exon 8 and two in exon 2 of *RETGC-1*, were identified in affected individuals from three LCA families. Subsequently, additional LCA-related mutations were identified in *RETGC-1* (Loyer et al., 1998). Mutations of *RETGC-1* were also shown to underlie the AD cone-rod dystrophy (CORD6) phenotype which maps to the same chromosomal region (Kelsell et al., 1998).

The human *RETGC-1* gene is 16kb long, contains 20 exons, encodes a protein of 1101 amino acids with a molecular weight of 119,274, and is conserved between species (Shyjan et al., 1992). The localisation of *RETGC-1* to chromosome 17p raised the possibility that a defect in this gene might be responsible for the retinopathy exhibited in the ADRP-SA1 family.

Phosphatidylinositol transfer protein (PITPN): Unlike the previous two candidates discussed, PITPN is not directly involved in the visual transduction cascade (as outlined in chapter 1). Instead, PITPN is involved with the regulation of membrane transport within photoreceptor cells (Vihtelic et al., 1993). A working model for the proposed function of PITPN was established with rdgB, the *Drosophila* homologue (figure 3.1). It has been proposed that rdgB is required to transport proteins involved in phototransduction from the subrhabdomeric cisternae (SRC) to the photosensitive rhabdomeric membranes. PITPN is believed to stimulate membrane transport from the Golgi. The protein is membrane-bound (with six transmembrane domains) and possesses calcium-binding properties.

The *PITPN* gene is expressed in a variety of sensory and neuronal cells (Vihtelic et al., 1993) and is highly conserved between species. The human *PITPN* cDNA is 1368bp long (GenBank accession No. M73704) and, to date, no genomic DNA sequence of the gene has been published. It is known, however, that the gene contains an intron of about 1kb in its 3' untranslated region (personal communication with Dr David Hunt, London, UK).

PITPN maps to human chromosomal band 17p13.3 (Fitzgibbon et al., 1994). A mutation identified in the *Drosophila* rdgB gene (*rdgB* mutation) causes mutant flies to undergo photoreceptor degeneration (Hotta and Benzer, 1970). It has been proposed that the correct

conditions for phototransduction between the SRC and the rhabdomeres is not established in *rdgB* mutants (Vihtelic et al., 1993). Studies have indicated that the SRC degenerates before the rhabdomeres, which suggests that the rhabdomeres are gradually lost as a result of inadequate maintenance by the SRC (Matsumoto-Suzuki et al., 1989).

In view of the postulated function of the PITPN protein as well as the existence of a retinal degeneration homologue in *Drosophila*, it is conceivable that defects in PITPN (and hence in the membrane transfer process), could be involved in human retinal diseases. The possible involvement of the *PITPN* gene in the pathology of the ADRP-SA1 phenotype was therefore investigated.

Pigment epithelium-derived factor (PEDF): PEDF is believed to influence the development and maintenance of the neural retina (NR) (Tombran-Tink et al., 1995). The gene (*PEDF*) is, however, expressed in the RPE. PEDF is a major secretory product of human RPE cells into the interphotoreceptor matrix (IPM). In the IPM, the PEDF protein possesses both neurotrophic and neuronal survival activities. Its loss may produce cell senescence (Tombran-Tink and Johnson, 1989). Although most of the cellular PEDF protein product is secreted into the IPM, it is also found to be associated with cytoskeletal structures. It co-localises with actin, suggesting that PEDF could be involved in the assembly and/or stability of cytoskeletal structures (Tombran-Tink et al., 1995).

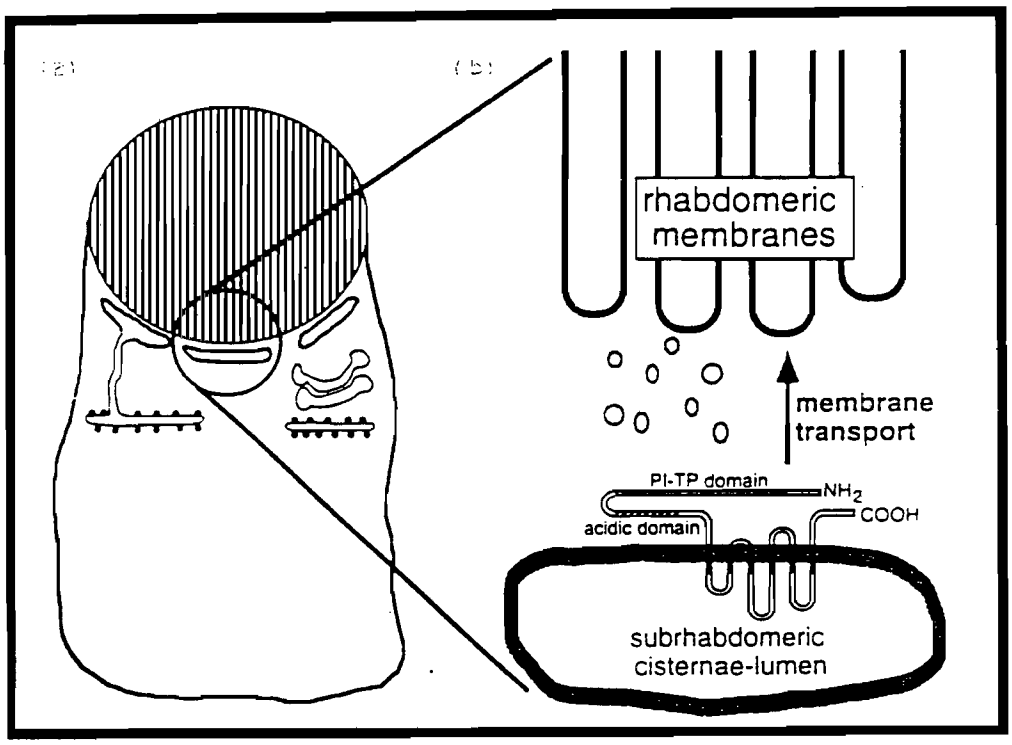


Figure 3.1. Working model of the function of the rdgb protein in the photoreceptor cell.

(a) A *Drosophila* photoreceptor cell showing various subcellular structures. The subrhabdomeric cisternae (SRC) is often connected to the rough endoplasmic reticulum by tubules. It is believed that integral membrane proteins such as rhodopsin pass through the SRC en-route to the rhabdomeric membranes.

(b) An expanded view of the interface between the SRC and the rhabdomeric membranes. The rdgB protein is situated within the SRC membrane with the PITP and acidic (calcium-binding) domains within the cytoplasm. The PITP and acidic domains are at these positions to respond to visual transduction triggers such as intracellular calcium levels. This subcellular localisation suggests that the rdgB protein regulates membrane transport from the SRC to replenish rhabdomeric proteins and phospholipids.

PEDF was localised to the short arm of chromosome 17 by fluorescent in situ hybridisation (FISH) analysis (Tombran-Tink et al., 1994). The gene is conserved between higher vertebrate species, is expressed in almost all human cell types and belongs to the serpine gene family (Steele et al., 1993). The serpines are a group of serine protease inhibitors. The PEDF reactive site, however, has a low sequence homology to the reactive sites of other proteases. It is therefore not thought to behave as a classical antiprotease.

The *PEDF* gene consists of eight exons which are transcribed into a 1.5Kb long mRNA sequence. The mRNA, in turn, is translated into a 50kDa protein of which several isoforms exist. The presence of these isoforms indicate, that different post-translational modifications take place within different cell types. Furthermore, the various isoforms could be associated with the different proposed functions of the PEDF protein (Tombran-Tink et al., 1995).

The proposed wide range of functions of PEDF in retinal cells, together with the location of the coding sequence on chromosome 17p, renders this a good candidate for the disorder segregating in the ADRP-SA1 family. It may be postulated that a disruption of the RPE-NR relationship could cause photoreceptor degeneration. *PEDF* was therefore screened intensively for potential pathogenic mutations in ADRP-SA1.

β-arrestin 2 (ARRB2): β-adrenergic receptor kinase (βARK) and its cofactor β-arrestin play an important role in determining homologous desensitisation of G-coupled receptors (Calabrese et al., 1994). β-arrestin quenches phosphorylated β-2-adrenergic receptors.

Both βARK and β-arrestin are members of multigene families. To date, three members belonging to the arrestin/β-arrestin gene family have been identified: arrestin (*SAG*), β-arrestin 1 (*ARRB1*) and β-arrestin 2 (*ARRB2*) (Wilson and Applebury, 1993). The gene, *ARRB2*, has been mapped to chromosome17p13 by fluorescent *in situ* hybridisation (Calabrese et al., 1994).

Although ARRB1 and ARRB2 are localised predominantly in neuronal tissues and in the spleen (Attramadal et al., 1992), they have also been shown to be expressed in a wide range of tissues (Calabrese et al., 1994). However, studies have shown that the β-arrestins act in concert with βARK to regulate G protein-coupled neurotransmitter receptors (Attramadal et al., 1992), of which rhodopsin is a member.

The genomic structure of *ARRB2* has not yet been elucidated, however, it encodes a cDNA sequence of approximately 1.2kb (GenBank AF106941). The proposed function of ARRB2 and the localisation of its coding sequence to the short arm of chromosome 17 validated the investigation of the gene as a candidate for the ADRP phenotype segregating in ADRP-SA1.

A comprehensive range of techniques was used to investigate the candidates described.

3.2. MATERIALS AND METHODS

The RP13 disease-causing gene was localised to chromosome 17p (Greenberg et al., 1994b). Various genes mapping to the same chromosomal region were investigated using a range of PCR-based techniques to test their candidacy for involvement in the RP13 phenotype in ADRP-SA1.

3.2.1. POLYMERASE CHAIN REACTION

Peripheral blood (10ml) was obtained from family members by venipuncture and genomic DNA was extracted using the Genomix™ blood scale-up kit [*Talent*, Italy] (Appendix A, section A.1).

PCR amplifications were performed in reactions containing 0.2μg of genomic DNA as templates; 50mM KCl, 10mM Tris-HCl pH8.3, 1.5mM $MgCl_2$, 200μM of dATP, dGTP, dTTP and dCTP; 1μCi of α-[^{32}P]dCTP (for amplification of microsatellite markers and omitted for single-stranded conformational polymorphism (SSCP) analysis); 10-20 pmol of each primer and 1 unit of *Taq* polymerase [*GibcoBRL*, UK], in a final volume of 25μl. Amplifications were carried out at 94°C for 3 min, followed by 30 cycles at 94°C for 30 seconds, 55°C to 60°C for 30 seconds, 72°C for 40 seconds and a final extension of 5 min at 72°C in an Omnigene [*Hybaid*, UK] thermal cycler machine.

PCRs to which radioactivity was incorporated, 20 µl of formamide loading buffer (Appendix B, section B.3) was added to each reaction after amplification. Samples were denatured for 3 min at 95°C and chilled on ice for 10 min, followed by electrophoresis through 6% denaturing polyacrylamide gels (Appendix B, section B.4). The gels were dried onto 3MM paper [*Schleicher and Schuell*, Germany] on a slab gel drier [*Hoefer Scientific Instruments*, USA] for 1-2 hrs at 80°C and exposed to X-ray [*Agfa*, Germany] for 16-32 hrs at –70°C. Non-radioactive products were used for SSCP analyses (section 3.2.3.).

3.2.2. LINKAGE ANALYSIS

Where intragenic microsatellite markers or SSCPs were available or identified, two-point linkage analyses were performed between the marker alleles and the RP13 disease locus. In order to produce a composite genetic map of flanking markers and the candidate genes, multipoint linkage analyses were performed between the tested intragenic and flanking markers using the LINKAGE software package Ver.5.03 (Lathrop and Lalouel, 1984). To conform to the number of alleles accommodated by the software, the number of alleles for each marker was reduced to five, ensuring no loss of information in the family. Lod scores between the markers and the ADRP locus were calculated using recombination fractions from 0.00 to 0.25 and the RP gene was assumed to be completely penetrant. A gene frequency of 0.001 was assumed for the disease locus.

3.2.3. INVESTIGATION OF *RCV1*

3.2.3.1. SSCP Screening

DNA from members of ADRP-SA1 was screened for a pathogenic mutation in the three exons of *RCV1* by SSCP analysis (Appendix A, section A.5). Genomic DNA was extracted from blood samples and the exons and flanking sequences amplified using oligonucleotide primers received from Dr Carol Freund (Baltimore, USA) (table 3.1.). Exon 1 was investigated using two overlapping PCR fragments because of its large size and to accommodate the size limitation for SSCP analysis of 200-300bp (Grompe, 1993).

SSCP loading buffer (Appendix B, section B.5) was added to each PCR product in a 1:1 ratio. The samples were denatured for 2 min at 95°C and then chilled on ice for 5 minutes. Ten µl of the denatured sample was electrophoresed through a non-denaturing 0.5% MDE™ gel matrix [*FMC BioProducts*, USA] at 5-12W for 16-18 hrs followed by silver staining to visualise the DNA banding patterns [Lohmann et al., 1992] (Appendix A, section A.5.3).

Table 3.1. Primer sequences used to amplify the three *RCV1* exons.

Exon	PCR Primers 5'-3'	Product Size (bp)	Annealing Temp. (°C)
1a	gctcacaccagccttgagc ccgcggtggtcatgtgcag	335	56
1b	catcacccagcagcagttc gcagctgcagcaggggac	293	56
2	tgagtagatttttgtgtacagtg gcagctgcagcaggggac	262	50
3	gatacattcacactgggatac tcacgggtgtcgtgtgagtg	252	56

3.2.3.2. Microsatellite PCR

The microsatellite marker *D17S945* was reported to be located within the 3' untranslated region of *RCV1* (Wiechmann et al., 1994b). To ascertain whether *RCV1* segregates with the ADRP disease phenotype, DNA from family members of ADRP-SA1 were genotyped with this intragenic marker. Two point lod scores were calculated as described (section 3.2.2.).

In order to refine the genetic localisation of *RCV1*, DNA from family members was genotyped with an additional 12 genetically ordered microsatellite markers spanning the RP13 chromosomal region. The order of the markers on the chromosome 17p genetic map was reported to be: tel- *D17S926 – D17S849 – D17S1529 – D17S1528 – D17S831 – D17S829 – D17S1353 – D17S938 – D17S796 – D17S960 – D17S786 – D17S804-* cen (Gyapay et al., 1994). A series of two point lod scores were calculated between each of the 12 microsatellite markers and the *RCV1* intragenic marker, *D17S945.*

3.2.4. INVESTIGATION OF *RETGC-1*

3.2.4.1. Direct PCR Sequencing

A 675bp genomic DNA fragment isolated during the initial characterisation of *RETGC*-1 (Oliveira et al., 1994), contained two complete and two partial exons of the gene. This candidate genomic fragment was assessed for DNA alterations between affected and unaffected members of ADRP-SA1 using direct PCR sequencing analysis. Oligonucleotide primer sequences (F -

5'cggtcaacaccgcctcgcgcatg3' and R - 5'cgggtgggatctcctgcaggctg3') used to amplify the fragment were the generous gift of Dr Steven Pittler (Alabama, USA). PCR fragments were electrophoresed through 2% agarose [Sigma, USA] gels, the bands were excised and purified using the QIAquick protocol and sequenced, using the Sequenase version 2.0 DNA sequencing kit (Appendix A, section A.6) as recommended by the manufacturer [USB, USA]. Sequencing reaction products were analysed on 6% PAGE gels and visualised by autoradiography. Fragments were compared between unaffected and affected individuals.

Investigation of *RETGC-1* using the direct PCR sequencing approach was extended to the entire coding sequence of the gene. In order to facilitate this analysis three pairs of oligonucleotide primers were designed from the cDNA sequence of the gene (GC1, GC2 and GC3) (table 3.2).

Peripheral blood (10ml) was obtained from affected and unaffected family members by venipuncture and the lymphocytes, separated by centrifugation, were then transformed with Epstein-Bar virus (EBV). Total RNA was extracted from the transformed lymphoblasts (Appendix A, section A.2). The RNA was reverse transcribed using RT PCR (Appendix A, section A.2.1) followed by amplification of the overlapping *RETGC-1* cDNA fragments. The fragments were directly sequenced in order to screen for potentially pathogenic mutations.

Table 3.2. Primer sequences used to amplify and screen *RETGC-1*.

Fragment	PCR Primers 5'-3'	Product Size (bp)	Annealing Temp. (°C)
GC1	gccctgtgactcctcacc acacccatctgccacctg	1178	55
GC2	ctccccactctttggcac ccctctgcaccacttcct	1247	55
GC3	gcaagaagtgtgccgcag atgggacagggagcaggg	1194	56

3.2.5. INVESTIGATION OF *PITPN*

3.2.5.1. Microsatellite Analysis

As a first step towards considering the possible involvement of *PITPN* in the ADRP-SA1 disease phenotype, DNA from affected and unaffected subjects in the family was genotyped with the marker *D17S5*, at the *PITPN* gene locus (personal communication with Dr David Hunt, London, UK). PCRs were carried out with the incorporation of radioactively labelled dCTP (as described in section 3.2.1) and two-point lod scores were calculated from the resultant genotypes on autoradiographs (section 3.2.2).

3.2.5.2. Restriction Fragment Length Polymorphism (RFLP) Analysis

The genomic DNA from the ADRP-SA1 family was tested for significant rearrangements within the *PITPN* gene. Genomic DNA from a subset of affected and unaffected individuals was restricted with a battery of nine restriction endonuclease enzymes (*AluI, BglII, Csp45I, DraI, HaeIII, MspI, TaqI, XhoI and PvuII*). The digestion products were transferred onto

hybond N^+ nitrocellulose filters (*Amersham,* South Africa) using the conventional Southern blot method (Appendix A, section A.4.1). The filters were probed with a *PITPN* cDNA PCR probe which was amplified using a pair of primers (PITPN-F/PITPN-R) (table 3.3.) designed to amplify the 1.5kb cDNA fragment. Southern blot membranes were prepared, according to protocols in Appendix A, section A.4 (Vandenplas et al., 1984). The membranes were hybridised overnight at 65°C with the [α^{32}P]dCTP-labelled probe (Appendix A, section A.4.2), 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, overnight.

3.2.5.3. Direct PCR Sequencing

In order to screen for pathogenic mutations in *PITPN*, oligonucleotide primers (PITPN-F/PITPN-R) were designed to amplify the 1.5kb cDNA sequence (table 3.3). The resulting PCR fragment was sequenced directly, from both ends. In order to expedite the analysis, additional nested primers (table 3.3.) were used to amplify the gene (Lotery et al., 1996). These facilitated the amplification of smaller fragments within the 1.5kb fragment for sequence analysis.

Total RNA was extracted from Epstein-Barr virus-transformed lymphoblasts of an affected and an unaffected member from ADRP-SA1 (Appendix A, section A.2). The RNA was reverse transcribed and fragments of the *PITPN* cDNA were amplified by PCR using nested primers. The amplified fragments were sequenced in order to screen for potentially pathogenic mutations (section 3.2.4.1.).

Table 3.3. Oligonucleotide primers used to amplify and sequence *PITPN* cDNA.

Oligonucleotide Primers	Product Size (bp)	Annealing Temp. (°C)
PITPN-F 5'-agcagagcacgacgaagac-3' PITPN-R 5'-ggggaacacacagaaatgg-3'	1500	55
PITPN-F 5'-agcagagcacgacgaagac-3' PITPN5-R 5'-gcaggtggtagatcttgtgtgtgtac-3'	300	55
PITPN4-F 5'-cgttattacaaatgagtacatgaaagaag-3' PITPN-R 5'-ggggaacacacagaaatgg-3'	840	55
PITPN2-F 5'-tgaccgtcaagttcaagtggtgggg-3' PITPN-R 5'-ggggaacacacagaaatgg-3'	540	55

3.2.5.4. Characterisation of The Intron-Exon Boundaries of *PITPN* for SSCP Analysis

Towards characterising the genomic structure of *PITPN* for further mutation analysis of the intron/exon boundaries, sequence comparisons were performed between the cDNA gene sequence (M73704) and data emerging from the high through-put genome sequence (htgs) database, using the BLAST function at the National Institute of Biotechnology Information (NCBI) world wide web (www) site.

3.2.6. INVESTIGATION OF *PEDF*

3.2.6.1. RFLP Analysis

A *Rsa*I RFLP in *PEDF* had previously been reported with a 3.5kb polymorphic band, and three additional constant bands at 1.7kb, 1.3kb, and 1.0kb (Tombran-Tink et al., 1994). DNA from subjects within the ADRP-SA1 family was tested for the *RsaI* RFLP using the

conventional Southern blot method and radioactively labelled probe hybridisation (Appendix A, section A.4). The 1.5kb cDNA PCR product, amplified using oligonucleotide primers P1/499 (5'-cctcagtgtgcaggcttagag-3' and 5'-ttgtatgcattgaaaccttacagg-3'), was used to probe the filters. The variant 3.5kb allele was seen in several individuals thereby facilitating analysis of the pattern of allele segregation within the family.

In order to test for any significant rearrangements within the *PEDF* gene, genomic DNA from a subset of affected and unaffected ADRP-SA1 individuals was restricted with a battery of nine restriction endonuclease enzymes (*AluI, BglII, Csp45I, DraI, HaeIII, MspI, TaqI, XhoI and PvuII*). The digestion products were transferred onto Hybond N^+ nitrocellulose filters (*Amersham*, South Africa) using the conventional Southern blot method. The radioactively labelled 1.5kb cDNA PCR product, was used to probe the filters (as described in section 3.2.5.2).

3.2.6.2. SSCP Analysis

The eight exons of *PEDF* were screened for the pathogenic alteration segregating in ADRP-SA1 using SSCP analysis (section 3.2.3.1). Eight pairs of oligonucleotide primers (table 3.4.) received from Drs Gerald Chader and Joyce Tombran-Tink (Washington, USA), were used to amplify the exons of the gene. The resulting PCR fragments included the intron-exon boundaries. In the initial screen of the PCR fragments, a section of ADRP-SA1 was investigated for altered SSCP banding patterns. Where SSCP alterations were observed, the investigation was extended to include all available family members. PCR fragments

exhibiting alterations were sequenced directly (as described in section 3.2.4.1.) in order to establish the precise DNA sequence change underlying the SSCP alteration.

In order to assess whether DNA sequence alterations effected changes at the protein level, nucleotide database comparisons were performed against all available non-redundant protein data sets at the NCBI using BLAST (Altschul et al., 1990).

Two point lod scores were calculated, as described in section 3.2.2. between the ADRP locus and the SSCP alterations observed in *PEDF*. The putative promoter region was screened for a pathogenic mutation using SSCP analysis. Five pairs of oligonucleotide primers were designed to produce overlapping PCR fragments spanning the 1.0kb length of the *PEDF* promoter region (table 3.5.).

3.2.6.3. Refining The *PEDF* Map Position

A series of two- and multipoint linkage analyses were performed towards fine mapping *PEDF* using the SSCP polymorphism in exon 4 as a fixed marker for the gene. The genetic order of the microsatellite markers in the region, as published by Genethon (Gyapay et al., 1994) and confirmed during this investigation (chapter 2), are as follows: cen – *D17S829* – ***D17S831*** – *D17S1528* – ***D17S1529*** - *D17S849* - *D17S926* - tel. The RP13 critical interval as defined by multipoint genetic linkage analyses (chapter 2) spans a 3cM interval between markers, *D17S1529* and *D17S831*.

Table 3.4. Oligonucleotide primer pairs used to amplify the exons of *PEDF*.

Exon	Primer sequences 5'-3'	Product size (bp)	Annealing Temp. (°C)
1	atcacagaatccaaattgagtgcag tcttttctgctccctggagtgcccc	277	65
2	gaaggggtagcggggcagtgcagt gttccctgtcctgtttcccgtgctg	237	62
3	cagagtccctgtgcatctctgcgag tcagccacgtttacgcagaggccac	336	70
4	tgagtatagtgtctgtgttctggga aagaccccccccagcctgcagcatgg	310	62
5	gctctcaaagacgggatgcttgctg agggctacagagtaagactccatcg	354	68
6	cagcatggcgccactgtctttctgg taccctgttttgcttctctatcctc	273	65
7	ggaaggcagctcctggctgtgtctg cacagtggaaggcccaaagaccctg	362	70
8	cccttggttggggtgttggggaagg tgtcctgtggaatctgctgtccctc	386	68

Table 3.5. Oligonucleotide primers used to amplify the promoter region of *PEDF*.

Fragment	PCR primers 5'-3'	Product size (bp)	Annealing Temp (°C)
T1	ggatcacctgaggtcagga ttaaaccttaggtcaatgt	220	55
T2	agagtgagtctccactggaa cgtcaccataaccaccatct	158	55
T3	atggtgacgggaaagaactg ggagtctgaggcaggagaat	224	55
T4	ctgcctcagactcccaagta aagaccagcctggccaa	247	55
T5	aggctggtcttgaactcctga ctctaagcctgcacactgag	332	55

Multipoint linkage analyses were carried out where the SSCP in *PEDF* exon 4 was moved up to and between the 3 fixed markers *D17S849*, *D17S1529* and *D17S831*. A θ value of 0.5 was used as the starting point away from *D17S849* and then 4 equal increment values were used to move in on the fixed marker loci.

3.2.6.4. PCR Sequencing

Considering the fact that SSCP analysis has a sensitivity of 80-85% (Grompe, 1993), it is conceivable that the technique had failed to resolve a potential disease-causing DNA aberration in ADRP-SA1. In light of this reservation together with the genetic fine mapping of *PEDF*, which deemed the gene an excellent candidate at the RP13 locus, the entire cDNA was sequenced directly, as previously described. The *PEDF* cDNA was sequenced using two pairs of nested oligonucleotide PCR primers (table 3.6).

Total RNA was extracted from Epstein-Barr virus-transformed lymphoblasts of both an affected and an unaffected member from ADRP-SA1, as previously described in section 3.2.4.1. The RNA was reverse transcribed with a reverse transcriptase and *PEDF* cDNA was amplified by PCR and directly sequenced in order to screen for potentially pathogenic mutations.

The intron-exon boundaries of the *PEDF* were also sequenced from the eight genomic DNA fragments amplified during the SSCP screening of the exons (table 3.4.).

Table 3.6. Oligonucleotide primers used to analyse the cDNA sequence of *PEDF*.

Oligonucleotide Primers 5'-3'	Product size (bp)	Annealing Temp. (°C)
cctcagtgtgcaggcttagag ttgtatgcattgaaaccttacagg	1500	58
ctgggagcggacgagcgaac tggggacagtgaggaccgcc	667	55

3.2.7. INVESTIGATION OF *ARRB2*

3.2.7.1. Sequencing of *ARRB2* cDNA

In order to screen for pathogenic mutations in *ARRB2*, oligonucleotide primers were designed to amplify and sequence the 1160bp of cDNA. The primers used were ARRB2-F 5'tcttcaagaagtcgagcc 3' and ARRB2-R 5'agtcgtcatccttcatcc 3'.

Total RNA was extracted from Epstein-Barr virus-transformed lymphoblasts of both an affected and an unaffected member of ADRP-SA1 (Appendix A, section A.2). The RNA was reverse transcribed and *ARRB2* cDNA was amplified by PCR and directly sequenced in order to screen for potentially pathogenic mutations (section 3.2.4.1.).

3.3. RESULTS

3.3.1. ANALYSIS OF *RCV1*

SSCP analysis: SSCP analysis of the four PCR-amplified fragments used to screen the exonic regions of *RCV1* revealed no altered DNA banding patterns between affected and unaffected subjects within a nuclear section of the ADRP-SA1 family.

Microsatellite analysis: To further investigate *RCV1* as the possible causal gene, DNA from members of the ADRP-SA1 family was genotyped with the intragenic microsatellite marker, *D17S945*. Two-point linkage analysis between the marker and the ADRP-SA1 disease phenotype produced a maximum lod score of 1.64 at $\theta = 0.10$ (table 3.7.). This result excludes a 10cM genetic distance on either side of *RCV1*.

Table 3.7. Pair-wise lod scores between *D17S945* and the ADRP-SA1 phenotype.

	Recombination fraction (θ)					
	0	**0.05**	**0.1**	**0.2**	**0.3**	**0.4**
D17S945	-α	1.43	1.64	1.42	0.85	0.24

In order to fine map *RCV1* and facilitate more accurate future investigations at the *RCV1* locus, a series of two-point linkage analyses was performed between *D17S945* and 12 genetically ordered DNA markers spanning a region of 22cM containing the RP13 locus. The

results obtained (table 3.8.) indicated that *RCV1* was most closely linked to the markers *D17S796* and *D17S938*. The findings placed *RCV1* 9 cM centromeric to the RP13 critical interval. These results confirmed and substantiated the exclusion of *RCV1* from being causative of the ADRP-SA1 disease phenotype.

Table 3.8. Pair-wise lod scores between *D17S945* at the *RCV1* gene locus and chromosome 17p microsatellite markers.

Recombination fraction (θ)

D17S945 *vs*	**0**	**0.05**	**0.1**	**0.2**	**0.3**	**0.4**
D17S926	-α	-3.21	-1.48	-0.26	0.02	0.02
D17S849	-α	-0.50	0.32	0.66	0.47	0.17
D17S1529	-α	1.14	1.48	1.41	0.91	0.31
D17S1528	-α	0.92	1.34	1.19	0.63	0.13
D17S831	-α	-0.29	0.63	0.88	0.51	0.11
D17S829	-α	1.24	1.39	1.11	0.61	0.17
D17S1353	3.22	3.28	2.98	2.08	1.07	0.28
D17S938	5.04	5.05	4.69	3.53	2.08	0.66
D17S796	4.97	4.52	3.98	2.80	1.63	0.57
D17S960	2.28	2.25	2.09	1.53	0.80	0.18
D17S786	4.75	4.27	3.74	2.60	1.46	0.49
D17S804	3.02	2.81	2.54	1.87	1.11	0.40

3.3.2. ANALYSIS OF *RETGC-1*

PCR sequencing: A 675bp genomic PCR fragment (isolated during the initial characterisation of *RETGC-1*), was sequenced in order to screen for the pathogenic alteration in the DNA of affected family members. Sequence analysis of the fragment revealed no sequence variations between affected and unaffected individuals from the ADRP-SA1 family.

As no intragenic variants were detected with which to track the gene in the family, the entire coding sequence of the gene was sequenced (after subsequent characterisation of the cDNA) using overlapping PCR fragments. No sequence variations were detected between the DNA of affected and unaffected subjects in the ADRP-SA1 family.

3.3.3. ANALYSIS OF *PITPN*

***Microsatellite analysis*:** In order to test *PITPN* for linkage to the ADRP-SA1 phenotype, DNA from family members was genotyped with the marker *D17S5* which is closely linked to the gene. While the disorder tracked consistently with one of the marker alleles, most of the meioses were uninformative. Two-point linkage analysis between the marker and the disease phenotype produced a very low positive lod score of 0.9 at θ = 0.00. The gene could not be excluded on this basis.

***Southern blot analysis*:** In the absence of other known tightly linked markers, the *PITPN* gene sequence was tested for gross rearrangements or polymorphisms in the DNA of affected individuals using Southern blotting and hybridisation to a labelled probe. The banding patterns observed using the nine restriction endonuclease enzymes listed in section 3.2.5.2 were consistent between affected and unaffected individuals. The results indicated that there were no major detectable genomic rearrangements in the *PITPN* gene of affected subjects in the family.

***PCR sequencing*:** As *PITPN* could not be excluded as the ADRP-SA1 disease gene, the entire 1.5kb cDNA was sequenced. A C → T substitution at position 1127 of the cDNA sequence in an unaffected unrelated control individual was observed using oligonucleotide primers listed in table 3.3. The sequence change resulted in the creation of a *PvuII* restriction enzyme site. The change was not detected in the DNA from family members on restriction enzyme analysis. No other sequence variations were observed between affected and unaffected subjects.

The genomic structure of PITPN and SSCP analysis: The genomic structure of *PITPN* was elucidated by performing database comparisons. The *PITPN* cDNA sequence (M3704) was found to be homologous to a large genomic sequence file (accession number AC006405). The AC006405 sequence of 164kb contained almost all of the *PITPN* cDNA sequence, except for the first 236bp at the 5' end and 68bp at the 3' end of the sequence. The genomic sequence file is incomplete and this would therefore account for the gaps in the sequence. Eight exons were distinguishable (table 3.9.). Following the identification of 8 intron/exon boundaries, mutation analysis was performed by SSCP analysis. Oligonucleotide primers derived from the intronic sequences flanking the exonic fragments were designed to amplify the exons of the gene (table 3.10.). No disease-causing mutations were identified in this analysis.

Table 3.9. The intron-exon boundaries of *PITPN*.

Exon	5' Boundary	3' Boundary	Size (bp)
1	caactatga**gagaaaa**	**acatctactc**cattcag	50
2	gacaggaaa**tcatagt**	**ggacgtctc**catacgcc	145
3	ggggcgata**tcgtttca**	**cgtcttggc**cactcacg	93
4	aagaaggtc**ttactcat**	**ctcttacac**tcatcatc	84
5	aagggtgtc**cacgtatt**	**agtcgttcc**atcctagg	83
6	taaagagag**tcctaat**	**ggttaacct**tccatggt	78
7	aaagattac**gtcgttct**	**gtaggtatt**ccattcatc	112
8	ggaaagata**tcgttctc**	**gacctactt**cattcacc	124

The bases in bold represent the 5' and 3' ends of each exon.

Table 3.10. Oligonucleotide primers designed to amplify the *PITPN* exons from genomic DNA.

Exon	Primer Sequences 5'-3'	Product Size (bp)	Annealing Temp. (°C)
1	actcgtctaacgtgtcggat gtcgaatcggttaccagtgt	145	55
2	ctcttcccaggcacaactct gtgtgtgttttcggtcgtag	235	55
3	gggtacttcttcgacctaga gaccggattaggccttgtg	261	55
4	ggtcattacgggtagaggtc gaccccgaaaacccttgatg	189	55
5	ccttacgctcaaggatccac ttccggtgggtgcagaaat	204	55
6-7	gttggggttaaggtgttaaa gttgtgtgtcgtcagtg	412	55
8	ggaaacggaagacgcaagat agattaactctcgggggtct	198	55

3.3.4. ANALYSIS OF *PEDF*

RFLP analysis: The *RsaI* RFLP within *PEDF* was genotyped in the ADRP-SA1 family to test for linkage as described in section 3.2.6.1. On visual examination of the resulting allele segregation pattern, no recombinant meioses were observed, however, the genotypes were mostly uninformative. It was therefore not possible to conclude whether an association between the disease phenotype and the gene existed. As linkage results did not conform to the exclusion criteria for linkage analysis, the genomic DNA of affected individuals was tested for any significant rearrangement within *PEDF* using a battery of restriction endonuclease enzymes (section 3.2.6.1). No gross rearrangements of banding patterns were observed between affected and unaffected individuals.

SSCP analysis: The exons and intron/exon boundaries of the *PEDF* gene were screened for SSCP alterations in the DNA of affected and unaffected members of ADRP-SA1. SSCP variants were detected in four of the eight PCR fragments analysed (table 3.11.). SSCP analysis of the extended ADRP-SA1 family proved that each of the four fragments segregated in a Mendelian fashion with no observed recombinant events. Direct PCR sequence analysis of the variant fragments revealed two base substitutions in exon 4 and one each in exons 1, 3 and 7 (table 3.11.).

Table 3.11. Base substitutions identified in *PEDF* on SSCP analysis of the exons.

Exon	Codon	Substitution
1	16 bases into intron 1	g → a
3	111	tga → tca
4	132	ccc → cgc
4	130	act → acc
7	321	tac → tat

Database searches suggested that while the substitutions in exons 3, 4 and 7 reside in the coding region of the tested PCR fragments, they do not incur amino acid changes at the peptide level. These findings indicated that the changes in these exons are not disease-causing, but are non-deleterious polymorphisms. The base substitution underlying the SSCP change observed in the exon 1 PCR fragment proved to be located 16 bases into intron 1. In order to ascertain whether this substitution was disease-causing, 56 unaffected unrelated control chromosomes were tested for its presence using SSCP analysis. The intron 1 base change was identified in 15 of the chromosomes tested (figure 3.2.).

The SSCP polymorphisms identified were further analysed to ascertain their usefulness in testing for linkage between the ADRP phenotype and *PEDF*. Visual examination of the allele segregation of the SSCPs in *PEDF* within ADRP-SA1 showed that the exons 1 and 4 changes were the

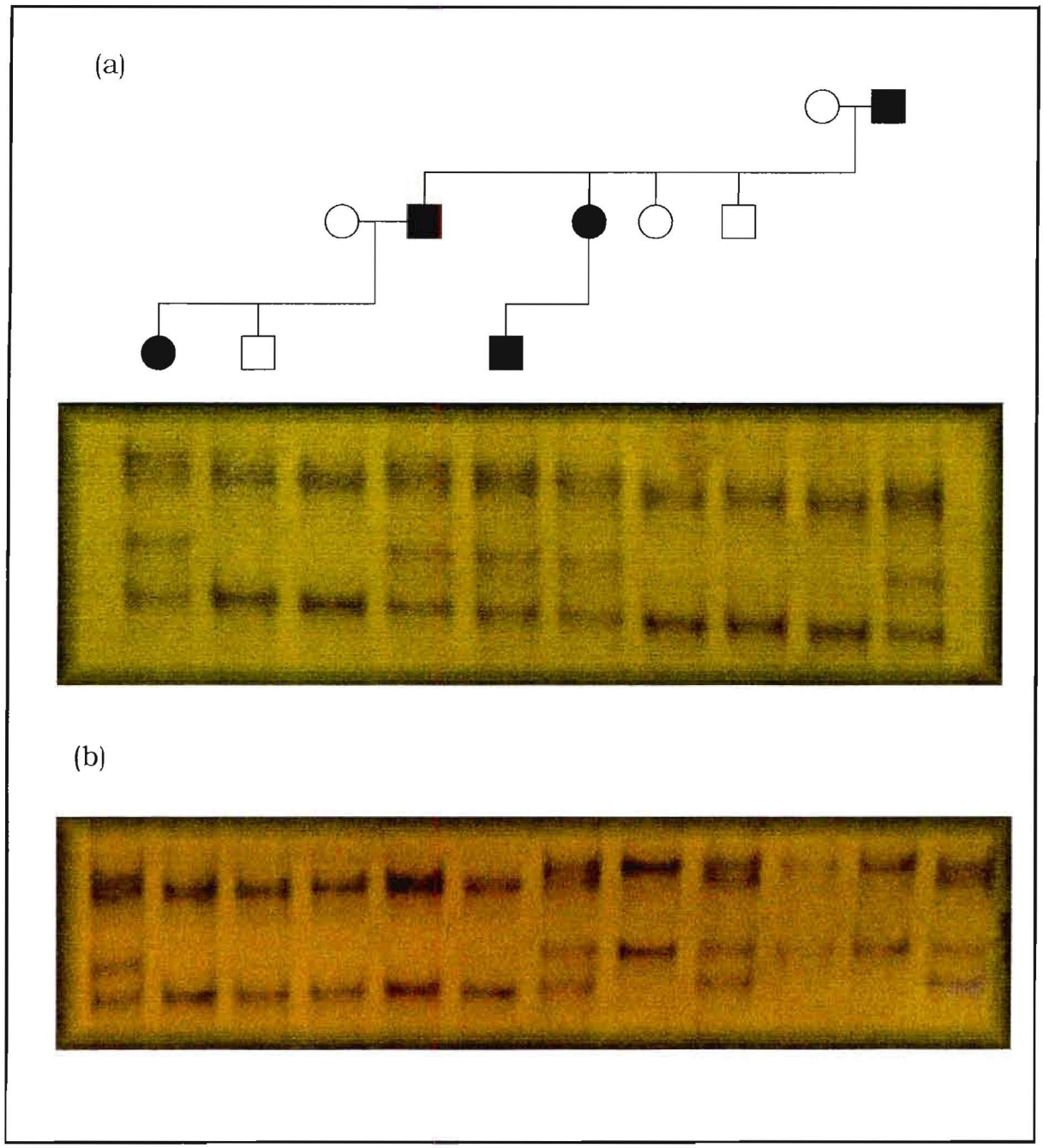


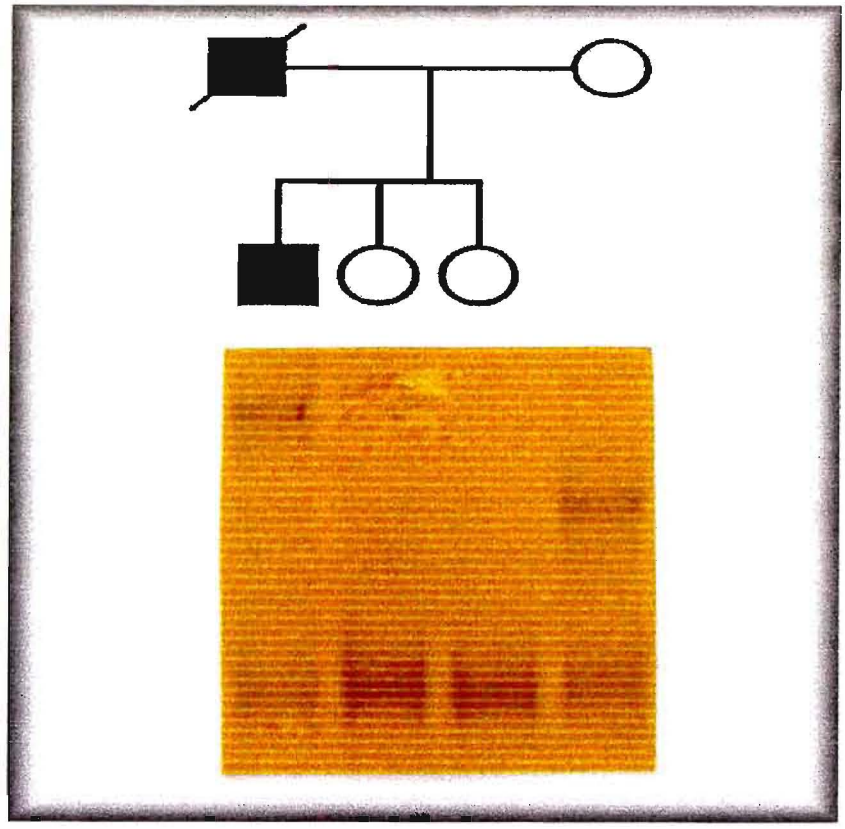
Figure 3.2. Silver stained SSCP gels showing (a) the Mendelian inheritance of the *PEDF* exon 1 polymorphism in a small branch of the ADRP-SA1 family and (b) the same change in unaffected unrelated control individuals.

most polymorphic and exhibited no recombinant meioses. Interestingly, the recombinant meioses that defined the critical genetic interval harboring the ADRP-SA1 disease gene (in individual VI-1, chapter 2, figure 2.5.), was informative and not recombinant for the exon 1 polymorphism. Two-point linkage analysis between the disease locus and the polymorphisms in exon 1 and 4 produced maximum lod scores of 4 and 7.1 respectively, at a $\theta = 0.00$. These findings strongly suggested that the *PEDF* gene lies within the RP13 genetic interval.

SSCP analysis of the five overlapping fragments spanning the promoter region of the gene did not show any altered banding patterns.

***Genetic mapping of PEDF*:** To confirm and substantiate preliminary results which suggested that *PEDF* lies within the RP13 critical interval, the gene was fine mapped with respect to previously ordered markers in the region. Two-point linkage analyses between the SSCP polymorphism in exon 4 of *PEDF* and the markers, *D17S1529, D17S1528* and *D17S831*, produced maximum lod scores of 6.53, 6.26 and 5.36, respectively (each at $\theta = 0.00$). The pooled results obtained from a series of multipoint linkage analyses are presented in figure 3.3. *PEDF* was estimated to be closest to marker *D17S1529*, with a maximum lod score of 6.71 (Goliath et al., 1996) within the RP13 critical interval.

(a)



(b)

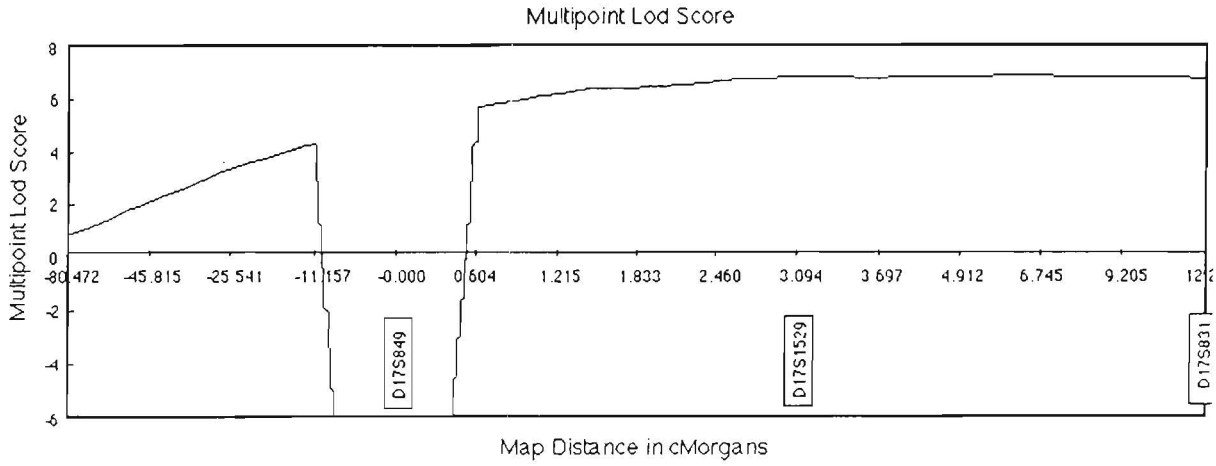


Figure 3.3. (a) Mendelian pattern of inheritance of the *PEDF* exon 4 SSCP polymorphism. (b) Multipoint map illustrating the pooled multipoint linkage results using the exon 4 polymorphism to mark the *PEDF* gene.

***Sequence Analysis*:** In order to exclude *PEDF* from the disease phenotype with a high level of confidence, the entire cDNA sequence and intron/exon boundaries were sequenced directly. Except for the polymorphic sequence variations identified during SSCP analysis of the exons (which were amplified from genomic DNA), no additional variations were observed. Interestingly, affected individual IV-17 was consistently heterozygous for the polymorphism in exon 4 (as revealed on genomic DNA as well as cDNA sequence analysis) (figure 3.4). This finding confirms that both alleles were sequenced during the cDNA sequence analysis and reflects expression of both alleles.

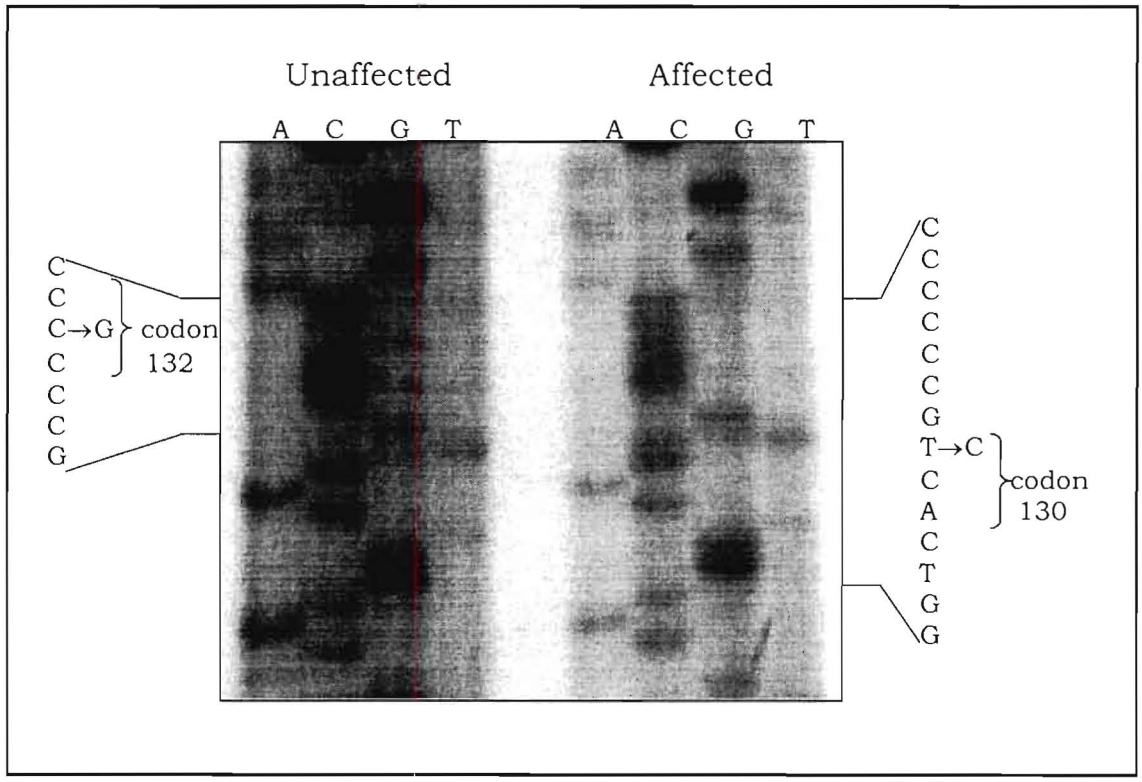


Figure 3.4. Diagram showing heterozygosity in the cDNA sequence of affected individual IV-17 around the *PEDF* exon 4 polymorphism.

3.3.5. ANALYSIS OF *ARRB2*

PCR sequencing: Since the intron-exon boundaries of *ARRB2* have yet to be established, the cDNA was sequenced in an ADRP patient and an unaffected family member from ADRP-SA1 in order to screen for pathogenic mutations. In total, 800bp of the 1160bp cDNA was sequenced but no pathogenic sequence variations were detected.

During the sequencing of the *ARRB2* cDNA, according to a then newly published radiation hybrid map of 17p13.3, the gene was shown to map between the microsatellite markers *D17S1828* and *D17S796*. This interval lies 4cM proximal to the refined RP13 locus, thus excluding *ARRB2* from being the gene which underlies the disease phenotype in ADRP-SA1.

3.4. DISCUSSION

Recently, substantial progress has been made in determining the genetic basis of monogenic retinal disorders. Mutations are identified on a regular basis in new genes responsible for some form of retinal degeneration. To date, in the ADRP form of human RDs alone, 12 genes have been localised to various chromosomal regions (chapter 1). Molecular genetic studies have identified disease-causing mutations associated with ADRP in 6 genes, while the remaining 6 genes which have been mapped, have yet to be identified (http:www.sph.uth.tmc.edu/RetNet/). The first two genes which were identified encode the membrane proteins, *RHO* and *peripherin/RDS* (McWilliam et al., 1989, Farrar et al., 1991). RHO initiates the phototransduction cascade (Yau, 1994) and peripherin/RDS plays an important role in maintaining the structural integrity of the photoreceptor discs (Kemp et al., 1992). More recently, an additional four genes were shown to be associated with ADRP. These include, (i) the transcription factor gene, *NRL* (14q), which is involved in the regulation of photoreceptor genes (Bessant et al., 1999), (ii) the gene underlying the RP1 phenotype (*RP1*) at the chromosome 8q locus (Sullivan et al., 1999), a retina-specific gene which regulates *RHO* expression, (iii) *RETGC-1* on chromosome 17p which was shown to underlie a dominant cone-rod dystrophy, which is a common variant of RP (Perrault et al., 1998), and (iv) *CRX* (on 19q), which encodes a photoreceptor-specific transcription factor that regulates *RHO* expression (Clarke et al., 2000). The remaining six ADRP-associated loci

have only been mapped to date. Among the latter anonymous loci, is RP13, which resides in a 3cM genetic interval on chromosome 17p13.3 (described in chapter 2) (Goliath et al., 1995).

In an attempt to identify the disease-causing gene at the RP13 locus, the 17p13.3 encompassing chromosomal region was interrogated for functionally significant genes. Genes which were located on 17p13 included; *RCV1*, *RETGC-1*, *PITPN*, *PEDF* and *ARRB2*. Comprehensive investigation of the candidate genes suggest that aberrations in these were unlikely to be responsible for the ADRP phenotype in ADRP-SA1. It was worth noting, however, that the inability to detect a pathogenic alteration could be due to one of several reasons, e.g. the unsuccessful selection of the right candidate genes for the analysis, the methods employed, or both. The most recently mapped retinal gene chromosome 17p13, *AIPL1*, (Sohocki et al., 1999) was excluded as a possible candidate, because of its reported map position centromeric to the RP13 critical interval (Sohocki et al., 1999).

RCV1 was excluded with a high level of confidence based on the observation of two recombinant meioses between an intragenic marker (*D17S945*) and the disease phenotype. As both recombinants were observed in individuals affected by RP, phenotypic misclassification due to reduced penetrance could be rejected. Furthermore, additional linkage analyses facilitated the genetic mapping of *RCV1* to a chromosomal region centromeric to the RP13 critical interval. This work was a refinement on the initial localisation of the gene to chromosome

17p, which was done by *in situ* hybridisation analysis (Bayes et al., 1995). The genetic mapping of *RCV1* corroborated the negative linkage results. It is interesting to note, that despite its key role in the phototransduction pathway, RCV1 has not yet been implicated in a human hereditary retinal disease. A number of possible explanations exist. The *RCV1* gene could have a mutation rate so low that the form of RD due to a defect in this gene would occur very rarely. A second possibility is that RCV1 is functionally redundant, where another protein has the same function. If this is the case, then individuals with *RCV1* mutations would not manifest a recognisable disease phenotype.

Since *RETGC*-1 had not been fully characterised, direct sequence comparison analysis of the cDNA of an affected and unaffected member of ADRP-SA1 was carried out. Results did not reveal any pathogenic alterations. These findings suggested that *RETGC-1* was not the gene responsible for the disease phenotype in the family. Further evidence against *RETGC*-1 involvement was subsequently provided by a group investigating Leber's congenital amaurosis (LCA1), a form of retinal degeneration. The LCA1 gene was genetically fine mapped to a 1cM critical interval (9cM centromeric to the RP13 critical interval) between markers *D17S938* and *D17S1353* defined by three critical recombinant events (Camuzat et al., 1996). The identification of *RETGC-1* mutations associated with the LCA1 disease phenotype placed the gene into that same critical interval. Although unequivocal proof of exclusion was not found in the current study, the localisation data indicated a low probability that the ADRP-SA1 disease phenotype was due to a

mutation in *RETGC-1*.

As the *PITPN* gene sequence had not been fully characterised, the cDNA sequence of the gene was investigated using direct sequence analysis. No disease-causing variants in affected individuals from the ADRP-SA1 family were detected. A genomic clone (AC006405) identified to be homologous to the *PITPN* cDNA sequence facilitated the characterisation of eight intron-exon boundaries. SSCP analysis of each exon and intron-exon boundary revealed no pathogenic sequence changes in the DNA of affected members of the ADRP-SA1 family. Even though a *Drosophila* homologue exists, where mutations have been shown to underlie photoreceptor degeneration (Hotta and Benzer, 1970), no human disease association has been reported to date.

In the search for the pathogenic alteration segregating in ADRP-SA1, PCR-SSCP analysis of the eight exons of *PEDF* elucidated five polymorphic alterations. These variants facilitated allele segregation analysis in the family as well as the genetic fine mapping of *PEDF* with respect to genetic markers which had been ordered at the RP13 locus (Goliath et al., 1996). No recombinant events were observed between the disease and an SSCP in exon 4. The observation that the disease gene and *PEDF* were tightly linked, was confirmed with a series of multipoint analyses, which placed *PEDF* close to the telomeric RP13-flanking marker, *D17S1529*. Based on the compelling linkage data presented in this chapter, *PEDF* was the most promising of the candidate genes investigated. Nevertheless, despite intensive sequence based

investigation, a disease-associated mutation was not identified in the *PEDF* gene. The possibility that a pathogenic mutation may result in a null allele, was an obvious concern. cDNA analyses for mutations is limiting in such cases since only the non-mutated allele may be present in the emerging sequence data. This theory was rejected when an individual heterozygous for the SSCP in exon 4 of genomic DNA, was shown to remain heterozygous for the same polymorphism on cDNA sequence analysis (figure 3.4.). This finding indicated that the promoter region of both alleles was functional. It was concluded that although *PEDF* is a prime candidate for the RP13 locus, the molecular genetic techniques utilised were unable to reveal any disease-associated DNA alteration.

No mutation was detected upon direct sequencing of the *ARRB2* cDNA. Furthermore, radiation hybrid mapping data obtained at Genemap98 at the NCBI, subsequently placed *ARRB2* out of the RP13 critical interval. For these reasons the gene was not investigated any further.

Although the five candidate genes discussed in this chapter have been screened extensively using a combination of molecular genetic techniques, no pathogenic genetic changes were found. Each one of them, however, remains a candidate for the other forms of RD localised to chromosome 17p. *RETGC-1* already has been reported to underlie LCA1 (Perrault et al., 1996) and CORD6 (Kelsell et al., 1998; Gregory-Evans et al., 2000).

The findings presented in this chapter including:

- the genetic fine mapping of the genes *RCV1*, and *PEDF*;
- the elucidation of polymorphic variants within the *PEDF* and *PITPN* genes; and
- the characterisation of the intron-exon boundaries of *PITPN*.

These findings will assist in future investigations into disorders involving RD and RPE dysfunction linked to chromosome17p.

Methods for the identification of new candidate disease genes are being developed and should generate additional candidates at the RP13 locus. The first two ADRP genes identified *RHO* and *peripherin/RDS* were obvious candidates for retinal degeneration because of their function in the visual phototransduction cascade. It is now becoming clear though, that any gene that co-localises with a disease phenotype could be a candidate for that disorder. Researchers around the world are characterising genes from cDNA libraries from various tissues and mapping them to specific chromosomes using techniques such as FISH and RH mapping. In many instances the function of these genes are unknown. Hence the candidacy of these genes when localised to the same chromosomal region as a disease phenotype, cannot be ruled out. Hence, the assumption that only retinal specific genes will underlie the various forms of retinal degeneration has been discarded. Examples include the ubiquitously expressed *RPGR* gene which has been reported to be associated with XLRP (Meindl et al., 1996), and the *ABCR* gene causative of Stargardt macular dystrophy, which is expressed in rod cells while the disorder affects mainly cone cells (Allikmets et al., 1997b). To expedite the elucidation of disease-causing genes, several

strategies have been developed towards identifying potential candidate genes for inherited retinal disorders. A method based on the use of subtractive and differential hybridizations from RD mouse retina, has been used in the identification of several retinal disease genes (Bowes et al., 1989). When mapped onto mouse chromosomes, these genes become candidates for retinal disease mapping to syntenic human chromosomal regions. Examples of genes identified in this way include *cGMP-PDE* β (Bowes et al., 1990) which led to the identification of defects in the human homologue shown to underlie ARRP (McLaughlin et al., 1993), *peripherin/RDS* (Travis et al., 1989) and *TULP1* (Hagstrom et al., 1998). The most recent RD gene identified using this method was the RP1 gene (*RP1*) (Sullivan et al., 1999). Another advantage of comparing human-mouse syntenic regions is that the animal model allows for the functional investigation of the encoded protein.

In conclusion, 118 retinal disease loci have been identified and at least 55 of the genes at these loci with defects responsible for various forms of retinal disease involving photoreceptor loss have been identified (Clarke et al., 2000). Several of the genes were first considered as candidates for study because of their involvement in murine retinal disease, others because of their chromosomal location and other novel genes were characterised by positional cloning techniques. Based on reports of disease loci for which no gene has yet been found, more than twice as many genes remain to be identified in this genetically heterogeneous group of diseases. It has been predicted that in the future, the positional candidate gene approach will be the method of

choice in the search for disease-causing genes (Strachan and Read, 1996). Hence, in the search for the RP13 causal gene, further screening of positional candidates should be continued.

The fact that a mutation was not detected in the functionally significant genes investigated, led to the exploration of the physical map of the 17p13.3 chromosomal region and more detailed interrogation of the interval. Towards this end, and because there was no evidence of existing contigs, it was necessary to extend the project into physically mapping the region.

CHAPTER 4
PHYSICAL MAP SPANNING THE RP13 LOCUS

4.1. INTRODUCTION

Following the mapping of the RP13 locus to chromosome 17p13.3, 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 3cM critical interval containing RP13.

Genetic maps define markers along each chromosome, and hence are invaluable tools to the molecular biologist providing the means to localise and identify disease genes. The work presented in chapter two of this thesis relied upon genetic marker maps for linkage analyses, and chapter three on genomic gene maps. Genetic maps are constructed using statistical analyses based upon a particular data set. Gene maps are constructed using a combination of mapping techniques eg. FISH analyses for gross localisation as was done for the *PEDF* gene, prior to this study, (Tombran-Tink et al., 1994), and linkage analyses, as described for the *PEDF* gene and the RP13 locus in this study (Goliath et al., 1996). While these maps are invaluable in localising and fine mapping genes which cause disease, they are labile, in that the order of information on these maps is in flux. Physical maps which include clone and sequence contig maps, however, consist of a linked library of small overlapping clones or sequences representing a complete chromosomal segment. Information on the genetic and gene maps

available for chromosome 17p has been exhausted in the search for the disease gene in family ADRP-SA1. This chapter presents work carried out towards constructing a physical map at the RP13 locus with a combination of *in vitro* and *in silico* analyses.

In vitro analysis involves the construction of physical maps of genomic DNA fragments cloned into vectors that are maintained in yeast cells as artificial chromosomes (YACs) (Burke et al., 1987). One of the major advantages of these vectors is their ability to contain large segments of genomic DNA. The ordered fragments form contiguous DNA blocks called contigs. Significant time and effort has been invested in establishing genomic libraries that cover the entire genome in relatively small overlapping fragments. Currently, the resulting library of available human YAC libraries comprise of clones containing insert DNA which varies in size from 10,000 bp to 1 Mb. The physical clone contig covering a critical interval, is an important tool for the identification and cloning of genes causative of inherited disorders. There are some problems, however, that need to be considered when working with YAC clones. One disadvantage is that YACs (40-60%) may be chimaeric i.e. the clones may contain two or more fragments derived from non-contiguous segments of human insert DNA. A second problem is that some clones are unstable and rearrangements may result in the deletion of internal regions of the insert fragment (which could be counter productive in gene isolation experiments or in the construction of physical maps of specific chromosomal regions).

In order to overcome these problems, several alternative cloning vectors have been developed e.g. bacterial artificial chromosomes (BACs), P1-derived artificial chromosomes (PACs) and cosmids (containing a bacteriophage lambda cos site) which contain smaller insert sizes.

One of the goals of the Human Genome Project has been to determine the sequence of the 3 billion bases that make up the human genome, to store this information in databases and to develop tools for analysis of the data (http://www.ornl.gov/hgmis/project/about.html). Cloned human genomic fragments are sequenced in a shot-gun approach resulting in an array of sequences for each clone. The arrays are ordered on completion of the sequencing of the cloned fragment. The sequence information is then stored in electronic files and placed in databases. Each sequence file may not be complete and may consist of an array of unordered sequence fragments of a specific clone. The *in silico* analysis of the sequence files encompasses the field of bioinfomatics which involves the creation, development, and operation of databases and other computing tools to collect, organize, and interpret data. To date, a vast range of bioinformatics utilities have been developed to analyse and arrange the sequence files into ordered sequence contigs. The networked information of searchable databases of genetic data has become a powerful resource in the field of molecular genetics. Establishing the sequence of the entire genome in the form of sequence contigs using these available tools will be the ultimate form of physical map.

4.2. MATERIALS AND METHODS

4.2.1. INVESTIGATIONS *IN VITRO*

4.2.1.1. Detection of YACS by STS Screening of Pooled Libraries

In order to generate a physical map of the RP13 region, YAC libraries of the Imperial Chemical Industries (ICI), Imperial Cancer Research Fund (ICRF) and Centre d'Etude du Polymorphisme Humain (CEPH) were screened using standard PCR protocols with microsatellite markers around the critical interval namely *D17S1529*, *D17S1528*, *D17S831*, *D17S1798* and *D17S5* (chapter 2). The ICI and ICRF pooled YAC libraries were obtained through the HGMP Resource Centre (Hixton, UK), and the CEPH mega-YAC library from the Jean Dausset CEPH (Paris, France). PCR products were checked for size by comparison with a 1kb molecular weight marker [*Gibco*, UK] on 2% agarose [Sigma, USA] gels. The YAC clones which proved positive for one or more of the microsatellite markers were streaked on YEPD [*Gibco*, UK] plates (Appendix A, section A.7.1) with 1µl/ml ampicillin and incubated for 2 days at 30°C. Single colonies of each YAC were tested for their sequence tagged site (STS) content by performing colony PCRs in order to test for clonal rearrangement during cell division before YAC DNA extraction. Sterile toothpicks were used to pick the colonies, followed by inserting the toothpick into standard PCR reaction mixes for direct PCR. YAC DNA of positive colonies was extracted into solution for further manipulations, and into agarose plugs in order to maintain the integrity of the DNA (Appendix A, section A.7.2). The YACs were retested for their STS content by PCR amplification and product resolution on 2%

agarose gels.

4.2.1.2. Isolation of YAC Ends

In order to establish the end sequences of the clones identified, YAC end sequences were generated by the vectorette and fragment isolation method adapted from Riley et al. (1990). The cloned DNA was digested with a range of restriction enzymes (*AluI, EcoRV, PvuII and RsaI*) and ligated with a blunt-end vectorette cassette, as described. The resulting 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 are detailed in table 4.1.

Table 4.1. Oligonucleotides used in vectorette PCR.

Primer	Sequences 5'-3'
Top strand blunt cassette	aggagaggacgctgtctgtcgaaggtaaggaacggacgagagaagggagag
Universal bottom strand cassette	ctctcccttctcgaatcgtaaccgttcgtacgagaatcgctgtcctctcctt
Vectorette primer	tctcccttctcgaatcgtaaccgttcgtac
T7 primer	aatacgactcactatag
SP6 primer	gatttaggtgaccatatag

4.2.1.3. YAC Contig Construction

In order to arrange the YACs identified by pooled library screening into an ordered contig, a series of PCR-based STS content mapping tests were carried out. Standard PCR protocols were used. The YACs were screened for the following five STSs: *D17S1529, D17S1528, D17S831,*

D17S5 and *D17S1798*; the EST, PEDF3; and the YAC end sequences 38BE8-L, 38BE8-R, 25BH6-L and 25BH6-R (Stack et al., 1995) (table 4.2.).

Table 4.2. Details if STSs used in YAC contig construction.

STS	Sequence	Product Size (bp)	Annealing Temp. (°C)
PEDF3	cagagtccctgtgcatctctgcgag tcagccacgtttacgcagaggccac	336	70
38BE8-L	atatcaacagtgtgcaacccag tttccaccactgagcacaatac	153	57
38BE8-R	ccagtccaggcagtccac ttacggaattcctggcctc	117	57
25BH6-L	ccagaccaacagagagaaactctg ggcccagaattcaagttggttt	400	57
25BH6-R	gaggttaattgacttgcctagagcc cagccaaggtcgccttttttaa	70	57

4.2.2. INVESTIGATIONS *IN SILICO*

The increasing number and size of sequence databases available on the www facilitates the creation of sequence contigs across regions of interest. A series of bioinformatic applications were used in an effort to establish a sequence contig, the ultimate physical map, across the RP13 chromosomal region.

4.2.2.1. Database Searches and Sequence Comparisons

Sequence files which map to the RP13 chromosomal region were identified by performing homology searches against the nucleotide databases outlined in table 4.3., using the basic local alignment search tool, BLAST, at the National Center for Biotechnology Information

(NCBI). The nucleotide sequence databases were BLAST searched with the markers surrounding the RP13 interval, as well as with expressed sequence tagged sites (ESTs) and STSs placed into the interval by radiation hybrid mapping as outlined at GeneMap98 (NCBI). End sequences of homologous files were used to search the same databases for overlapping sequence files.

The STS content of the genomic files was tested by performing homology searches using the BLAST 2 SEQUENCES utility at the NCBI. Files were retrieved using the sequence retrieval utilities at EMBL and Genbank. Table 4.4. lists the universal resource locators (URLs) for the bioinformatics utilities used most frequently in the *in silico* analyses of the RP13 chromosomal region.

Table 4.3. Nucleotide databases used in *in silico* analyses.

Database	Abbreviation
Genome survey sequences	gss
High through-put genome sequences	htgs
Non-redundant nucleotide sequences	nr
Sequence tagged site database	dbSTS
Expressed sequence tagged site database	dbEST
Sequences released in the last month	month
STACK	STACK

Table 4.4. Bioinformatics utilities used in *in silico* analyses.

UTILITY	URL
BLAST2 Sequence retrieval	http://www.ncbi.nlm.nih.gov/blast/blast.cgi
EMBL Sequence retrieval	http://www.ebi.ac.uk/cgi-bin/emblfetch
GENBANK	http://www.ncbi.nlm.nih.gov/irx/genbank/query_form.html
BLAST 2 sequences	http://www.ncbi.nlm.nih.gov/gorf/bl2.html
BAC end sequence	http://www.tigr.org/tdb/humgen/bac_end_search/bac_end_search.html
GeneMap98	http://www.ncbi.nlm.nih.gov/genemap98/map.cgi?CHR=17
Genome sequencing	http://www.ncbi.nlm.nih.gov/genome/seq/
STACKsearcher	http://ziggy.sanbi.ac.za/stack/stacksearch.html

4.2.2.2. Identification of Retinal ESTs Mapping To The RP13 Interval

In order to identify any retinal ESTs within the critical interval, a database of the RP13 sequence files was created and compared with the eye subset of the STACK EST database using the STACKsearcher utility at the South African National Bioinformatics Institute (SANBI). The STACKsearcher allows you to perform a BLAST search of your query sequence/s against the STACK database. The STACK EST database consists of groups of aligned ESTs organised via tissue subdivisions as well as a whole body index.

4.2.2.3. Identification of New Microsatellite Markers

The identification of new polymorphic markers within a region of interest could prove useful in reducing the critical interval. Towards this end the RP13 sequence contig was searched with arbitrary sequences of di- and trinucleotide repeats using BLAST 2 SEQUENCES at NCBI.

4.3. RESULTS

4.3.1. PHYSICAL MAPPING *IN VITRO*

Identification of YAC clones mapping to the RP13 region: To identify clones residing in the RP13 chromosomal region three pooled YAC libraries (ICI, ICRF and CEPH) were screened using microsatellite markers bracketing the interval. A total of 4 YACs were identified as containing at least one of the genetic markers from the RP13 region. YAC clones 4X70-B3, 4X127-H12 and 4X54-A9 were identified from the ICRF library with markers *D17S1528*, *D17S831* and *D17S1798* respectively, and clone 34I-B6 from the ICI YAC library with marker *D17S1529* (table 4.5). In addition YACs 441D8, 38BE8, 416F9 and 21FH10 which map to the region around the marker *D17S5*, were obtained from Dr C. Inglehearn (London, UK) (table 4.5.).

YAC contig construction by STS content and integration of genetic and physical mapping data: In order to organise the 4 YACs identified by pool screening (4X70-B3, 4X127-H12, 4X54-A9 and 34I-B6) into an ordered contig, a series of PCR-based STS content mapping tests were carried out. The same strategy was used to integrate these 4 YACs with the 4 YACs around the marker *D17S5* (441D8, 38BE8, 416F9 and 21FH10). The *D17S5* YACs form part of a small contig, constructed by Stack et al. (1995). Details of the STS content mapping of the 8 YAC clones are presented in table 4.5. and illustrated in figure 4.1. YACs 416F9 and 38BE8 are each homologous for the non-polymorphic STS 38BE8-L as well as the polymorphic marker *D17S5*.

The marker, *D17S1528,* is present in YAC 38BE8 but not in 416F9. The markers *D17S5, D17S831* and *D17S1528* are present in 441D8 while this YAC does not contain the STS 38BE8-L. The STSs PEDF2 and PEDF3 (second and third exon of the *PEDF* gene) were present in 4X70-B3 alone, thereby specifying the physical orientation of the markers. No further YACs could be identified to close the gap between the YAC 4X70-B3 and 34I-B6, by screening the pooled YAC libraries. The UK group, headed by Dr Chris Ingelhearn was, however, able to close the gap with three PAC clones 268-A16, 300-H3 and 222-B19 (figure 4.1). These findings were confirmed in this study.

Table 4.5. STS content of YAC clones.

YAC	SOURCE	SIZE (KB)	*D17S1529*	PEDF3	38BE8-R	*D17S1528*	25BH6-L	*D17S831*	*D17S5*	25BH6-R	38BE8-L	*D17S1798*
34I-B6	ICI	225	+	0	0	0	0	0	0	0	0	0
4X70-B3	ICRF	~1000	0	+	+	+	+	+	+	0	0	0
4X127-H12	ICRF	365	0	0	0	0	0	+	+	0	0	0
4X54-A9	ICRF	NA	0	0	0	0	0	0	0	0	0	+
441D8	CEPH	530	0	0	0	+	+	+	+	0	0	0
416F9	CEPH	NA	0	0	0	0	0	0	+	+	+	0
38BE8	Zeneca	265	0	0	+	+	0	0	+	+	+	0
21FH10	Zeneca	200	0	0	0	0	+	0	+	0	0	0

Markers are listed in their most likely physical order from the telomere to centromere. Clone sizes of the last four YACs were taken from Stack et al. 1995 and the first four were obtained through personal communication from Dr. C. Inglehearn (UK). Polymorphic STSs are in italics (NA-not analysed).

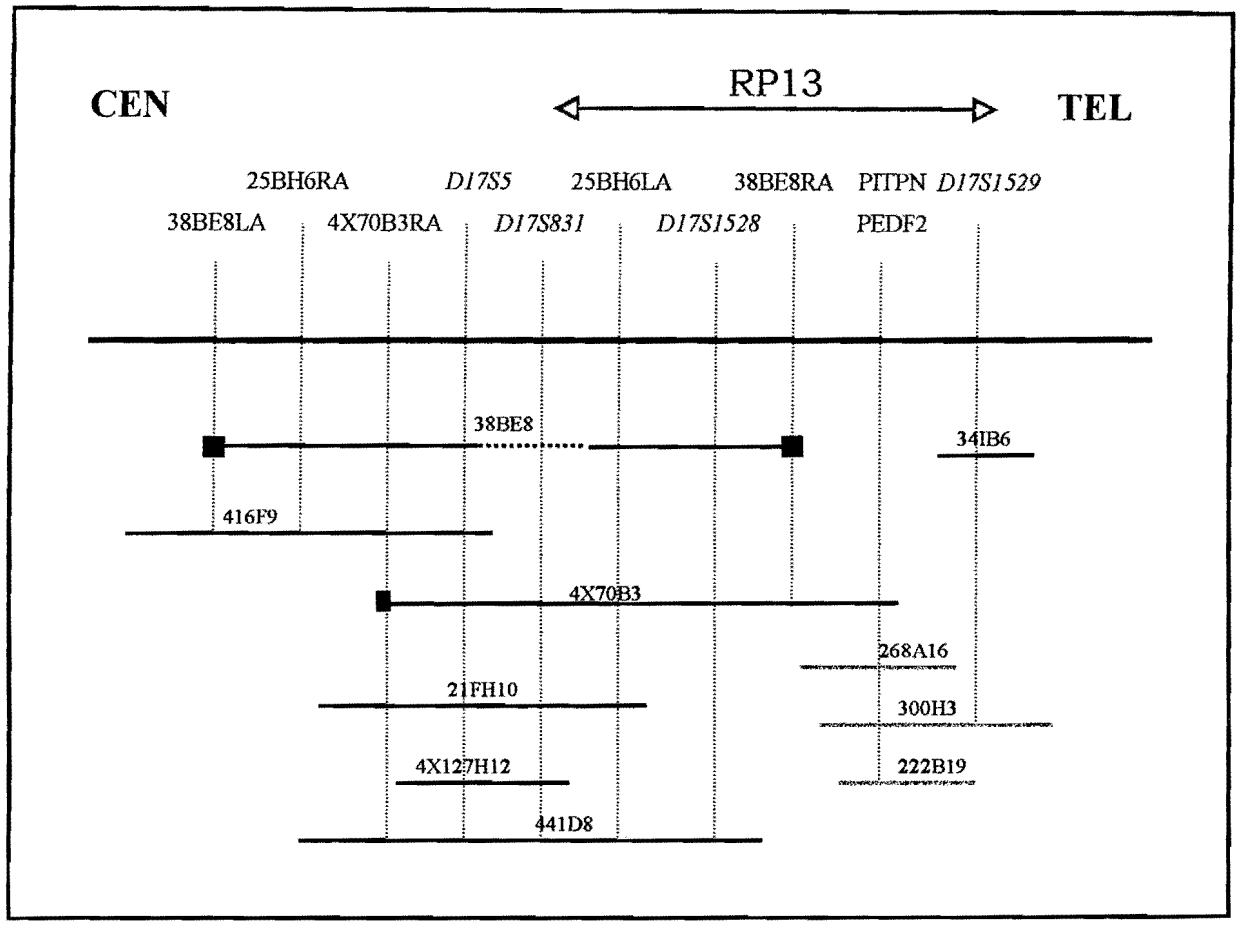


Figure 4.1. An STS content physical clone contig spanning the RP13 chromosomal region.
The red arrow and markers represent the RP13 interval and bracketing markers. End clones are represented by black squares. Black and purple lines represent YAC and PAC clones respectively. Polymorphic STS are italicised.

4.3.2. PHYSICAL MAPPING *IN SILICO*

Identification of sequence files mapping to the RP13 region: In order to compliment and enhance the physical clone contig at the RP13 interval, construction of a physical sequence contig of the region was initiated. Sequence databases were BLAST searched with markers bracketing the RP13 interval to identify sequence files residing in the same chromosomal region. A total of 8 genomic sequence files were identified in the nucleotide databases as containing at least one of the genetic markers from the RP13 region (table 4.6.). Six of the sequence files identified were incomplete, each consisting of several unordered fragments (AC006435, AC0021705, AC007873, AC006405, AC002093, AC015884). The remaining 2 sequence files are completely sequenced (AC002316 and AC005696).

Sequence assembly construction by STS content and integration of genetic and physical mapping data: In order to organise the 9 sequence files identified in the R13 chromosomal region into sequence contigs, a series of sequence comparisons and EST/STS content investigations were carried out between files.

Using the technique of radiation hybrid (RH) mapping many ESTs and genes have been mapped to various chromosomal regions (http://www.ncbi.nlm.nih.gov/genemap98/). This technique involves the use of a panel of somatic cell hybrids, with each cell line containing a random set of fragments of irradiated human genomic DNA in a hamster background. Twenty-nine ESTs have been placed into the RH

interval which includes the RP13 critical region (figure 4.2). Sequence comparisons between the RP13 sequence files and each of these candidate ESTs were carried out. The sequence alignment results placed several of these ESTs onto the RP13 sequence files as well as several STSs and genes (table 4.7). Sequence files AC002316 and AC005696 form a small sequence assembly which overlaps by 15 633bp. Distal to this assembly, sequence files AC002093 and AC006405 also overlap. Figure 4.3. illustrates the sequence contig placed against the genetic and clone contig maps of the region as discovered during the course of this study. Sequence file, AC005696, contains the markers *D17S1845* and *D17S1798*, 146.433kb apart on the sequence contig and 1cM on the genetic map.

Table 4.6. Sequence files identified in the RP13 chromosomal region.

ACCESSION No.	SIZE (kb)	IDENTIFYING MARKERS
AC002316	88.071	*D17S1845*
AC005696	177.411	*D17S1845, D17S1798*
AC007873	214.644	PEDF
AC002093	133.687	PITPN
AC006405	184.122	PITPN
AC015884	190.910	*D17S1529*
AC021705	190.765	*D17S379*
AC006435	135.363	*D17S379*

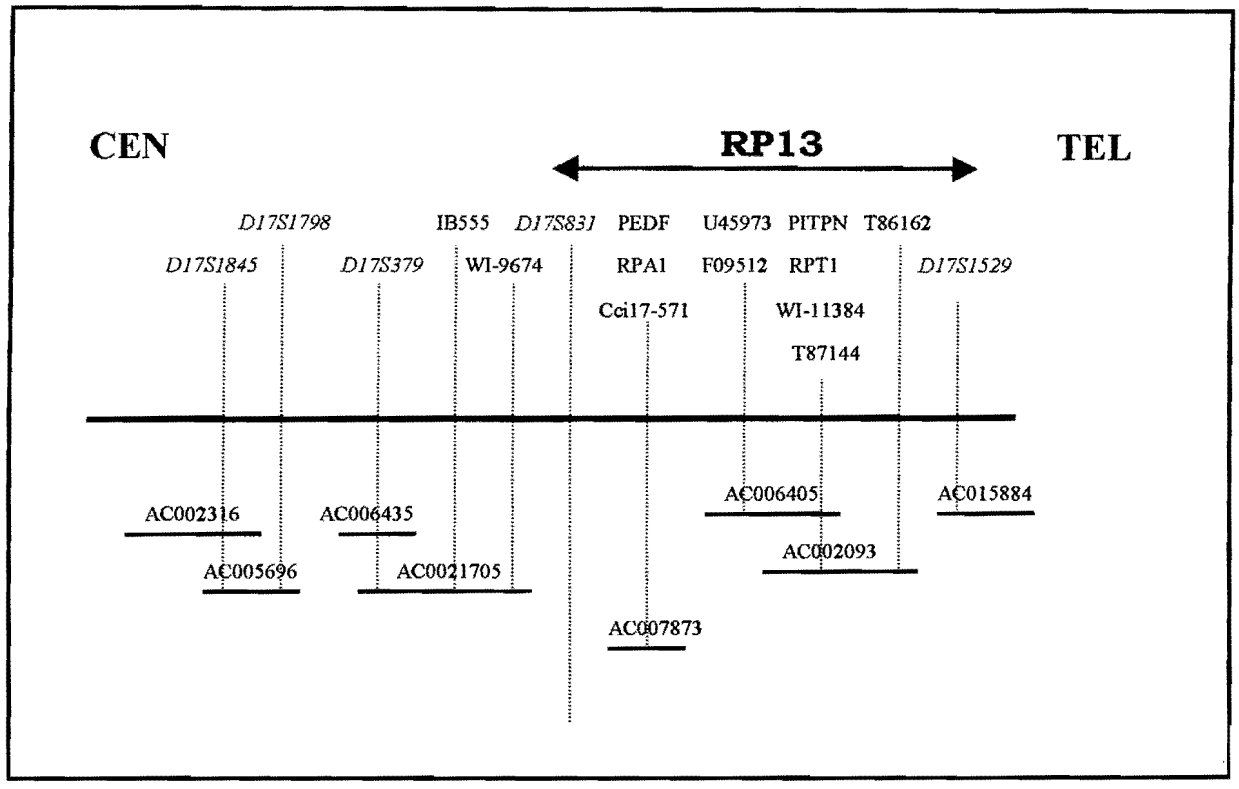


Figure 4.2. A partial STS content physical sequence contig and expression map of the RP13 chromosomal region.

The ESTs are unordered within the interval.
AC002316 = 88 071bp
AC005696 = 177 411bp
AC006435 = 135 363bp
AC0021705 = 190765bp
AC007873 = 214 644bp
AC006405 = 163 035bp
AC002093 = 133 687bp
AC015884 = 190910bp

Table 4.7. Genes/ESTs/STSs placed on the sequence contig in this study.

Gene/EST/STS	Sequence file
F09512	AC006405, (AC002093?)
T86162,T83770 T87144,T86163, WI-11384	AC006405, AC002093
Phosphatidylinositol (4,5) biphosphate	AC006405
D17S1845	AC002316, AC005696
D17S1798	AC005696
PEDF	AC007873
RPA1	AC007873
Cci17-517	AC007873
PITPN	AC006405, AC002093
RPT1	AC006405, AC002093
T96691, F19292, T98677	AC002093
D17S379	AC021705, AC006435
IB3555	AC021705
WI-9674	AC021705
D17S1529	AC015884
PRP8	AC002093
WI-17501	AC015844

Retinal ESTs mapping to the RP13 sequence contig: The retinal ESTs identified in the STACK EST database are detailed in table 4.8.

Table 4.8. Retinal ESTs placed on the partial RP13 sequence contig.

EST	SEQUENCE FILE	GENE
AA318345	AC006405	*PITPN*
AA317269	AC006405	*PITPN*
T30609	AC002093	*PRP8*
AA317401	AC005696	Miller-Dieker lissencephaly disease gene
W22489	AC005696	Human retina cDNA

Microsatellite markers identified: A $(CA)_n$ repeat was identified in the region of overlap between sequence files AC006405 and AC002093. A sequence comparison performed between the two sequence files showed that the dinucleotide repeat was polymorphic between them. BLAST searching the STS database with the repeat, including the flanking region, proved that the repeat had not been previously identified. PCR primers designed on either side of the repeat region (RPT1-F 5'gacctgttcccaggaatgtg 3', RPT1-R 5'aaaaggcaacaaggcatttg 3') were used to amplify the region and to genotype the subjects within the ADRP-SA1 family. Five alleles were observed with the same allele segregation pattern as that of the telomeric bracketing marker *D17S1529* (figure 2.6., chapter 2). Hence, the RP13 interval could not be genetically refined any further using this marker.

4.4. DISCUSSION

The AD form of retinitis pigmentosa was mapped to chromosome 17p (chapter 2) within the interval defined by markers, *D17S1529* and *D17S831* (Goliath et al., 1995). This chapter described the construction of a physical map of the RP13 chromosomal region using a combination of *in vitro* and *in silico* analyses.

Physical maps are used to isolate and characterise individual genes and other DNA regions of interest and provide the substrate for DNA sequencing. The lowest- resolution physical map is the chromosomal or cytogenetic map and the more detailed YAC or cosmid contig map represents the order of overlapping DNA fragments spanning the genome. The ultimate physical map of the human genome, however, is the complete DNA sequence.

The physical clone contig constructed across the RP13 interval comprises 7 YACs and 3 PACs. The contig confirms the reported order of loci on the genetic map and allows for the placement of markers relative to each other that had not been resolved previously. The order of STSs highlights which of the genes are positional candidates and which are not. Ultimately, according to this study the *PEDF* and *PITPN* genes remain candidates at the RP13 locus.

Much progress has been made towards sequencing the entire human genome resulting in the establishment of large sequence databases.

Examination of these sequence databases for large-scale comparative analysis of genome sequence data is becoming the most important application of bioinformatics for the characterisation of candidate disease genes. Using these bioinformatics utilities to interrogate the sequence databases, a partial sequence contig of the RP13 chromosomal region was constructed. The sequence contigs confirm corresponding areas on the physical clone contig. Two of the original candidate genes investigated at the RP13 locus have been placed onto the electronic sequence contig (*PITPN* and *PEDF*). Although no disease-causing mutations were identified in either of these genes, (chapter 3.3.3. and 3.3.4.), their precise physical mapping should facilitate the molecular genetic investigations of other retinal disorders mapping to the same chromosomal region. An additional candidate gene, *PRP8*, was identified by *in silico* mapping (chapter 4.3.2.). The gene is retinally expressed and resides on the sequence contig constructed within the RP13 critical interval. Several other ESTs that were previously unmapped were placed onto the contig.

The sequence databases provide DNA fragments that can be screened not only for markers known to be in a region of interest, but also for additional polymorphisms. The identification of new polymorphisms improves the genetic map of the region with the possibility of further refining the gene location. Indeed, a $(CA)_n$ repeat region was identified within two overlapping sequence files at the RP13 locus. Comparison of the repeat regions between the two sequence files showed that the region was polymorphic. The repeat region identified in sequence file,

AC006405 was two (CA) units longer than that in sequence file, AC002093. Following the design of primers on either side, the repeat was amplified and the subjects within the ADRP-SA1 family genotyped (chapter 4.3.2.). The allele segregation pattern of the marker proved to be the same as seen for the telomeric flanking marker, *D17S1529*. This result therefore, did not facilitate the narrowing of the RP13 interval.

It is known that telomeric chromosomal regions are the most gene-rich areas of the genome (Lese et al., 1999). Consequently, closing the gaps in the physical maps of these regions has received much attention. It is also believed that genetic maps of telomeric chromosomal regions are expanded with respect to the corresponding physical maps. The RP13 interval covers a genetic distance of 3cM (chapter 2) at the telomeric region of chromosome 17p. In genetic terms this should extrapolate to a physical distance of approximately 3 megabases. However, a genetic distance of 1cM (1megabase) separates the DNA markers *D17S1845* and *D17S1798* at the centromeric end of the RP13 interval on the genetic map of the region. In this study these markers were both placed onto the sequence file AC005696 (which was completely sequenced) with a physical distance of about 150kb between them. This observation of apparent enhanced recombination at the telomeric end of chromosome 17p confirms a previous study suggesting the same phenomenon (Martens et al., 1998). In this previous reported study it was shown that terminal regions of human chromosomes are characterised by a series of T_2AG_3 repeats and that the number of repeats at chromosome 17p telomeres were shorter than that for the

average telomere length (Martens et al., 1998). Taken together these findings may indicate that the 3cM RP13 interval which, in general genetic terms, should extrapolate to a physical distance of approximately 3 megabases, could actually traverse a much smaller physical distance. If this is the case then it would be foreseeable that with the current release of draft sequences of the entire genomic sequence, that the RP13 interval could be completely sequenced in the not too distant future.

The YAC/PAC contig, together with the partial sequence-contig, forms a solid foundation for the elucidation of the RP13 disease-causing gene. The establishment of a transcriptional (EST) map of the region commenced in this study further facilitates this goal. The identification of candidate ESTs and genes on the physical contigs of the RP13 interval will highlight new candidate genes for ADRP, and other genetic disorders that map to this region of chromosome 17p13.3. The *PITPN* and *PEDF* genes were placed in the RP13 interval both genetically (chapter 3) and physically (chapter 4). Intense investigation of these genes, however, revealed no pathogenic alterations in the ADRP-SA1 family. Furthermore, according to the GeneMap98 database at the NCBI, other genes in the RP13 interval include the *PRP8*, human phosphatidylinositol (4,5) biphosphate, myosin-1 beta, alpha-2-plasmin inhibitor and the replication protein A1 genes. Although these genes are expressed in several tissues their candidacy for the RP13 gene has not been excluded. As discussed in chapter 3, the tendency to study only functionally significant genes in a region associated with disease, is

gradually being abandoned. This shift in focus, is due to the elucidation of disease-associated alterations in genes either with an unknown function or no clear functional significance to the disorder being studied. Examples include the ubiquitously expressed *RPGR* gene reported to be associated with XLRP (Meindl et al., 1996) and *TIMP3*, a member of the TIMP gene family, has been shown to be causative of Sorsby fundus dystrophy (Weber et al. 1994).

Clearly the rapidly expanding genome sequencing projects generate enough data with which to overwhelm researchers. Fortunately, the bioinformatics tools available create an excellent environment for searching and exploring this ever expanding "sequence universe." In the future, the complete integration of the sequence and clone contigs presented here will be useful aids in identifying new candidate genes and ESTs within the RP13 interval.

CHAPTER 5

GENERAL DISCUSSION

Identification of the disease-causing genes is an important step toward understanding inherited retinal degenerative diseases. Currently, in the field of inherited RD, major progress has been made in identifying the underlying causal genes. With subsequent molecular and cell biological investigation yielding insights into the pathogenic mechanisms underlying this group of disorders, it is likely that this perspective in disease aetiology, will facilitate the development of therapeutic measures. The rate at which these genes are being identified continues to increase with the constantly improving genetic and physical maps.

This dissertation describes the progress towards identifying the disease-causing gene segregating in one of the largest ADRP families recorded in South Africa. Using the positional candidate approach, the causal gene was localised to the short arm of chromosome 17 (17p13.1) (Greenberg et al., 1994b). The successful mapping followed the exclusion of large chromosomal regions which were previously reported to be either linked to ADRP, or which contained other functionally significant candidate genes. The novel, and at that stage, unique South African locus, designated RP13, was the first to be identified amongst a large cohort of SA RD families under investigation in a single family (chapter 2). The

identification of RP13 confirms that the positional candidate approach is a powerful method for mapping disease genes. The same approach was subsequently used in the mapping of another novel SA ADRP gene to the long arm of chromosome 17 (RP17) (Bardien et al., 1995).

Following initial genetic mapping between markers situated 23cM apart, the RP13 locus was fine mapped to a 3cM critical interval between the microsatellite markers, *D17S1529* and *D17S831,* within the telomeric chromosomal band, 17p13.3, (Goliath et al., 1995). The genetic interval could not be reduced any further due to the lack of additional microsatellite markers within the RP13 critical region on the genetic map and no additional meioses being available for investigation.

Following the localisation of RP13 in the SA family, the disorder was reported in two additional ADRP families (from the USA and UK, respectively) and was mapped to the same locus at 17p13.3 (Kojis et al., 1996; Tarttelin et al., 1996). The disorder in all three families is characterised by an early onset form of ADRP. Furthermore, the common British ancestry of both the SA and USA RP13-linked families led to the hypothesis that the disease in all three families was due to a founder mutation. Genetic analysis, however, comparing linkage data in the SA and UK families revealed distinct disease-associated haplotypes (the family from the USA was not available for testing). This finding indicates that the phenotype in the disparate families might be a result of different pathogenic mutations. However, it is possible that the different disease-associated haplotypes may indicate a very "old"

underlying pathogenic mutation where recombination events over many generations might have increased the likelihood of tightly linked markers being separated. The eventual identification of the disease-causing gene/s and pathogenic mutation/s will clarify this issue.

It is possible that the molecular demography underlying inherited RD in SA may be different to that in other geographic regions of the world. This theory is proposed because, of the entire cohort of SA RP families being investigated, two novel loci have been reported to date; RP13 (Greenberg et al., 1994) and RP17 (Bardien et al., 1995). Furthermore, the disorder in only a minor number of families has been shown to map to each of these loci (three at each locus). In addition, although international findings suggest that rhodopsin gene mutations account for 20% of ADRP, subjects which form part of the research cohort in the laboratory of Human Genetics at UCT exhibit a decreased incidence of rhodopsin gene mutations as reported by Roberts et al. (2000).

Subsequent to the identification of the RP13 locus on chromosome 17p13, several other retinal disorders have been reported to map to the same chromosomal region, but to distinct loci. These include LCA1 (Camuzat et al., 1995), CORD5 (Balciumiene et al., 1995), SLS (Rogers et al., 1995), CACD (Lotery et al., 1996), CORD6 (Kelsell et al., 1997), and LCA2 (Hameed et al., 2000) (chapter 2). The specific defect in the *RETGC-1* gene has since been identified for LCA1 and CORD6 (Perrault et al., 1996, Kelsell et al., 1998) and for SLS in the fatty aldehyde dehydrogenase gene (Rogers et al., 1995).

Six functionally significant retinal genes map to the short arm of chromosome 17 (*RCV1*, *RETGC-1*, *PITPN*, *PEDF*, *ARRB2* and *AILP1*). The retinally expressed gene *AILP1*, which was most recently mapped to chromosome 17p was not considered a candidate for the RP13 disease gene due to its map position being outside of the critical interval (Sohocki et al., 1999). The first five genes were tested as possible disease-causing candidates in the ADRP-SA1 family. The molecular genetic techniques used did not reveal potentially pathogenic mutations in any of these genes. In addition, the *RCV1* gene was excluded on the basis of recombinant events between the disease phenotype and an intragenic polymorphic marker. The *RETGC-1* and *ARRB2* genes were further excluded by the subsequent genetic fine mapping of these genes, which placed them into a different genetic interval to that of the RP13 locus (Camuzat et al., 1996 and GeneMap98). Although the remaining two candidate genes, *PITPN* and *PEDF*, map to the RP13 critical interval, extensive interrogation of these genes did not reveal any pathogenic alterations. The data emerging from the analysis of the genes, markers and loci, collectively used during this study, contributed significantly to the integrity of the genetic map of the 17p13 chromosomal region. Furthermore, the identification of intragenic polymorphisms in certain instances has and will in the future, allow for the rapid investigation of these genes in other retinal disorders that map to the same chromosomal region (chapter 2).

The various retinal phenotypes linked to the chromosome 17p region could be the result of mutations in one or several of the 17p13 retinally-expressed genes. The absence of a potential pathogenic alteration in the functionally significant positional candidate genes investigated in this study so far, suggests that the causal gene is likely to be an as yet unknown retinally-significant gene on chromosome 17p.

The RP13 critical interval spans a genetic distance of 3cM, which may initially appear to be too large for the application of conventional positional cloning techniques. However, the locus resides at the telomeric end of chromosome 17p, a condensed physical region with respect to the corresponding genetic map (Martens et al., 1998). In light of this, physical mapping strategies were deemed feasible across the 3cM interval towards identifying the novel gene. A physical clone contig that spans the RP13 region was constructed with YAC and PAC clones. This contig consists of overlapping clones and contains both flanking markers (*D17S1529* and *D17S831*), that define the RP13 critical interval. The contig provides the scaffolding upon which new genes can now be localised and identified.

In conjunction with the physical mapping strategies using cloned DNA (*in vitro* analysis), bioinformatics techniques (*in silico* analysis) were also applied in this study. Together with the physical clone contig, a partial physical sequence contig was constructed with large sequence files retrieved from various substantial sequence databases available on the www. Sequence comparisons between the RP13 sequence files and EST

databases were undertaken in an attempt to generate a transcription map of the interval. This approach was also carried out using an eye cDNA database (STACK). The findings emerging from the partial sequence contig confirms and extends information obtained in areas of the clone contig. Using this strategy a number of retinal ESTs and cDNAs were identified which are now potential disease-causing genes for RP13. A future extension of this study would be the investigation of the newly identified retinal candidate genes at the RP13 locus. Furthermore, both the physical clone and sequence contigs of the RP13 interval require intensive investigation. As bioinformatics has become widely used in the characterisation of candidate and disease genes, both *in vitro* and *in silico* technologies need to be undertaken during further investigations towards identifying the RP13 disease-causing gene.

The establishment of the disease-associated haplotype has several implications for the ADRP-SA1 family. In SA, over the past twenty years, increased public awareness of genetic disorders and research has led to an increased need for informed genetic counselling. This type of service requires a clearly defined diagnosis and detailed knowledge of the molecular genetics of a known inherited condition. In particular, accurate assessment of presymptomatic individuals in families with RD is becoming available for a range of retinal dystrophies (Jay and Evans, 1996) allowing for more informed counselling and educated decisions to be made by patients, guardians and caregivers, respectively. The disease-associated haplotype segregating in this SA family, could now

be used in the development of a predictive test for at-risk individuals. Using linkage analysis with the conserved disease-associated haplotype, asymptomatic individuals could be offered the opportunity to be informed of their risk for developing the condition. Since such a proposed test would be performed using linkage analysis and not be based on the precise disease-causing mutation, an increased or decreased relative risk would be inferred. A presymptomatic testing programme of this nature would only be established after wide consultation, ethical debate and under very specific guidelines (Greenberg et al., 1996). In the first instance, the age and psychological status of the individual would need to be considered. Furthermore, a psychological support mechanism would need be put in place in order to facilitate adaptation to the possible outcome of the test. Of utmost importance with presymptomatic testing, is the presence of thorough pre- and post-test counselling which would, amongst other things, endeavour to ensure that the individual understands the test and is prepared for the implications thereof, as fully as possible. Undoubtedly, an adult subject -based presymptomatic testing programme would assist those concerned in decision making and development of particular life skills and career training. The potential for prenatal testing also exists, upon request, should the need arise. This would, however, require greater deliberation and decision making.

Initially it was thought that only retina-specific genes would underlie retinal disease. An observation which emerged was that the genes responsible for RD may be distributed over three categories: (1)

abnormalities in genes encoding the visual cascade proteins, for example, *RHO*, *GNAT1* and α- and β-*PDE*, (2) abnormalities in genes encoding retinal structural components, such as *peripherin/RDS* and *ROM1*, each members of the tetraspanin gene family (Phelan and Bok, 2000), and (3) abnormalities in genes expressed in the RPE which include *ABCR*, *TULP1* and *CRX* (chapter 1). Defects from each category result in a pathogenic effect in the photoreceptor layer of the retina. However, as the effort of finding RD disease-causing genes progresses, it is interesting to note that a number of these preconceived ideas about the types of genes which could be involved in RD have to be revisited.

Recent reports have disproved the theory of only retina-specific genes underlying RD; for example Sorsby fundus dystrophy was found to be associated with *TIMP3*, a ubiquitously expressed gene. This observation therefore indicates that future research efforts would need to be broad based, making as few assumptions as possible. Although some genes involved in retinal disorders do not show expression that is restricted to the visual process and supporting anatomical structures, it seems likely that the highly specialised function of the retina must require a large number of retina-specifically expressed genes. Therefore, several studies are currently underway with the specific goal to isolate retina- and RPE-specific genes (Sohocki et al., 1999). Retinal gene isolation studies such as this may contribute to the endeavour of identifying the RP13 causal gene.

The study of animal models of retinal dystrophies has provided several candidate genes for investigation of RP in humans. This powerful field of research has also led to the identification of new genes and even new gene families. The identification of the gene defects responsible for two mouse models of RD, the RD (rd) mouse and the RD slow (rds) mouse, led to the screening of the human gene homologues β-*PDE* and *peripherin/RDS*. This in turn led to the identification of a number of β-*PDE* and *peripherin/RDS* disease-causing mutations in humans with retinal dystrophies. In addition to the spontaneous dystrophies, laboratory mice offer the opportunity for genetic manipulation and experimentation through the introduction of a foreign gene (transgenic animals) or by the targeted disruption of an endogenous gene (gene 'knock-out'). The study of animal models of human dystrophies therefore offers many obvious benefits. In addition to those already described, detailed histopathological, molecular and electrophysiological studies not possible in human subjects can be undertaken in animal models. Any significant findings that emerge will contribute to the overall understanding of disease mechanisms in the retina. Hence this method of identifying disease-causing genes has been and will continue to be widely used.

Present and future therapeutic modalities: Characterisation of the underlying genetic basis for disease is leading to new concepts of pathogenesis at the molecular and cellular levels. The corresponding expansion in the understanding of disease processes is also stimulating

interest in the development of therapeutic strategies. Although no satisfactory treatment for most inherited retinal dystrophies exists, several processes are currently being intensively investigated towards this end.

The manipulation of apoptosis: A particular dilemma in the development of therapy is the evidence which suggests that the metabolic defect caused by the underlying mutation does not cause photoreceptor cell death directly. Investigation of animal models of RD has revealed that photoreceptor cell death occurs by a common mechanism known as apoptosis or programmed cell death (Chong and Bird, 1999). The significance of this discovery is that this process of premature cell death, instead of being the primary underlying genetic defect, may be amenable to therapeutic manipulation. Apoptosis, which is an important part of embryonic development, is the genetically encoded potential of all cells and governs cell turnover. This process is activated, for example, for the removal of cells infected by viruses or those harbouring disease-causing mutations (Wyllie et al., 1980).

It has been observed that injection of growth factors into the eye reduces the rate of apoptosis in degenerating retina which results in long term survival of photoreceptors (Faktorovich et al., 1990). Studies have shown that the more effective growth factors are those which have a relatively specific influence on neural tissue, for example the neurotrophin growth factors (Borhani et al., 1993). The ciliary neurotrophic factor has been shown to delay photoreceptor

degeneration in rd mice and transgenic mice carrying a mutated rhodopsin gene (LaVail et al., 1998). This form of treatment of RD i.e. manipulation of the apoptotic process, has the advantage that it may be effective in diseases with unknown genetic mutations.

Gene therapy: Another therapeutic approach being investigated in the field of RD, is that of gene therapy. The aim of this type of therapy is the replacement of the defective gene with genes that express normally. Some promising observations in the field of RD therapy research have been noted. Photoreceptor cell death has been shown to be delayed in RD mice (with a β-*PDE* defect) with the use of subretinal injection of a recombinant replication defective adenovirus that contains the murine cDNA for the normal β-*PDE* (Bennett et al., 1996). More recently, subretinal injection of recombinant adeno-associated virus (AAV) encoding a peripherin/rds transgene into the rds mouse resulted in the stable generation of outer segment structures and formation of new stacks of discs containing both perpherin/rds and rhodopsin (Ali et al., 2000). This re-establishment of the structural integrity of the photoreceptor layer also results in electrophysiological correction. This study demonstrates for the first time that a complex ultrastructural cell defect can be corrected both morphologically and functionally by *in vivo* gene transfer. The longevity of transgene expression, however, requires further manipulation and investigation.

Artificial vision: The avenue of artificial vision in the management of retinal dystrophies is also being investigated. The concept of artificial

vision is based on the fact that in the retina of RP patients, photoreceptors at the periphery are damaged but the inner retina remains healthy. The particular application of this technology, if successfully developed, will be in restoration of vision in individuals in whom the photoreceptor cells have degenerated, as occurs in all RD patients with time. The intact inner retina is thus subjected to electrical stimulation in order to effect a visual sensation (Humayun et al., 1994). Various "prosthetic devices" are currently being investigated in Germany and in the USA, with evidence of minimal perception of light stimulus. It is likely that the enormous strides in research and development in the field of robotics, will lead to a better understanding of the interface between biology and electronics, which in turn, will improve this very promising area of visual restoration. Using this strategy no viable eye tissue is required, however, methods for permanent implantation of electrical devices may pose major technical problems.

Retinal cell transplantation: Research in the field of transplantation involves the replacement of lost photoreceptors or RPE cells with healthy ones. In early experiments fetal retinal tissue was shown to survive and differentiate, but failed to show a normal orientation in the retina (Aramant et al., 1988). RPE cell transplantation into the subretinal space delayed photoreceptor loss and retinal function in rats (Sheedlo et al., 1989). Although no visual improvements were recorded, these studies showed that the grafts survived in most patients.

Research efforts in the fields of ophthalmology, genetics and biology in general as well as biotechnology hold promising prospects for sufferers of RD and their descendants who may be at risk. Initially, the administration of an easily deliverable gene construct before any detectable signs of disease, may be a preferable means of avoiding manifestation of the disorder. Ultimately, the successful manipulation of damaged cells will restore the visual process but the cause of the degenerative process in the individual will need to be known if this is to be accomplished effectively. Therapies may therefore have to be tailored to each individual or family of individuals even with the same genetic cause of RD.

5.1. CONCLUDING REMARKS

The main findings resulting from this investigation are:

- Mapping of the disease locus in a large family with ADRP to a 3cM region on chromosome 17p13.3 (chapter 2),
- construction of a complete clone and partial sequence contig across the critical interval (chapter 4),
- exclusion of three candidate genes *RCV1*, *RETGC*, and *ARRB2* (chapter 3),
- genetic fine mapping of *RCV1* and *PEDF* (chapter 3),
- the physical placement of *PITPN* and *PEDF* on the physical contigs (chapter 4),
- SNPs detected in the *PITPN* and *PEDF* genes (chapter 3),

- the characterisation of intron-exon boundaries of *PITPN* (chapter 3). and
- preparation for the establishment of a potential presymptomatic test for at risk individuals in the family concerned.

These findings have far reaching implications for molecular research involving retinal genes on chromosome 17p. The definition of the RP13 locus has and may in the future, facilitate the localisation of the disorder in other previously unmapped families using the genetic markers shown to be tightly linked to the disease-causing gene.

The physical contigs provide a valuable resource for the cloning of the RP13 causal gene as well as other genes in this region. With the entire genome sequence being documented in electronic databases, it is possible that the field of biotechnology may be the investigative approach of choice towards characterising the disease-causing gene, with these contigs as the foundation.

In the search for disease-causing genes in the future, the positional candidate gene approach will very likely be the method of choice (Strachen and Read, 1996). To this end, the precise genetic localisation of the retinal genes in this study, will assist in establishing possible candidates in the investigation of retinal disorders fine mapped to the corresponding genetic intervals on chromosome 17p. In addition, the elucidation of intragenic SNPs within *PEDF* and *PITPN* as well as the characterisation of intron-exon boundaries within the latter gene, will

facilitate the future investigation of these candidates as possible causal genes underlying the chromosome 17p-linked retinal disorders.

Once the RP13 disease-causing mutation has been identified, the question of treatment for the RP13-linked families can be addressed. An animal model would then be useful to determine the exact mechanism of cell death in this form of RP. The work presented in this dissertation provides the foundation for future research such as this, which will have the potential to eventually contribute towards the development of a form of therapy for this large SA ADRP-SA1 family which formed the basis of this study.

APPENDIX A

Techniques:

A.1 DNA isolation from lymphocytes

A.2 RNA isolation from transformed lymphoblasts

A.3 DNA oligonucleotides

A.4 Southern blot analysis

A.5 Single strand conformation polymorphism analysis (SSCP)

A.6 DNA sequencing

A.7 YAC DNA analysis

A.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).

A.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, air-dried and finally resuspended in 20-50 µl distilled water.

A.2.1 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.

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

A.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

A.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.

A.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.

A.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 B) 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

A.4.3 Autoradiography

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

A.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:

A.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.

A.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.

A.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.

A.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).

A.7 YAC DNA ANALYSIS

A.7.1 YAC DNA ISOLATION

- 10 ml cultures were grown overnight in YEPD media containing Ampicillen (100 µg/ml).
- 100 ml broths were inoculated with 0.2 ml of the overnight cultures and grown 2 days at 30°C.
- The cells were pelleted at 3000 rpm for 5 min at 20°C and resuspended in 5 ml of solution 1.
- 20 µl of lyticase enzyme (10mg/ml) was added to the pellets and incubated for 1 hour at 37°C, shaking.
- The cells were pelleted gently at 1000 rpm for 10 min and the supernatent removed.
- The pellet was resuspended in 5 ml of solution 2 at 65°C for 10 min.
- The DNA was allowed to cool to room temperature and an equal volume of ethanol was added.
- The DNA was pelleted at 2000 rpm for 10 min.
- The DNA was resuspended in 2 ml TE, pH 7.4.

- 20 µl of Rnase (10µg/ml) was added to the DNA suspension and incubated at 37°C for 30 min.
- 40 µl of proteinase K (10µg/ml) was added and the mixture incubated at 65°C for 1 hour and allowed to cool to room temperature for about 15 min.
- The DNA was extracted 3x with phenol/chloroform and then once with chloroform.
- The DNA was precipitated with 1/10 sodium acetate and 2x ethanol, pelleted, dried and resuspended in 250 µl of TE.

A.7.2 YAC AGAROSE PLUGS

- 50 ml cultures were grown for 2 days in YEPD 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.

APPENDIX B

Media, Buffers and Solutions:

B.1 agarose loading buffer

B.2 TBE (1X)

B.3 formamide loading buffer

B.4 6% denaturing polyacrylamide gels

B.5 SSCP loading buffer

B.6 hybridisation solution for Southern blots

B.7 SCE

B.8 lysis solution

B.9 Solution D

B.10 Solution 1

B.11 Solution 2

B.12 TE

B.1 Agarose loading buffer

0.25% bromophenol blue

40% sucrose

B.2 TBE (1X)

0.089 M Tris

0.089 M Boric acid

0.002 M EDTA (pH 8.0)

B.3 Formamide loading buffer

80% formamide

10 mM EDTA (pH 8.0)

0.1% xylene cyanol

0.1% bromophenol blue

B.4 6% denaturing polyacrylamide gels

5.7% acrylamide

0.3% bis-acylamide

7.65 M urea

1X TBE

B.5 SSCP loading buffer

95% formamide

20 mM EDTA (pH 8.0)

10 mM NaOH

0.02% xylene cyanol

0.02% bromophenol blue

B.6 Hybridisation solution for Southern blots

1 M NaCl

1% SDS

5 % Dextran sulphate

10% PEG 6000

100 µg per ml denatured Salmon Sperm

B.7 SCE

1 M sorbitol

0.01 M sodium citrate

0.06 M EDTA (pH 7.0)

B.8 Lysis solution

10 mM Tris

1% n-Lauroyl sarcosine

0.45 M EDTA (pH 7.8)

B.9 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	

B.10 Solution 1

0.9M sorbitol

20mM EDTA

14mM beta-mercaptoethanol, pH 7.5

B.11 Solution 2

4.5M GuHCL

0.1M EDTA

0.15M NaCl

0.05% sarkosyl, pH 8

B.12 TE

10mM Tris HCL

1mM EDTA (pH 8.0)

APPENDIX C

C.1 Department of Human Genetics DNA consent form

C.2 Department of Human Genetics Clinical data form

C.1

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 specimens 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 co-operation 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.
7. The DNA bank may request relevant clinical details from my doctors

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)

- To be used for linkage analysis for members of my immediate family.
- 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.
- To be used without any restrictions.
- 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**

C.2.

CONSENT FOR PARTICIPATION IN RESEARCH PROGRAMME FOR RETINAL DEGENERATION DISORDERS

DEPARTMENT OF HUMAN GENETICS
FIRST FLOOR, ANATOMY BUILDING,
UNIVERSITY OF CAPE TOWN MEDICAL SCHOOL,
OBSERVATORY, 7925

(Please read both sections and sign BOTH if you agree with the conditions)

1. Information for Research Database

Patients with retinal degenerative disorders are likely to benefit from local and international research aimed at defining the cause of their condition. To facilitate treatments that will emerge from these research efforts, in the future, it is essential to establish and maintain a comprehensive database (a computer record) of all patients with these diseases. Should a treatment become available it can then be offered in a targeted manner to patients through the database. The treatments are likely to be very specific so we require as much detail as possible about your particular disease to enable us to keep useful information. You can be assured that patient confidentiality is a major concern and your information will only be available to research workers in this field. Your name will not be divulged. The purpose of the database is to assist the researchers in assessing the magnitude and biology of the problem in our country and to enable them to contact and counsel patients quickly should treatment be available.

Consent

I agree that my details may be released to researchers and authorise my doctor to release medical details to the Department of Human Genetics at the University of Cape Town provided that any information from such research will remain confidential.

Signature..... **Print Name**.....

2. Working together with the Retinal Preservation Foundation of South Africa (RP Foundation)

The RP Foundation has played an active role in assisting with research into many retinal eye diseases and continues to do so. The Foundation provides many services including counselling and provision of information to the patients and their families. The Foundation would like to keep the names and contact details of all patients with retinal degeneration in South Africa and offer you their services free of charge.

Consent

I agree that the attached details also be given to the RP Foundation provided that the information remains confidential

Signature..... **Print Name**.....

DNA SCREENING PROGRAMME OF RETINAL DEGENERATIVE DISORDERS

DEPARTMENT OF HUMAN GENETICS
FIRST FLOOR, ANATOMY BUILDING, MEDICAL SCHOOL, UNIVERSITY OF CAPE TOWN, OBSERVATORY, 7925
TELEPHONE: (021) 406-6467 FACSIMILE: (021) 448-0906

PATIENTS SURNAME ..... FIRST NAME .....

DATE OF BIRTH ..... TELEPHONE ..... FACSIMILE ....

ADDRESS:.....

SEX: M ☐ F ☐ ETHNIC GROUP: BLACK ☐ CAUCASIAN ☐ INDIAN ☐ MIXED ANCESTRY ☐ OTHER ☐

IN MY OPINION THE PATIENT HAS ONE OF THE FOLLOWING CONDITIONS:

RETINITIS PIGMENTOSA — **Retinal degeneration would appear to be:**
- **The diffuse form** ☐
- **Sectoral (regional) form** ☐

USHER SYNDROME — **Retinitis Pigmentosa and Congenital Hearing Loss:**
- **TYPE 1 (profound deafness)** ☐
- **TYPE 11 (mild to severe deafness)** ☐

MACULAR DEGENERATION
- **BEST DISEASE** ☐
- **FUNDUS FLAVIMACULATUS** ☐
- **PATTERN DYSTROPHY** ☐
- **SORSBY FUNDUS DYSTROPHY** ☐
- **STARGARDT DISEASE** ☐
- **FAMILIAL OR EARLY ONSET (under 55yrs)**
- **AGE RELATED MD** **Wet** ☐ **Dry** ☐

CONE & ROD DYSTROPHY ..... ☐

OTHER RETINAL DISORDERS (SPECIFY) .... ☐

MODE OF INHERITANCE

Dominant Inheritance ☐ **Recessive Inheritance** ☐ **Unknown but Familial** ☐
X-Linked Inheritance ☐ **Isolated Case** ☐
Number of family members affected: .....

AGE OF ONSET ..... **(years) VISUAL ACUITY: Left ..... Right .....**

PROGRESSION OF DISEASE: .....

OTHER CLINICAL FEATURES: .....

TESTS PERFORMED

ERG	☐	Colour Fundus Photographs	☐
Visual Acuity	☐	Fluorescein angiogram	☐
Visual Fields	☐	DNA Screening Programme	☐
Ishihara Test	☐		

FUNDUS EXAMINATION

****RIGHT EYE**

LENS: Yes / No Pseudophakic Cataract: /Aphakic : Yes / No
DISC: Normal ..... Waxy Pallor ..... Other .....
VESSELS: Normal ..... Attenuated .....Other .....

MACULA: Comments .....
Exudates Yes ☐ No ☐
Epiretinal membrane Yes ☐ No ☐
Granularity Yes ☐ No ☐
FOVEA: Appearance.....
PERIPHERAL RETINA:
Comment: .....
Bone spicule: Yes ☐ No ☐ Distribution: .....
Quality and distribution of pigment:
Pigment: ..... Depigmentation: .....
White dots .....
Quadrant ..... Periphery .....

VITREOUS: Normal Yes ☐ No ☐
Cellularity Yes ☐ No ☐
Vitreous Floaters Yes ☐ No ☐

****LEFT EYE**

LENS: Cataract Yes/No Pseudoaphakic/Aphakic Yes/no
DISC: Normal .....Waxy Pallor..... Other .....
VESSELS: Normal .....Attenuated ..... Other ....

MACULA: Comment: .....
Exudates Yes ☐ No ☐
Epiretinal membrane Yes ☐ No ☐
Granularity Yes ☐ No ☐
FOVEA: Appearance .....

PERIPHERAL RETINA:
Comment .....
Bone spicule: Yes ☐ No ☐ Distribution: .....
Quality and distribution of pigment:
Pigment: ..... Depigmentation: .....
White dots .....
Quadrant ..... Periphery .....

VITREOUS: Normal Yes ☐ No ☐
Cellularity Yes ☐ No ☐
Vitreous Floaters Yes ☐ No ☐

NAME OF DOCTOR: (Please Print) .....SIGNATURE:.....
TELEPHONE: ..... FACSIMILE :.....
DATE::.....

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