# Mutation analysis of important retinal candidate genes: progressing from research to diagnostic service

By

Lisa Jane Roberts BSc (Hons)

RBRLIS002



SUBMITTED TO THE UNIVERSITY OF CAPE TOWN

In fulfilment of the requirements for the degree of

MSc (Med) in Human Genetics

August 2006

Supervisor: Dr. G. Rebello

Co-Supervisors: A/Prof. J. Greenberg & Prof. R. Ramesar

Division of Human Genetics, University of Cape Town

# DECLARATION

I, Lisa Jane Roberts, hereby declare that the work on which this dissertation is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university. This study has been approved by the Research Ethics Committee of the Faculty of Health Sciences; reference number 196/2006.

I empower the university to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

Signature:

Signed by candidate

Date:

14 August 2006

## Dedication

To my parents - the most generous people I know - who lead by example

and, in doing so, continue to teach me the important stuff.

They are always supportive, never predictable,

and still offer to phone me back.

## Acknowledgements

I would like to express my sincere thanks to

- My supervisor, and friend, George Rebello, whose help, support and advice (even before I registered for this degree) has meant so much. Also to his family: Renate, Kate and Anne, for countless Friday night dinners and special times.
- My co-supervisor, Jacquie Greenberg, for listening. I am so grateful for her patience, kindness and encouragement.
- My co-supervisor, Raj Ramesar, for providing a challenging and interesting environment in which to grow and learn.
- My fabulous family, especially Stephen, Daryn, Nanna, Itala, Diana and Fred, who give me courage, make me laugh and are always in my corner.
- My friends outside the lab who checked in regularly and even bought supplies to keep me going.
- My friends and colleagues in the Division of Human Genetics, especially Janine S, Janine D, Alvera, Gemma and George, for providing hilarious conversations, chair ballet and a variety of other entertaining dances, as well as wonderful support. I cannot mention everyone but they all make it fun to come to work.
- My colleagues: Sister Lecia Bartman, for following up on family pedigrees and clinical information; Alvera Voster for her help with the MLPA technique; Annapurna Hazra for her assistance with the statistical calculations; JoJo Cheung and Don Ping for the translation of the Wang et al. publication (2005) and Nicki Tiffin for reading and providing feedback on parts of this thesis.
- Adri Winkler, the Postgraduate administration manager, who told me with kind words and great insight exactly what I needed to hear.
- All families with RDDs in South Africa, for their participation in the research programme. To Retina South Africa, the University of Cape Town, the South African Medical Research Council, and Technology and Human Recourses for Industry Programme (THRIP, South Africa) for supporting this research programme

# Table of Contents

# List of Figures

# List of Tables

# Abbreviations

| | |
|---|---|
| °C | degree Celcius |
| µl | microlitre |
| ADRP | autosomal dominant retinitis pigmentosa |
| Ala | alanine |
| APS | ammonium persulphate |
| Arg | arginine |
| ARRP | autosomal recessive retinitis pigmentosa |
| BLAST | basic local alignment search tool |
| bp | basepairs |
| cDNA | complementary DNA |
| cGMP | cyclic guanosine monophosphate |
| cM | centimorgan |
| Cys | cysteine |
| dbSNP | SNP database |
| DCX | doublecortin |
| dHPLC | denaturing high performance liquid chromatography |
| DNA | deoxyribonucleic acid |
| dNTPs | deoxynucleotide triphosphates |
| EDTA | ethylenediaminetetra-acetic acid |
| ER | endoplasmic reticulum |
| ERG | electroretinogram |
| fwd | forward |
| g | gram |
| Glu | glutamic acid |
| GPCR | G-protein coupled receptor |
| $H_2O$ | water |
| His | histidine |
| Ile | isoleucine |
| IVS | intervening sequence |
| kb | kilobase |
| KCl | potassium chloride |
| kDa | kilodalton |
| LCA | Leber congenital amaurosis |
| Leu | leucine |
| Lys | lysine |
| MAP | microtubule-associated protein |
| Mb | megabase |
| Met | methionine |
| mg | milligram |
| $MgCl_2$ | magnesium chloride |
| min | minute |
| ml | millilitre |
| MLPA | multiplex ligation-dependent probe amplification |
| mM | millimolar |
| mRNA | messenger RNA |

# Abbreviations continued

| | |
|---|---|
| NCBI | National Centre for Biotechnology Information |
| ng | nanogram |
| PCR | polymerase chain reaction |
| PDA | piperazine diacrylamide |
| Phe | phenylalanine |
| pmol | picomole |
| Pro | proline |
| RDD | retinal degenerative disorder |
| rev | reverse |
| *RHO* | rhodopsin |
| RNA | ribonucleic acid |
| RP | retinitis pigmentosa |
| *RP1* | retinitis pigmentosa1 |
| RPE | retinal pigment epithelium |
| *RPE65* | retinal pigment epithelium-specific protein 65kDa |
| SA | South Africa |
| sec | second |
| Ser | serine |
| SNP | single nucleotide polymorphism |
| SSCP | single stranded conformational polymorphism |
| $T_a$ | annealing temperature |
| TBE | tris borate EDTA buffer |
| TEMED | NNN'N'- tetramethylethylenediamine |
| Thr | threonine |
| Tris | tris[hydroxymethyl]aminomethane |
| Tyr | tyrosine |
| U | units |
| UCT | University of Cape Town |
| UK | United Kingdom |
| USA | United States of America |
| UTR | untranslated region |
| UV | ultraviolet |
| V | volts |

# Abstract

Approximately one third of all human inherited disease includes defects of the eye. Retinal degenerative disorders (RDDs) are a group of diseases characterised by photoreceptor cell death in the retina and consequent vision loss. The Division of Human Genetics at the University of Cape Town (UCT) has samples archived in the RDD DNA database from over 1000 South African families. The research in this Division currently involves mutation screening of retinal candidate genes, with the goal of identifying the causative genetic mutation in each of the families registered in the database, in order to facilitate future therapeutic intervention.

The purpose of this study was to determine the distribution and clinical utility of mutations in important candidate genes in a subset of South African RDD patients. To this end, three important retinal candidate genes were selected and screened in appropriate patient cohorts. The mutation analysis included screening for large deletions which is a novel approach in the study of RDDs. The screening of Rhodopsin (*RHO*) in 61 individuals, retinitis pigmentosa 1 (*RP1*) in 70 individuals and retinal pigment epithelium-specific protein 65kDa (*RPE65*) in 87 individuals led to the identification of 10 families for whom a molecular diagnostic service can now be provided. For most families the amount of useful information available without further research is minimal, however for four of the families, therapeutic interventions may be possible, now or in the near future.

In addition to the pathogenic mutations found, 17 single nucleotide polymorphisms (SNPs) were identified during this study. Furthermore, a significant association between ethnicity and the frequency of the high and low risk alleles of two of these SNPs (that may modify the phenotype of RDDs) was shown. This information may be useful in providing diagnostic or prognostic indicators in the future.

The utility of RDD research should not be trivialised as it identifies families who may benefit from current interventions or be eligible for possible therapeutic trials, eliminates gene candidates in families, and is necessary for understanding the disease (which in itself is a requirement for development of therapies).

# 1 Introduction

## 1.1 The retina and vision

Vision is the process whereby incoming photons of light are converted into neural signals that are transmitted to the brain where they are processed into a representation of the image being viewed. Vision is mediated by the retina, a transparent tissue lining the posterior segment of the eye (Figure 1.1). Light passes through the cornea and is directed onto the retina by the lens.

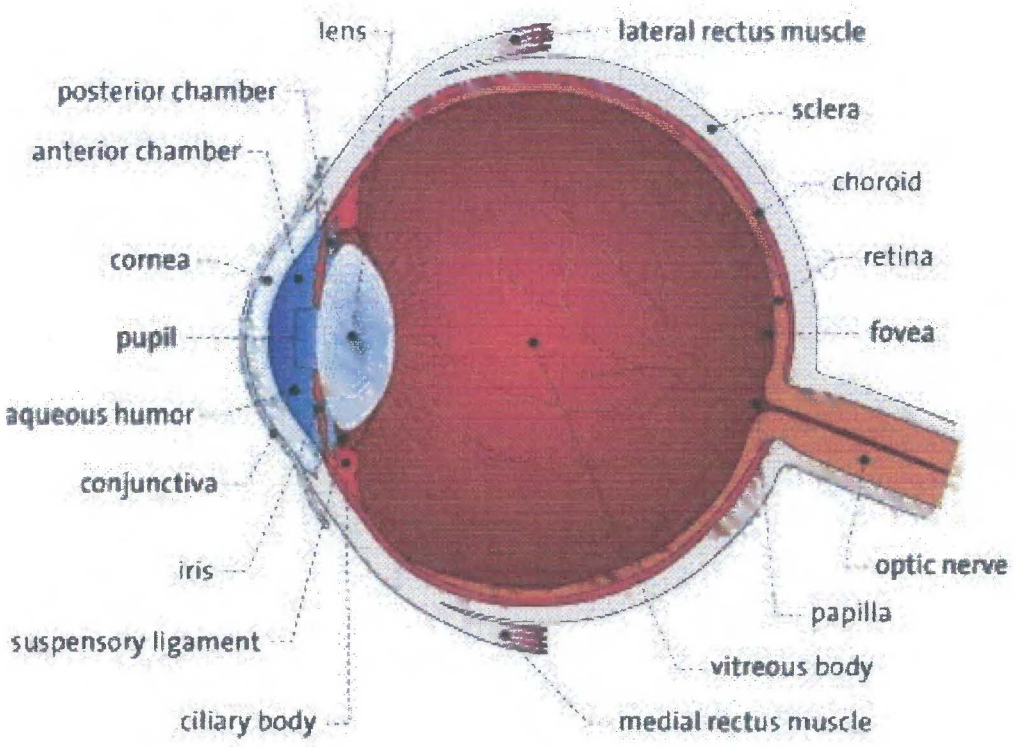



**Figure 1.1** A diagrammatic cross section of the human eye, showing the position of the retina and other important components involved in vision.

Reproduced from the Rodenstock Vision Care Products website: http://www.rodenstockusa.com/con_disease.asp

The retina of an adult human eye contains approximately 92 million rod photoreceptor cells and 5 million cone photoreceptor cells [1]. These photoreceptors are the site of the phototransduction cascade, the pathway where photons of light are converted into chemical signals that are transported via the optic nerve to the brain and ultimately result in visual cortex stimulation (Figure 1.2). Although they use the same basic mechanism for phototransduction, rod cells allow monochromatic vision in dim light, while cone cells allow colour perception and detailed vision in bright light [2].

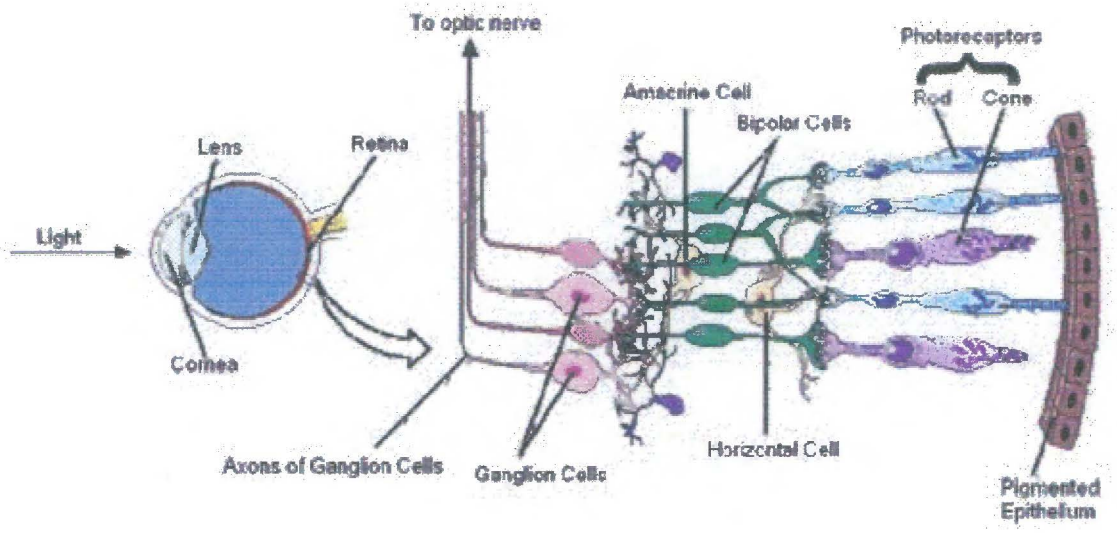



**Figure.1.2** A diagram showing the layers of the retina involved in the phototransduction cascade.

Reproduced and adapted from the SmartDraw website:
http://www.smartdraw.com/examples/medical/images/retina.gif
Light passes from left to right, through the neural cells (ganglion, amacrine, bipolar, horizontal and photoreceptor cells), and the corresponding chemical signal passes from right to left, from the photoreceptor to the optic nerve.

The photoreceptor cells are in close contact with the retinal pigment epithelium (RPE). The human retina contains approximately 5 million of these RPE cells, arranged in a single-cell layer between the photoreceptors and the choroid. The RPE functions to maintain the photoreceptor cells by providing nutrients and metabolites and phagocytosing waste products [1, 2].

## 1.2 Retinal degenerative disorders

Approximately one third of all human inherited disease includes defects of the eye [3]. Retinal degenerative disorders (RDDs) are characterised by photoreceptor cell death in the retina and consequent vision loss. Over 166 genes have been implicated in RDDs, of which 116 have been identified [4]. Remarkable genetic, phenotypic and clinical heterogeneity have been observed in the study of RDDs:

- Genetic heterogeneity refers to the fact that different mutations can lead to the same clinical characteristics or phenotype (in this case, a specific RDD). Genetic heterogeneity can be further defined as either allelic or locus heterogeneity: allelic heterogeneity describes different mutations at the same locus causing the same phenotype; locus heterogeneity describes mutations at different loci causing the same phenotype. RDDs display both allelic and locus heterogeneity.
- Phenotypic heterogeneity refers to the fact that one defective gene may cause different phenotypes in different individuals.
- Clinical heterogeneity refers to the same mutation causing different phenotypes in different individuals [5].

Retinal dystrophies can be either progressive or stationary (non-progressive). Rod dystrophies can present as stationary nightblindness (due to rods simply not functioning adequately) or nightblindness which can progress to a loss of peripheral vision (due to subsequent rod death). The loss of peripheral vision can, in turn, lead to total blindness (due to a secondary loss of cones). Cone dystrophies present with a loss of central vision. Some RDDs can also be associated with extra-ocular disease and are thus classified as syndromic [6]. These issues all confound the study of retinal dystrophies as the clinical boundaries separating diagnoses frequently depend on the judgment of the ophthalmologist.

A molecular confirmation of diagnosis is also complicated by the fact that inherited RDDs can segregate in autosomal dominant, autosomal recessive, X-linked, digenic (diallelic/ triallelic) or mitochondrial modes, with, or without, reduced penetrance [6].

### 1.2.1 Retinitis pigmentosa

Retinitis pigmentosa (RP) is the most common RDD, affecting approximately 1 in 3500 people [3]. RP is a group of retinal diseases characterised by nightblindness, progressive loss of peripheral visual fields and eventual loss of central vision. Total blindness occurs in 30% of cases. A definitive clinical feature of RP is the "bony spicule" appearance of pigmentary deposits resulting from RPE cells migrating into the degenerating retina (Figure 1.3). Other features include constriction of retinal arterioles, waxy pallor of optic discs and a reduced electroretinogram (ERG). Cataracts, astigmatism and myopia often develop [3, 7].

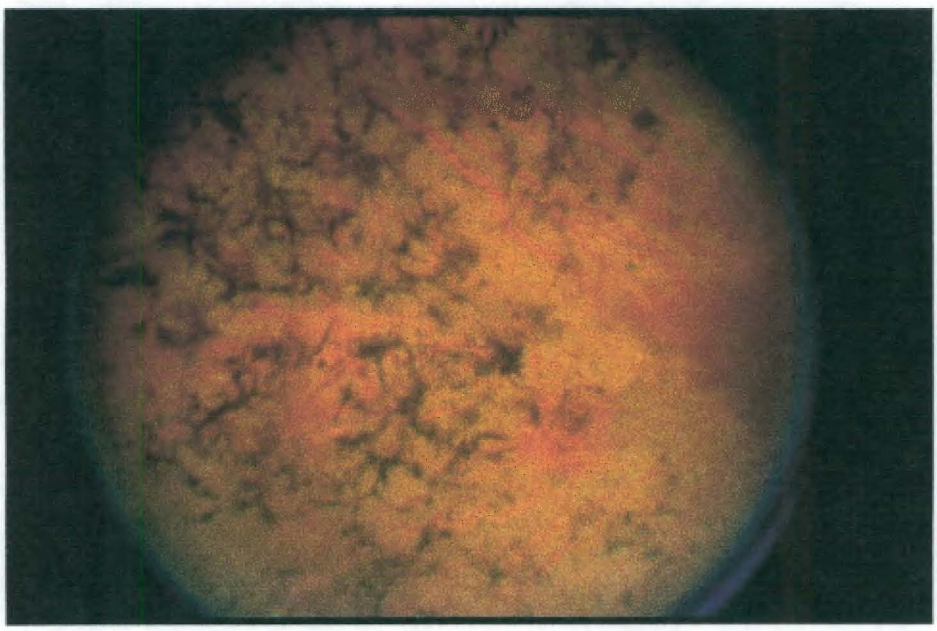


**Figure 1.3** A fundus photograph of a retina exhibiting the characteristic pigmentary deposits of RP, which appear as dark web-like structures.

Reproduced from website of the John A. Moran Eye Centre at the University of Utah: http://insight.med.utah.edu/opatharch/retina/retinitis_pigmentosa.htm

Although there is a wide range of phenotypes with respect to disease severity (with variable age of onset and disease progression), the age of onset can generally be correlated to the mechanism of inheritance: X-linked RP has the earliest age of onset, followed by autosomal recessive, with autosomal dominant having the latest age of onset [8].

Half of RP cases appear to be sporadic (but could be recessive or X-linked) and it is estimated that 20% of RP is autosomal dominant, 20% is autosomal recessive and 10% is X-linked. Digenic RP does occur but is rare. Although RP can be manifest as part of a syndrome, most RP is non-syndromic [3, 7].

Approximately 40 genetic loci have been implicated in non-syndromic RP [3, 4]. Genes implicated in the phototransduction cascade and visual cycle (Vitamin A metabolism) generally cause recessive RP; genes encoding photoreceptor structural proteins, transcription factors and splicing factors generally cause dominant RP. The rest of the genes are classified as miscellaneous (have unknown functions or are involved in intracellular transport, cell adhesion or signalling) and are harder to group, causing dominant, recessive, X-linked and syndromic RP [3].

#### 1.2.1.1 Autosomal dominant retinitis pigmentosa

Autosomal dominant retinitis pigmentosa (ADRP) can be classified into two main clinical types: Type 1 or "diffuse" ADRP, and type 2 or "regional" ADRP [9]. In type 1 ADRP there is diffuse and severe loss of rod function with cone function being preserved. In individuals with type 1 ADRP, onset of symptoms, including nightblindness, occurs early (in the first decade of life). Type 1 disease occurs in approximately 20% of ADRP cases. In type 2 ADRP, there is regional loss of both rods and cones. In individuals with type 2 ADRP, onset of symptoms begins later (in the second or third decade of life) and can vary within or between families, with asymptomatic mutation carriers being reported.

Besides the clinical heterogeneity observed in ADRP, genetic heterogeneity also occurs. Seventeen genes have been implicated in autosomal dominant retinitis pigmentosa (ADRP), of which 15 have been cloned [4].

The genes causing ADRP can be divided into the following functional categories:

- Genes encoding structural proteins of the photoreceptors: peripherin/RDS (*RDS*), retinal outer segment protein 1 (*ROM1*), and rhodopsin (*RHO*).
- Genes encoding transcription factors: cone-rod homeobox transcription factor (*CRX*) and neural retinal leucine zipper (*NRL*).
- Genes encoding splicing factors: pre-mRNA processing factor 8 (*PRPF8*), pre-mRNA processing factor 3 (*PRPF3*), pre-mRNA processing factor 31 (*PRPF31*) and RP9 (*RP9*).
- Genes encoding proteins important for photoreceptor development: retinitis pigmentosa 1 (*RP1*) and retinal fascin homolog 2 (*FSCN2*).
- Genes encoding enzymes: inosine monophosphate dehydrogenase 1 (*IMPDH1*), and carbonic anhydrase 4 (*CA4*).
- Miscellaneous: semaphorin 4A *(SemA4A)* and guanylate cyclase activating protein 1B (*GUCA1B*).

#### 1.2.1.2 Autosomal recessive retinitis pigmentosa

Autosomal recessive retinitis pigmentosa (ARRP) is thought to be the most prevalent form of RP. The symptoms of ARRP often overlap with other retinal diseases, like Leber congenital amaurosis. Once again there is both clinical and genetic heterogeneity observed in this disease - 24 loci have been implicated in ARRP, for which 19 genes have been identified [3, 4, 7, 10].

The genes identified for ARRP can be divided into the following functional groups:

- Genes encoding proteins important in the visual cycle: ATP-binding cassette transporter (*ABCA4*), retinal pigment epithelium-specific protein 65kDa (*RPE65*), lecithin retinol acyltransferase (*LRAT*), retinaldehyde binding protein 1 (*RLBP1*) and retinal G-protein coupled receptor (*RGR*).
- Genes encoding proteins important in the phototransduction cascade: rhodopsin (*RHO*), phosphodiesterase alpha subunit (*PDE6A*), phosphodiesterase beta subunit (*PDE6B*), s-arrestin (*SAG*), cyclic nucleotide gated channel alpha 1 (*CNGA1*) and cyclic nucleotide gated channel alpha 1 (*CNGB1*)
- Genes encoding proteins for cell-cell adhesion or signalling: C- mer proto-oncogene tyrosine kinase (*MERTK*), crumbs homologue 1 (*CRB1*) and usherin (*USH2A*).
- Miscellaneous: tubby like protein 1 (*TULP1*), nuclear receptor subfamily 2 group E3 (*NR2E3*), retinitis pigmentosa 1 (*RP1*), neural retina leucine zipper (*NRL*) and ceramide kinase-like protein (*CERKL*).

### 1.2.2 Leber congenital amaurosis

Leber congenital amaurosis (LCA) accounts for at least 5% of all inherited retinal dystrophies and is considered to be the most severe RDD [11]. LCA is predominantly an autosomal recessive disorder, although autosomal dominant cases have been noted.

LCA is a bilateral congenital blindness diagnosed by a severely diminished or absent ERG before the age of one year [12]. The clinical abnormalities vary from a normal fundus to a pigmented fundus, and a range of symptoms including typical RP, a granular appearance of the RPE, attenuation of retinal vessels and optic atrophy have been noted. Associated clinical symptoms can include nystagmus (roving eye movements), cataracts, photophobia, a

sluggish pupillary reaction, digito-ocular stimulation (eye-poking or rubbing), psychomotor retardation and developmental delay [13-15].

It has been proposed that LCA is caused by three pathological processes [15] :

- Aplasia (abnormal formation of photoreceptors)
- Degeneration (atrophy of the photoreceptors and RPE)
- Dysplasia (biochemical dysfunction, resulting in no primary damage to the photoreceptors or RPE).

Genetic heterogeneity underlies the clinical heterogeneity observed in LCA, with 10 genes having been identified so far [4, 16]. Each of these gene products vary greatly in function (from transcription factor to protein transport or photoreceptor morphogenesis), and cannot be grouped functionally. The genes identified are guanylate cyclase (*GUCY2D*), aryl hydrocarbon interacting protein (*AIPL1*), tubby like protein 1 (*TULP1*), retinal pigment epithelium-specific protein 65kDa (*RPE65*), cone-rod homeobox transcription factor (*CRX*), inosine monophosphate dehydrogenase 1 (*IMPDH1*), lecithin retinol acyltransferase (*LRAT*), crumbs homologue 1 (*CRB1*), retinol dehydrogenase 12 (*RDH12*) and RPGR interacting protein (*RPGRIP1*).

Five of the genes causing LCA have also been implicated in ARRP. In addition to this genetic overlap, a clinical one has been observed. It has therefore been suggested that LCA and early onset ARRP may represent a continuum of phenotypes [16].

## 1.3 Development of therapeutics

The eye is the ideal organ for gene-based therapies – it is small, self contained and isolated from circulation by the blood-ocular barriers [17].

Recessive RDDs (such as LCA and ARRP) generally arise through gene mutations causing a loss of functional product. These disorders are thus good candidates for gene replacement therapies (where a copy of the gene that makes a functional product is introduced). In ocular gene replacement therapy the eye is accessible, requires less viral vector to achieve gene transfer, and the exposure of other organs to these viral vectors is reduced. The most successful ocular gene therapy to date is that of the *RPE65* gene (which causes ARRP and LCA). In 2001, it was reported that wild type *RPE65* restored vision when it was delivered in an adeno-associated viral vector by means of subretinal injection to the eye of the LCA canine [18]. In 2005 the same group reported that a single subretinal treatment restored visual function in approximately 90% of cases and that after three years the responses continued to remain stable [19]. They also noted that RPE65 was not expressed in tissues other than the treatment site and that no deleterious effects were observed as a result of the therapy. The therapy has also been successful in both naturally occurring [20] and genetically engineered [21] mice lacking RPE65. A cohort of patients with LCA due to *RPE65* mutations has been identified by clinical and molecular profiling, and subjects are being recruited for a Phase I trial [22].

Dominant RDDs (such as ADRP) generally arise through gene mutations causing a gain of function, and the mutant protein becomes damaging. Treatment is therefore more complicated, requiring that the mutant protein be eliminated or reduced, whilst allowing the wild type protein to be expressed [17, 23]. The use of a ribozyme, an RNA-cleaving molecule that can be targeted to the mutant mRNA, has reduced photoreceptor degeneration in the rat model of a rhodopsin mutation [24].

Two main challenges have arisen in RDD therapeutics:

- The heterogeneity of mutations causing RDDs has resulted in impractical mutation-dependent approaches.
- The retina is terminally differentiated, and gene therapy can not restore lost photoreceptor cells because no new photoreceptor cells are being produced. These therapies are only successful in halting progression of disease. (RPE65 therapy restores vision in LCA canines as the mutant protein is expressed in the RPE and causes no loss of photoreceptors initially). Therapy must therefore be administered before cell death takes place [25].

For these reasons, research into the use of neural stem cells to restore the retina [26], the direct inhibition of the apoptotic pathways [27] and the use of chaperones to manipulate misfolded proteins and thereby reduce apoptosis [28] is important. Improved understanding of the molecular mechanisms of disease is certainly required before restoring vision becomes a reality. It is anticipated that further understanding of the pathogenesis of RDD gene mutations may lead to the identification of more generic approaches to therapy.

## 1.4 RDD research in South Africa

The Division of Human Genetics at the University of Cape Town (UCT) has had an interest in the genetics of RDDs since 1972 [29] and the RDD project was initiated in 1990 [30]. There are currently samples archived in this database from over 1000 South African families.

The samples have been collected from families throughout South Africa (SA) by genetic nurses in collaboration with the Department of National Health and Population Development and the lay support society, Retina South Africa. A clinical co-ordinator maintains contact with the families, updating clinical information, extending pedigrees and referring individuals to ophthalmologists for confirmation of diagnoses.

Recent RDD research in SA has resulted in the identification of the positional candidate genes on chromosomes 17p and 17q. These loci were identified in two large immigrant families, and were subsequently identified as the *PRPF8* and *CA4* genes, respectively [31, 32].

The Division of Human Genetics at UCT is currently involved in the routine screening of families with RDDs for mutations in candidate genes. The ultimate goal of this screening is to identify the causative genetic mutation in each of the families registered in the UCT RDD database, in order to facilitate management and future therapeutic intervention. Once a mutation has been identified in a family, members of that family are offered a diagnostic service, in order to determine each individual's mutation status.

The priority groups for the screening are the individuals with the most severe form of disease, and/or the individuals with RDDs most likely to be amenable to therapy in the near future. The logical extension of this approach is to use the above mentioned criteria, selecting candidate genes reported in the biomedical literature as most commonly causing RDDs.

## 1.5 Candidate genes selected for this project

### 1.5.1 Rhodopsin (*RHO*)

The *RHO* gene was the first gene to be associated with RP [33, 34]. Mutations in *RHO* have subsequently been shown to be the most common cause of RP, and account for around 30% of all ADRP cases internationally [4, 7, 8] and as much as 50% of cases in the UK [35].

*RHO* is expressed in the rod photoreceptor cells. Rods consist of an outer segment and an inner segment (Figure 1.4). The inner segment contains the metabolic machinery of the cell, and the outer segment consists of an array of membranous discs which are continually shed into the RPE and renewed.

These discs function in maintaining the structure of the rod outer segment as well as housing the components of the visual transduction pathway [1]. RHO comprises more than 90% of the protein content of the disc membranes [36].

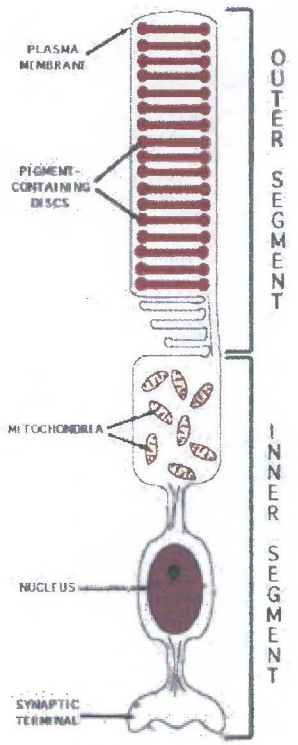



**Figure 1.4** A diagram showing the structure of the rod photoreceptor, with its inner and outer segments.

Reproduced from the website of the Department of Chemistry, Washington University: http://www.chemistry.wustl.edu/~edudev/LabTutorials/Vision/Vision.html

*RHO* encodes a G-protein coupled receptor (GPCR) which acts in the first stage of the signal transduction cascade in rods, mediating vision in dim light. The GPCR is a heptahelical transmembrane protein, opsin, coupled to the G-protein transducin. RHO is inactive when covalently linked to the chromophore 11-*cis* retinal. When a photon of light is absorbed by the retina, the 11-*cis* retinal isomerises to all-*trans* retinal, which causes a conformational change of RHO to the active form, metarhodopsin II. This in turn activates transducin, which triggers a biochemical cascade that ultimately causes a drop in the cGMP concentration. This decrease in cGMP levels leads to hyperpolarisation of the rod cell membrane, which generates a nerve impulse to the brain (visual cortex stimulation) [36-38].

Up to 150 pathogenic mutations have been identified in *RHO* [37], the two most common mutations being Pro23His and Pro347Leu. *RHO* mutations predominantly cause ADRP, though some cases of ARRP have been reported as being due to *RHO* mutations (Figure 1.5).

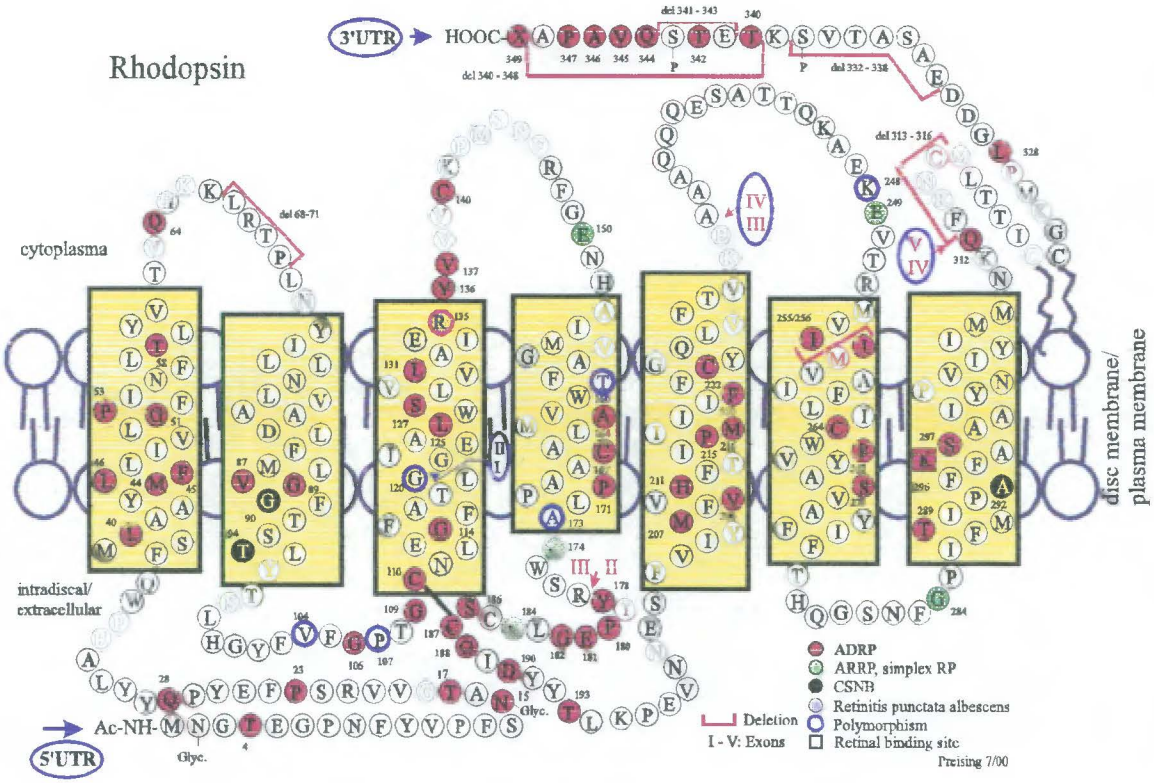



**Figure 1.5** A diagram showing the distribution of pathogenic mutations of the heptahelical transmembrane RHO protein.

Reproduced from the Retina International mutation database:
http://www.retina-international.org/sci-news/rhomut.htm

The disease-causing mutations identified in the *RHO* gene affect the extracellular, transmembrane and intradiscal domains of the RHO protein. *RHO* mutations are classified as either Class I or Class II mutations. Class I mutations occur predominantly near the carboxyl terminus of the protein or the first transmembrane domain and account for 15% of the mutations. Class I mutations result in a protein that resembles wild type RHO in yield, forms a functional chromophore with 11-cis retinal, and translocates to the plasma membrane as normal [39, 40]. This class of mutation affects the post-Golgi trafficking, and RHO is not directed towards the photoreceptor outer segment but accumulates in the cell body or extracellular vesicles. Class II mutations

occur in the extracellular, transmembrane and intradiscal domains and account for 85% of the mutations. This class of mutation causes misfolding of the protein, which cannot form a functional chromophore and accumulates in the endoplasmic reticulum (ER) [39, 40].

Recently, a new system of classification has been proposed by Michael Cheetham and colleagues from the Institute of Ophthalmology at the University College London [40]. This system divides RHO mutations into six classes which are not mutually exclusive, and proposes that mutations with no biochemical or cellular defect remain unclassified. The new classes proposed are:

- Class I: Fold normally but are not transported to the outer segment
- Class II: Misfold, do not reconstitute with 11-cis-retinal and are retained in the ER
- Class III: Affect endocytosis
- Class IV: Affect stability of the protein and posttranslational modification, but not necessarily through misfolding
- Class V: Show an increased activation rate for the G-protein (transducin)
- Class VI: Constitutively activate opsin in the absence of chromophore and light

Regardless of the classification system used, RHO misfolding emerges as one of the main causes of ADRP [37, 39]. It is also likely that dominant *RHO* disease-causing alleles are generally gain of function mutations, causing cell death through various mechanisms. Future therapy is thus dependent on accurate molecular diagnosis and classification of each mutation [40].

### 1.5.2 Retinitis pigmentosa 1 (*RP1*)

*RP1* was the first locus reported to be associated with RP. This locus was assigned to chromosome 1 in 1977 [41], although this finding was later retracted and the locus re-assigned to chromosome 8 [42, 43]. Twenty years later, the retinitis pigmentosa 1 gene (*RP1*), also called "Oxygen regulated protein 1" (*ORP-1*), was identified and mutations therein were found to cause ADRP [43, 44]. In 2005, homozygous mutations in *RP1* were reported to cause ARRP in three consanguineous Pakistani families [45], although there have been no other reports of recessive mutations in this gene. *RP1* mutations are thought to cause 4-10% of ADRP [43, 46] with the Arg677X mutation alone accounting for 2% of ADRP. This is the third most common mutation contributing to ADRP after the 2 *RHO* mutations; Pro23His and Pro347Leu [43].

The RP1 protein is expressed exclusively in the outer segment portion of the axoneme in the photoreceptor cells. The microtubule-based axoneme begins in the inner segment and passes through the cilium into the outer segment. The function of the axoneme is unknown, but it may be required to stabilise the membrane discs [47, 48].

Pathogenic *RP1* mutations generally cause frameshifts or truncations of the protein and cluster in a region spanning approximately 550 codons of the last exon (exon 4). Mutant mRNA appears to escape nonsense-mediated decay and the truncated protein is expressed. This mutant protein localises correctly to the axoneme in mice but does not demonstrate the concentration in this area, which the wild type does. In addition, the outer segments show incorrectly oriented discs that are not stacked into their proper arrays [47].

The N-terminus (exons 2 and 3) of RP1 has 2 tandem domains with homology to the microtubule-binding domains of doublecortin (DCX). These domains have been shown to bind to microtubules from the retina and enhance tubulin polymerisation. When these domains were removed in mice mutants, the axonemes were shorter than normal and the RP1 was

mislocalised towards the photoreceptor inner segment. RP1 is therefore the first photoreceptor microtubule-associated protein (MAP) identified. MAPs regulate microtubules and are thus indirectly responsible for determining cell shape [48].

These findings indicate that the N-terminal of RP1 with the DCX domains functions to control and stabilise the photoreceptor axoneme and localise the protein correctly. The C-terminal of RP1 could then link the discs to the axoneme (either directly or by interacting in a protein complex at the junction between the outer segment and cilium) in order to correctly orientate and stack the discs of the photoreceptors. Mutations at the C-terminal would thus cause a loss of connection between the discs and the axoneme, resulting in the defective disc stacking observed. This disorganisation of the photoreceptor outer segment would eventually cause cell death [47, 48].

A wide range of disease onset and severity is caused by *RP1* mutations, from patients only 11 years old experiencing nightblindness, to asymptomatic carriers at age 66 [49, 50]. Despite this clinical heterogeneity, heterozygous mutation carriers can generally be said to exhibit type 2 ADRP (later age of onset) while homozygous carriers are more severely affected and have an earlier age of onset [45, 50, 51].

The clinical heterogeneity observed suggests other genetic or environmental factors may modify the phenotype of *RP1* mutations. It has recently been proposed that there are interactions between *RHO* and *RP1* with one variant in *RHO* (IVS4-23G>A) and two in *RP1* (N985Y and C2033Y) acting together to increase the risk of developing RP. In addition, a single nucleotide polymorphism (SNP) in the 5' untranslated region (UTR) of *RHO* ("-26G>A" in the reference but hereafter called "g.269A>G") is associated with an increased risk of RP, while R872H in *RP1* is associated with a decreased risk of RP [52].

### 1.5.3 Retinal pigment epithelium-specific protein 65kDa (*RPE65*)

The retinal pigment epithelium-specific protein 65kDa (RPE65) was cloned in 1993 [53] and the gene encoding this 61 kDa protein expressed in the RPE was characterised in 1995 [54]. Mutations in *RPE65* are responsible for approximately 2% of ARRP and 16% of LCA [55].

RPE65 functions in the visual retinoid cycle, which is the process by which RHO is regenerated. As discussed previously, RHO consists of the apoprotein opsin, attached to a chromophore, 11-*cis* retinal. During visual transduction the 11-*cis* retinal is isomerised to all-*trans* retinal. Regeneration of 11-*cis* retinal is essential for visual function and takes place via the visual retinoid cycle (Figure 1.6) [56].

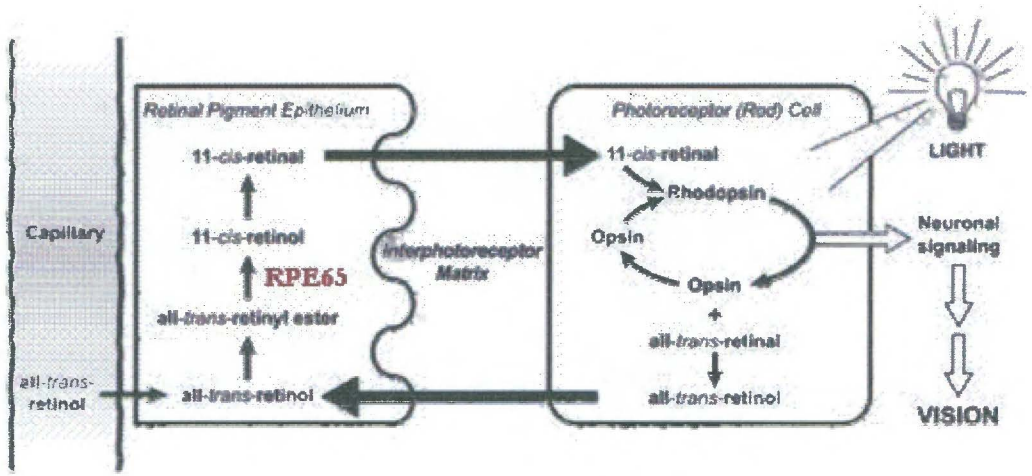



**Figure1.6** A diagram showing the regeneration of 11-cis retinal via the visual retinoid cycle, and the stage at which RPE65 functions in this cycle.

Reproduced and adapted from the website of the Linus Pauling Institute, at the Oregon State University: http://lpi.oregonstate.edu/infocenter/vitamins/vitaminA/visualcycle.html

It has recently been shown that RPE65 is an isomerohydrolase enzyme, converting all-*trans* retinyl ester to 11-*cis* retinol [56]. This explains previous findings that *RPE65* knockout mice [57] and mice with a nonsense mutation in *RPE65* [58] lack 11-*cis* retinal and functional RHO, but accumulate opsin and all-*trans* retinal esters.

The blindness associated with *RPE65* mutations is biochemical, meaning it is initially due to a lack of visual pigment. The RPE itself is healthy, although photoreceptors eventually start to degenerate as a secondary event. *RPE65* mutant mice show normal retinas initially, with photoreceptor outer segments becoming shortened only after seven months and disappearing completely after 27 months [58]. It has been suggested that in *RPE65* null mutants, continuous light-independent opsin activity is responsible for retinal degeneration although the process by which this occurs has not been identified [59]. The lack of immediate degeneration makes *RPE65* an excellent candidate for therapy, and as mentioned previously, several groups have reported that *RPE65* gene delivery generates isomerohydrolase activity and restores vision in canine and murine models of disease [18-22].

As with *RP1* and *RHO*, modifier effects have been reported in *RPE65* [60-62]. The Leu450Met variant in *RPE65* was found to protect against light damage and reduce retinal degeneration by modulating RHO regeneration. Light damage to photoreceptors is caused by excess absorption of photons by RHO. The Leu450Met variation leads to a reduced level of RPE65, which in turn causes a reduced capacity for RHO regeneration, fewer photons can be absorbed and light damage susceptibility is reduced.

A founder mutation in *RPE65*, Tyr368His, was described as causing an early onset severe retinal dystrophy in an isolated Dutch population [63]. The founder effect in South Africans descended from Dutch settlers has been reported for other diseases, and this gene is an excellent candidate for an investigation of this nature [64, 65].

## 1.6 Aims of this project

The primary aim of this project is to determine the distribution and clinical utility of mutations in important candidate genes in a subset of South African RDD families. The secondary aims are to identify families in the UCT RDD database for which a molecular service can be offered, and to assess the information which could be provided to the families with regard to their relevant mutation(s).

The proposed experimental plan is as follows:

1. To examine the DNA of selected families in the RDD database for mutations in three important candidate genes as described in the biomedical literature.
2. To develop diagnostic tests for returning results arising from the research to the families.
3. To assess all the available information, including frequency, genotype-phenotype correlations and molecular pathogenesis of the mutations identified.

Once a family has been identified as having a pathogenic mutation, a diagnostic assay will be developed for that family. The relevant diagnostic assay will be performed on the family to ensure that the test is reproducible and sensitive, and that the mutation co-segregates with the disease in the family and follows the expected pattern of inheritance. The diagnostic test will only be made available to individuals belonging to that family, as the high level of mutational heterogeneity precludes the offering of this mutation-specific diagnostic test to other individuals with RDDs. The mutations will be assessed in terms of molecular mechanism of disease, population frequency and genotype-phenotype correlations (using published literature) to determine the clinical utility of identifying genetic changes in South African families with RDDs.

# 2 Materials and Methods

## 2.1 Patient recruitment and cohort selection

No new patients were recruited specifically for the mutation screening portion of the project. All screening was performed on DNA archived in the UCT RDD DNA bank, which has been collected for the broader RDD project of the Division of Human Genetics. As mentioned previously, affected individuals and their family members are referred to the Division of Human Genetics from throughout SA. Biological material, relevant clinical details and demographic information from over 1000 families are currently archived in the database. Informed consent was obtained according to the tenets of the Declaration of Helsinki (2000). The consent forms and patient information sheets used for recruiting these families were approved by the UCT research ethics committee in 1999 (Ref. 196/99), and this specific research project was approved in 2006 (Ref. 196/2006).

It should be noted that due to a historical ascertainment bias the ethnic breakdown of the samples archived does not reflect the population distribution. Due to lack of resources in rural areas, individuals referred by clinics and private doctors in urban areas were more frequently recruited, resulting in a preponderance of Caucasian individuals. This limitation is currently being addressed. Samples were classified as being derived from Caucasian, Black, Indian or Mixed Ancestry population groups. "Caucasian" refers to people of Western European origin, mainly Dutch, French, German and British; "Black" refers to people of indigenous Black African origin and "Indian" refers to people whose ancestors settled in SA from the Indian subcontinent. The "Mixed Ancestry" group (sometimes termed "Coloured") is the most complex, comprising individuals whose ancestry is a mixture of Caucasian, Black African, Khoi-San (Hottentots and Bushmen), Malay (Javanese and Sumatran), Madagascan and West African [66]. The ethnic breakdown of the cohorts selected for mutation analysis of the candidate genes is shown in Table 2.1.

For the initial mutation screening, DNA of one affected individual from each family in the selected cohort was screened. A cohort of 61 individuals was selected for screening of *RHO*. Of these individuals, 47 were classified as having ADRP and 14 were classified as having ARRP. None of the individuals selected had any causative RDD mutation previously identified. For the *RP1* mutation screen, a cohort 70 individuals was selected, all of whom were classified as having ADRP. Of these individuals, 13 had mutations identified in other RDD genes including five with *RHO* mutations. These individuals were initially included to investigate the possible effect of modifier alleles, and evidence supporting this approach has recently been reported [52]. For the *RPE65* mutation screen, a cohort of 87 individuals was selected. Of these individuals, 18 were classified as having LCA and 69 as having early onset ARRP (with an age of onset younger than 15 years). None of the individuals selected for *RPE65* screening had causative mutations identified in other genes.

In addition to the conventional screening of candidate genes for genetic variations, a novel approach for insertion/deletion screening was undertaken. For the multiplex ligation-dependent probe amplification (MLPA) screen to analyse the *RHO*, *RP1* and *RPE65* genes for gross deletions, a cohort of 50 individuals was selected. Of these individuals, 34 were classified as having ADRP, 14 with LCA and two with early onset ARRP. Of these individuals, three were included despite having a causative mutation identified: one individual with a 10bp deletion in *RP1* was included to determine whether a deletion of this small size could be identified by MLPA, and the two individuals with ARRP were included as only one mutant allele had been identified in *RPE65* in each of these individuals.

**Table 2.1** The ethnic breakdown of the cohorts selected for mutation analysis of candidate genes.

| Screen | Caucasian | Black | Indian | Mixed Ancestry | Other (Taiwanese) | Total |
|---|---|---|---|---|---|---|
| *RHO* | 43 | 9 | 2 | 7 | 0 | 61 |
| *RP1* | 45 | 17 | 2 | 6 | 0 | 70 |
| *RPE65* | 66 | 7 | 10 | 3 | 1 | 87 |
| MLPA | 27 | 18 | 2 | 2 | 1 | 50 |

If a pathogenic mutation was detected in an affected individual, DNA from other family members recruited for the research was tested to determine whether there was co-segregation of the variant with the phenotype.

For the population frequency analysis of a novel variant, DNA from a cohort of 50 individuals was screened for that variant. The control cohort selected for this purpose was matched for ethnic group to the test sample in which the variant was first detected. These control individuals were not assessed for the presence of RDDs.

## 2.2 Gene annotation and primer design

The final annotations of the candidate genes were based on the genomic sequences downloaded from the National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/) and the Ensembl Genome Browser (http://www.ensembl.org/). The annotations were based on the version of the genome available on 1 November 2005. Approximately 5kb upstream and downstream of each gene were included, and the sequence files were converted into annotated forms using a perl script designed by Dr. G. Rebello (personal communication). The final annotated sequences of the three genes can be seen in Appendix 1, which serves as the reference for all the sequence-based information in this project, including primers and MLPA probes used, and all sequence variants detected.

All the primers used for mutation screening were selected prior to the start of this project by other researchers. The primers for *RHO* and *RP1* had been reported previously [9, 67]. The primer sequences were aligned onto the gene sequences to confirm their location. For *RHO* and *RPE65*, primers spanned each of the exons including the intron/exon boundary. Large exons (greater than 500bp in length) were divided into smaller, overlapping fragments. Small exons with short intervening sequences were combined into a single fragment by the positioning of the primers. For *RP1*, primers amplified three overlapping fragments spanning the mutation hotspot (as described by Bowne *et al.* [67]) in exon 4 of the gene.

After much experimental optimisation, three primer pairs were found to be unacceptable, namely *RHO* exon 5, *RPE65* exon 10 and *RPE65* exons 11-13. Professor T. Dryja provided assistance and advice with the sequences of the primer pair spanning *RHO* exon 5. The primer pairs spanning *RPE65* exon 10 and exons 11-13 were redesigned using the following criteria:

- The primers are at least 60bp into the intron, from the intron/exon boundary.
- Primers are approximately 20 nucleotides in length and have % GC contents between 45 and 55%. Forward and reverse primers have similar melting temperatures (1°C apart) in the range of 55°C - 62°C.
- Primers forming problematic heterodimers, homodimers and hairpins are avoided.
- Primer pairs amplifying unwanted sequences, as assessed by BLAST [68] are avoided.

For the purpose of primer design, a combination of bioinformatics tools was used, namely Primer3 [69], Integrated DNA Technologies' OligoAnalyzer version 3.0 (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/), Oligocalculator (http://bioinformatics.org/JaMBW/3/1/9/index.html) and NCBI BLAST [68]. The final primers used for the mutation screens of the three

candidate genes are listed in Table 2.2 and their positions on the annotated sequences can be viewed in Appendix 1.

**Table 2.2** Final PCR primer sequences used for mutation screening.

| Gene | Exon | Forward primer (5' → 3') | Reverse primer (5' → 3') |
|---|---|---|---|
| ***RHO*** | 1A | TTCGCAGCATTCTTGGGTGG | AGCAGGATGTAGTTGAGAGG |
| | 1B | CAACTTCCTCACGCTCTACG | CATTGACAGGACAGGAGAAG |
| | 2 | CCGCCTGCTGACTGCCTTGCAG | GCTTCTTCCCTTCTGCTCAGTG |
| | 3 | TTGGCTGTTCCCAAGTCCCT | TCCAGACCATGGCTCCTCCA |
| | 4 | TCACGGCTCTGAGGGTCCAG | GAGTAGCTTGTCCTTGGCAG |
| | 5 | ACTCAAGCCTCTTGCCTTCC | GCCACAGAGTCCTAGGCAGG |
| ***RP1*** | 4F | TGCTCAGTGTGGTTTAACAAAAC | CTATGGAAATTCTTGGAAATCG |
| | 4G | GGAAGACCTCCAGAAAAGTGATAC | CATTCCTCTCAAATACCCAGATG |
| | 4H | CCAAAGATTTTTATGCACCG | CAATTTACCACACTCGTTTCATTTC |
| ***RPE65*** | 1 | GAGAGCTGAAAGCAACTTCTG | ATAGCACATTTATCATGAATCCATG |
| | 2 | CTATCTCTGCGGACTTTGAGC | GCCAGAGAAGAGAGACTG |
| | 3 | GGCAGGGATAAGAAGCAATG | CTGAGTTCAGAGGTGAAAAC |
| | 4-5 | CTGTACGGATTGCTCCTGTC | GAACATCACCTAGCACTGTG |
| | 6 | TATAATGTATCTTCCTTCTCTCAAC | CTCACAATACAGTAACTTTCTCAC |
| | 7-8 | AAATAAGAGGCTGTTCCAAAGC | TTAAACACATCTTCTTCAGAATCAC |
| | 9 | GTACACTTTTTTCCTTTTTAAATGCATC | GTTTTAGATGTGATTCAGATTGAGTG |
| | 10 | TGCCTGTGCTCATGTTTGAC | TGAGAGAGATGAAACATTCTGG |
| | 11-13 | GTTTGAATTCTTTCCTGCTCAC | CTAACATACAGAACTGCAGTAAG |
| | 14 | AGTCAGAAAAAGAAGTCAGGTC | ATTGCTTGCTCAACTCAGTGC |

## 2.3 Mutation Screening

All the standard recipes used for mutation screening are described in Appendix 2.

### 2.3.1 Polymerase chain reaction

Polymerase chain reaction (PCR) had to be optimised for each set of primers. Generally, PCR was performed in a 25µl or 50µl volume using 200ng genomic DNA, 10 or 20pmol fwd and rev primer, 0.2mM dNTPs, 1X buffer, 1.5mM magnesium chloride and 0.1 - 1U Taq DNA polymerase. The general thermal cycling conditions used were as follows: 95°C for 5 minutes, followed by 30 cycles of {94°C for 30 seconds, $T_a$°C for 30 seconds, 72°C for 40 seconds} and finally 72°C for 7 minutes.

Parameters altered to achieve optimal PCR included the amount of DNA template, final concentration of magnesium chloride ($MgCl_2$), annealing temperature ($T_a$) and the cycling conditions. The type of commercial Taq DNA polymerase used, and its corresponding buffer, was also a factor in the optimization process. Invitrogen™ Taq (Invitrogen™ Life Technologies, UK), Biotaq (Bioline Limited, UK) or GoTaq® (Promega, USA) were used. The 10X Invitrogen™ buffer contains no $MgCl_2$ so it could be added as needed; the 10X Biotaq buffer contains potassium chloride (KCl) so if $MgCl_2$ was required an in-house buffer was used; and the 5X GoTaq® buffer contains 7.5mM $MgCl_2$.

Each *RHO* PCR had to be optimised twice, once for traditional single stranded conformational polymorphism analysis (SSCP), restriction enzyme digests and automated sequencing, and once for SSCP using the Multiphor™ II electrophoresis system. The main parameter altered for the Multiphor™ SSCP PCR was the thermal cycling condition: the time for each stage was reduced and the cycling was generally as follows: 94°C for 3 minutes, followed by 28 cycles of {94°C for 15 seconds, $T_a$°C for 15 seconds, 72°C for

45 seconds}, and finally 72°C for 7 minutes. The final optimised PCR parameters for the two SSCP applications are listed in Table 2.3. All of these PCRs were performed in a final volume of 25µl.

**Table 2.3** Optimised conditions for PCR of *RHO* exons for two methods of analysis.

| Application | Exon | DNA | Taq | $MgCl_2$ | Cycling |
|---|---|---|---|---|---|
| **Traditional SSCP & sequencing** | 1A | 100ng | 0.5U Biotaq | 0 | 94°C - 3 min {94°C - 30 sec, 55°C - 30 sec, 65°C - 40 sec} X 30, 72°C -7 min |
| | 1B | 100ng | 0.5U Invitrogen™ Taq | 1.5mM | 94°C - 3 min {94°C - 30 sec, 60°C - 30 sec, 72°C - 40 sec} X 30, 72°C -7 min |
| | 2 | 100ng | 0.5U Invitrogen™ Taq | 1.5mM | As above |
| | 3 | 200ng | 0.5U Biotaq | 0 | As above |
| | 4 | 200ng | 0.5U Invitrogen™ Taq | 1.5mM | As above |
| | 5 | 200ng | 0.5U Biotaq | 1mM | As above |
| **Multiphor™ II SSCP** | 1A | 200ng | 0.5U Invitrogen™ Taq | 1.5mM | 94°C - 3 min {94°C - 15 sec, 55°C - 15 sec, 65°C - 45 sec} X 28, 72°C -7 min |
| | 1B | 200ng | 0.5U Invitrogen™ Taq | 1.5mM | 94°C - 3 min {94°C - 15 sec, 58°C - 15 sec, 72°C - 45 sec} X 28, 72°C -7 min |
| | 2 | 200ng | 0.5U Invitrogen™ Taq | 1mM | 94°C - 3 min {94°C - 15 sec, 60°C - 15 sec, 72°C - 45 sec} X 28, 72°C -7 min |
| | 3 | 200ng | 0.1U Biotaq | 0 | As above |
| | 4 | 200ng | 0.5U Biotaq | 2mM | 94°C - 3 min {94°C - 30 sec, 60°C - 30 sec, 72°C - 40 sec} X 30, 72°C -7 min |
| | 5 | 200ng | 0.5U Invitrogen™ Taq | 1.5mM | 94°C - 3 min {94°C - 15 sec, 60°C - 15 sec, 72°C - 45 sec} X 28, 72°C -7 min |

Only one optimised PCR condition was required per amplicon of the *RP1* and *RPE65* genes.

For the *RP1* gene, PCR amplification was undertaken in a final volume of 50µl using the following general conditions: 0.2mM dNTPs, 1X Invitrogen™ Buffer, 1.5mM $MgCl_2$, 1U Invitrogen™ Taq and 200ng genomic DNA. Glycerol was added to the reaction for two of the amplicons to a final concentration of 1% and the final concentration of primer was altered for one amplicon. Thermal cycling conditions were as follows: 95°C for 5 minutes, followed by 30 or 35 cycles of {94°C for 30 seconds, 50°C for 30 seconds, 72°C for 40 seconds} and finally 72°C for 7 minutes. The final optimised PCR parameters for *RP1* are listed in Table 2.4.

**Table 2.4** Summary of the requirements for optimal PCR amplification of the *RP1* fragments of interest.

| Fragment | Primer | Glycerol | No. of cycles |
|---|---|---|---|
| 4F | 25pmol | 1% | 30 |
| 4G | 20pmol | 1% | 35 |
| 4H | 20pmol | 0 | 30 |

For the *RPE65* gene, PCR amplification was performed using the following general conditions: 0.2mM dNTPs, 1X Buffer (commercial buffer corresponding to the Taq used), and 200ng genomic DNA. Parameters altered to optimise the PCR included the final concentration of $MgCl_2$, the Taq used, and the reaction volume (which in turn affected the final amount of primer required). The parameters altered to achieve optimal *RPE65* amplification are listed in Table 2.5.

Touchdown PCR was used for all *RPE65* amplicons except exon 10 and exons 11-13. Touchdown cycling conditions were as follows: 95°C for 3 minutes, followed by 10 cycles with decreasing $T_a$ {94°C for 15 seconds, 60 → 55°C for 15 seconds, 72°C for 30 seconds}, 20 cycles of {89°C for 15 seconds, 55°C for 15 seconds, 72°C for 30 seconds} and finally 72°C for 5

minutes. Normal cycling was performed for the remaining 2 amplicons, the only difference being the $T_a$ of 50°C for exon 10 and 47°C for exon 11-13. The cycling conditions used were therefore 95°C for 5 minutes, followed by 30 cycles of {95°C for 30 seconds, $T_a$°C for 30 seconds, 72°C for 30 seconds} and finally 72°C for 5 minutes.

**Table 2.5** Summary of the optimal PCR parameters used for amplification of the *RPE65* exons.

| Exon | $MgCl_2$ | Taq | Reaction volume | Primer |
|---|---|---|---|---|
| 1 | 2mM | 1U Invitrogen™ Taq | 50µl | 20pmol |
| 2 | 1.5mM | 0.5U Invitrogen™ Taq | 25µl | 10pmol |
| 3 | 1.5mM | 0.5U GoTaq® | 25µl | 10pmol |
| 4-5 | 1.5mM | 0.5U GoTaq® | 25µl | 10pmol |
| 6 | 2mM | 0.5U Invitrogen™ Taq | 25µl | 10pmol |
| 7-8 | 2mM | 0.5U Invitrogen™ Taq | 25µl | 10pmol |
| 9 | 2mM | 0.5U Invitrogen™ Taq | 50µl | 20pmol |
| 10 | 2mM | 1U GoTaq® | 50µl | 20pmol |
| 11-13 | 2mM | 0.5U Invitrogen™ Taq | 50µl | 20pmol |
| 14 | 1.5mM | 0.5U Invitrogen™ Taq | 50µl | 20pmol |

PCR products were analysed by electrophoresis after loading 5µl PCR product with 3µl agarose loading dye (Appendix 2) on 2% agarose gels containing ethidium bromide (0.4µg/ml) (Appendix 2). Gels were visualised by ultraviolet (UV) transillumination, and 0.5µg of DNA size standard was included on each gel (Appendix 2).

### 2.3.2 Single stranded conformational polymorphism analysis

Single stranded conformational polymorphism (SSCP) analysis is based on the principle that, under nondenaturing conditions, the sequence of a single stranded DNA molecule determines the three-dimensional conformation and hence the mobility. DNA samples are amplified over the region of interest, heat denatured and the single strands separated in a nondenaturing polyacrylamide gel. Separation is based on both size and conformation, so a difference in electrophoretic mobility, observed as a band shift, indicates a sequence variation. The sensitivity ranges from 70 - 95% and depends on the electrophoretic conditions used. The sensitivity is also inversely proportional to the size of the DNA fragment being analysed. Optimisation of parameters such as gel temperature, addition of glycerol or denaturing agents, duration of electrophoresis, gel polymer and electrophoresis buffer is often required [70-73].

DNA from a cohort of 47 individuals with ADRP and 14 with ARRP was screened for mutations in *RHO* using SSCP analysis. Screening had been started on some of the samples prior to the start of this project, but all results were re-analysed and the screening repeated and completed as necessary.

Screening of the cohort DNA began using a traditional SSCP technique with a vertical polyacrylamide gel system. However, this technique was found to require much experimental optimisation of electrophoresis conditions and multiple conditions were required to achieve mobility shifts in mutation-positive controls, as seen in Table 2.6. Generally, 10µl of PCR product of each sample was heat-denatured at 95°C for 5 minutes in an equal volume of SSCP loading dye (Appendix 2), and subsequently cooled on ice for 5 minutes. The denatured samples were run on two 10% polyacrylamide gels, one containing 7.5% urea and one containing 5% glycerol (Appendix 2). Polyacrylamide gels containing urea were run using 1.5X TBE electrophoresis buffer and gels containing glycerol were run using 0.5X TBE electrophoresis buffer (Appendix 2). As an alternative, 0.5X MDE™ gels

(BMA, USA) were run with or without 10% glycerol (Appendix 2), using 0.6X TBE electrophoresis buffer (Appendix 2). Electrophoresis was performed overnight and visualisation was by silver staining (Appendix 2).

**Table2.6** Summary of the optimised electrophoresis conditions for traditional SSCP of *RHO.*

| Exon | Mutation Control | Condition 1 | Condition 2 | Special consideration |
|---|---|---|---|---|
| 1A | Thr58Arg | Polyacrylamide + Urea, 6 Watts | Polyacrylamide + glycerol, 12 Watts | |
| 1B | Gly109Arg | Polyacrylamide + Urea, 6 Watts | MDE, 8 Watts | |
| 2 | None | Polyacrylamide + Urea, 6 Watts | MDE+ glycerol, 6 Watts | |
| 3 | Asp190Asn | MDE, 6 Watts | MDE+ glycerol, 12 Watts | |
| 4 | Lys296Glu | Polyacrylamide + Urea, 3 Watts | MDE+ glycerol, 4 Watts | Large (402bp) PCR product must be cut into 2 fragments using *BstEII* enzyme prior to SSCP |
| 5 | Pro347Leu | Polyacrylamide + Urea, 4 Watts | MDE, 1 Watt | |

DNA from three ADRP individuals and 14 ARRP individuals was screened using this traditional SSCP technique, however variations detected were frequently inconclusive or not reproducible and samples would occasionally not resolve. It was therefore decided to use a different electrophoresis system. The unresolved samples were re-screened where required, together with the rest of the *RHO* cohort using the Multiphor™ II electrophoresis system (Amersham Pharmacia Biotech, UK). The Multiphor™ II electrophoresis system is a two buffer-based, temperature regulated, horizontal gel system. This system has a mutation detection rate of 97.5%, functions optimally using a single defined SSCP condition and extends analysis to larger fragments (500-600bp) [74].

Generally, amplification products of samples were heat denatured as above, in an equal volume of Multiphor™ loading dye (Appendix 2). Three microlitres was loaded onto a 12% nondenaturing polyacrylamide gel (Appendix 2), and electrophoresis was performed on the Multiphor™ II electrophoresis unit, using tris borate electrophoresis buffer (Appendix 2). Initially all gels were electrophoresed at 15°C, for 90 minutes at 355 Volts. Visualisation was by silver staining (Appendix 2).

When the mutation-positive controls failed to resolve and/or the discrimination between wild type and variant sequences was difficult to interpret, the gel conditions were further optimised. Parameters altered to improve resolution included the duration of electrophoresis (minutes), the ratio of PCR product to loading dye prior to heat denaturation of the sample, and the temperature at which the electrophoresis is performed. The gel temperatures were controlled using a waterbath circulator (Labcon, SA) containing water and 1% SIERRA® antifreeze (Old World Industries, Inc., USA). The optimised electrophoresis conditions for the screening of *RHO* are summarised in Table 2.7.

**Table 2.7** Summary of the optimised electrophoresis conditions for Multiphor™ II SSCP of *RHO*.

| Exon | PCR product : Loading dye ratio | Duration of electrophoresis | Temperature of electrophoresis |
|---|---|---|---|
| 1A | 3µl : 3µl | 105 minutes | 5°C, 9°C and 12°C required |
| 1B | 3µl : 3µl | 105 minutes | 15°C |
| 2 | 3µl : 3µl | 90 minutes | 9°C |
| 3 | 1µl : 5µl | 120 minutes | 18°C |
| 4 | 1µl PCR : 2µl $H_2O$ : 3µl dye | 120 minutes | 5°C |
| 5 | 3µl : 3µl | 90 minutes | 9°C |

All samples exhibiting variant SSCP profiles were sequenced; however when numerous samples appeared to have the same SSCP profiles, one of these samples was selected as representative and sequenced. The restriction enzyme site altered by that sequence variant was determined, and groups of samples with similar SSCP profiles were tested for the presence of that variant using the restriction enzyme identified.

### 2.3.3 Denaturing high performance liquid chromatography

Denaturing high performance liquid chromatography (dHPLC) was performed using the WAVE® Nucleic Acid Fragment Analysis System (Transgenomic Inc, USA). In a dHPLC experiment DNA molecules are bound to a column, and are eluted by an increasing concentration of acetonitrile. Size-based separation of PCR products is achieved using nondenaturing conditions at 50°C on the WAVE®, whilst mutation detection is performed using partially denaturing conditions. Prior to mutation detection the amplicon sequences are analysed using WAVE® software. This software predicts the regions of each amplicon that have a 70% helical fraction (i.e. are 70% melted) at a specific temperature (Figure 2.1). Heteroduplexes have lower affinities for the column than homoduplexes and will elute earlier, therefore any variations in the melted portion of the amplicon will be detected. Several injections, each with a different set of parameters, are often required per amplicon to achieve melting across the entire fragment. Separation chemistry is based on the sequence and size of the fragment, and the analysis temperature [75, 76]. The mutation detection application of the WAVE® has a specificity and sensitivity exceeding 96%, and works optimally with fragments shorter than 500bp.

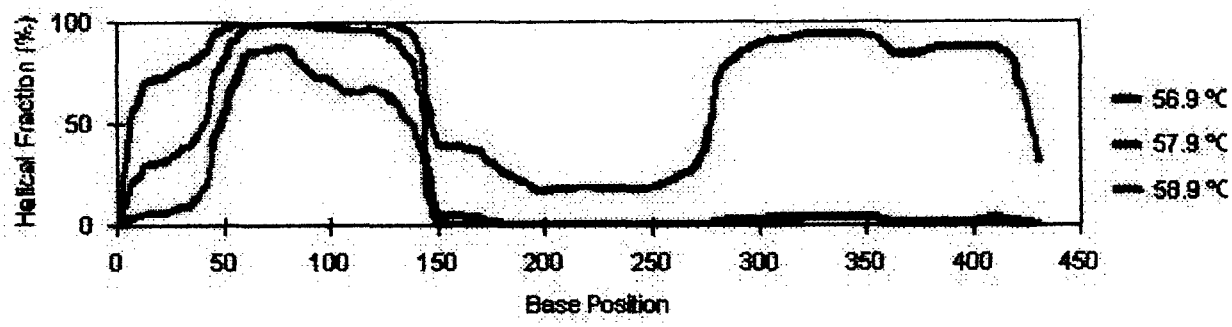



**Figure 2.1** Melting profile of *RPE65* exon 4-5, as predicted by the WAVE® software. Several melting domains are present in this fragment. The regions from 0 - 50bp and 270bp - 440bp have a 70% helical fraction at 56.9°C. The region from 50 - 150bp requires a temperature between 57.9°C and 58.9°C to obtain a 70% helical fraction. The region from 150bp - 270bp requires a lower temperature to achieve optimal melting (not shown).

DNA from 70 individuals was screened for mutations in the *RP1* hotspot, and DNA from 87 individuals was screened for mutations in the entire coding region of *RPE65* using dHPLC. Gradient parameters, temperatures and flow rates were developed empirically, based on the size and melting profile of each of the amplified fragments of *RP1* and *RPE65*. An aliquot of 7-15µl of each PCR product was heat-denatured at 95°C for 5 minutes, and heteroduplex formation was promoted by allowing the tube temperature to reach room temperature over 45 minutes. Each sample screened for *RPE65* was mixed with a wild type sample prior to heteroduplexing to ensure detection of homozygous variations. Samples were injected onto the dHPLC column and mutation detection was performed according to the optimal separation conditions listed in Table 2.8. A wild type control was included for each condition in every dHPLC experiment, for comparison with the test samples.

**Table 2.8** Optimised dHPLC conditions for mutation screening of *RP1* and *RPE65*.

| Amplicon | Temperature (°C) | Flow rate (ml/min) | Gradient duration (min) | Volume of PCR injected (µl) | Time shift (min) |
|---|---|---|---|---|---|
| RP1ex4F | 54 | 0.9 | 4.5 | 15 | 0 |
| | 56.1 | 1.5 | 2.4 | 15 | 0.5 |
| RP1ex4G | 54 | 0.9 | 4.5 | 10 | 0 |
| | 54.5 | 0.9 | 4.5 | 7 | 0 |
| | 57.5 | 0.9 | 4.5 | 10 | 1 |
| RP1ex4H | 55 | 1.5 | 2.4 | 7 | 0 |
| | 56 | 1.5 | 2.4 | 7 | 0 |
| | 57 | 1.5 | 2.4 | 7 | 0 |
| RPE65ex1 | 54.2 | 1.5 | 3 | 9 | 0 |
| | 57.2 | 1.5 | 3 | 9 | 0 |
| | 59.4 | 1.5 | 3 | 9 | 0 |
| | 60.7 | 1.5 | 3 | 12 | 0 |
| RPE65ex2 | 60 | 1.5 | 3 | 9 | 0 |
| | 62 | 1.5 | 3 | 12 | 0 |
| RPE65ex3 | 60.5 | 1.5 | 3 | 9 | 0 |
| | 61.5 | 1.5 | 3 | 9 | 0 |
| RPE65ex4-5 | 56.3 | 1.5 | 3 | 9 | 0 |
| | 57 | 1.5 | 3 | 9 | 0 |
| | 58.3 | 1.5 | 3 | 12 | 0 |
| RPE65ex6 | 54 | 1.5 | 3 | 9 | 0 |
| | 57 | 1.5 | 3 | 9 | 0 |
| | 58.7 | 1.5 | 3 | 12 | 0 |
| RPE65ex7-8 | 55.5 | 1.5 | 3 | 15 | 0 |
| | 56.6 | 1.5 | 3 | 15 | 0 |
| RPE65ex9 | 53 | 1.5 | 3 | 13 | 0 |
| | 55 | 1.5 | 3 | 13 | 0 |
| | 57.5 | 1.5 | 3 | 13 | 0 |
| | 59 | 1.5 | 3 | 13 | 0 |
| RPE65ex10 | 54.5 | 1.5 | 3 | 15 | 0 |
| | 56.5 | 1.5 | 3 | 15 | 0.5 |
| | 57.9 | 1.5 | 3 | 15 | 1 |
| RPE65ex11-13 | 56 | 1.5 | 2.6 | 15 | 0 |
| | 56.9 | 1.5 | 2.6 | 15 | 0.5 |
| | 58.5 | 1.5 | 2.6 | 15 | 1 |
| | 59.5 | 1.5 | 2.6 | 15 | 1.5 |
| RPE65ex14 | 53.4 | 1.5 | 3 | 7 | 0 |
| | 54.6 | 1.5 | 3 | 7 | 0 |
| | 57 | 1.5 | 3 | 7 | 0 |
| | 59.2 | 1.5 | 3 | 7 | 0 |

All samples exhibiting variant dHPLC elution profiles were sequenced. When numerous samples appeared to have the same variant profiles, one of these samples was selected as a representative and sequenced. The group of samples with similar dHPLC profiles was tested for the presence of that variant using a second dHPLC analysis to confirm whether they were in fact due to the same sequence variation. In this second dHPLC, termed a "WAVE mixing experiment", an equal volume of the PCR product being queried was mixed with that of the sequenced variant sample. This mixture was subjected to heteroduplex formation and dHPLC analysis with the appropriate gradient condition. Lack of additional peaks indicated that sequences of the PCR product being queried and the known sample were identical. This highly effective WAVE mixing experiment was found to be cost efficient when compared to direct sequencing of multiple samples with the same variant, or when restriction enzyme digests were not an option [77].

### 2.3.4 Sequencing

DNA samples that exhibited SSCP mobility shifts or variant dHPLC elution profiles were characterised by direct sequencing. PCR products were purified using the QIAquick® gel extraction kit, QIAquick® PCR purification kit or the QIAEX II gel extraction protocol (Qiagen, UK), according to the manufacturer's instructions and samples were eluted in a final volume of 30µl distilled water (SABAX water, Adcock Ingram, SA). Purified samples were sequenced in both forward and reverse directions, using the same primers used for the original PCR. Sequencing reactions were performed using the BigDye® terminator cycle sequencing kit version 3.1 (Applied Biosystems, USA). Sequencing reactions depended on the quality and sequence of each PCR product and were thus optimised on an individual basis. Optimisations included altering the amount of template, altering the amount of primer, and performing ½ or ¼ BigDye® reactions. The recommended reaction is as follows: 3 -10ng purified PCR product, 3 -10pmol primer and 1X BigDye® reaction mix in a 20µl final reaction volume.

Either of the following cycling conditions was used:

- 96°C for 1 min, followed by 25 cycles of (95°C for 45 sec; 60°C for 4 min)
- 96°C for 5 min, followed by 25 cycles of (96°C for 30 sec; 50°C for 15 sec; 60°C for 4 min)

After the cycle sequencing reaction, sequence products were purified using Centri-Sep columns (Princeton Separations, USA) according to manufacturer's instructions. Columns were re-used a maximum of 8 times; the columns were prepared using 900µl of 6.25% Sephadex G-50 fine (Amersham Biosciences, Sweden) as a column gel. Sequencing was resolved on an ABI Prism™ 377 or an ABI Prism™ 3100 automated sequencer (Applied Biosystems, USA).

The ABI Prism™ 377 is a polyacrylamide gel-based sequencing system, and 36cm gels were used. The gels were mixed in a 50µl volume containing 36% urea, 4% Acrylogel (BDH Laboratory Supplies, England) and 1X TBE buffer and were de-gassed by filtration prior to polymerization (Appendix 2). Purified, sequenced samples were dried to a pellet under a vacuum and then resuspended in 6µl loading dye (Appendix 2). Samples were heated at 95°C for 2 minutes and placed on ice, and 1.5µl of the sample/loading dye mix was loaded onto the gel. Analysis was performed using ABI Prism™ DNA Sequencing Analysis Software version 3.4. For sequencing using the capillary based ABI Prism™ 3100 system, 5µl of purified sequencing reaction was added to 7µl Hi-Di™ formamide (Applied Biosystems, USA) and heat denatured prior to running. Analysis was performed using ABI Prism™ DNA Sequencing Analysis Software version 3.7.

Sequences obtained were analysed for the presence of variations by using NCBI BLAST [68] or by aligning the sequences with a reference sequence using Clustal W [78] or BioEdit version 7.0.0 [79].

### 2.3.5 Restriction enzyme digests

Restriction enzymes digests were performed to verify the presence of a particular sequence variant in the DNA of control individuals and/or the family of the affected individual in whom the variant was initially detected. Variants were analysed using Webcutter 2.0 (http://rna.lundberg.gu.se/cutter2/) to determine whether they created or destroyed restriction enzyme sites in order for restriction fragment length analysis to be performed. Enzymes were purchased from Promega (USA), Roche Diagnostics (SA), Boehringer Mannheim (Germany) or New England Biolabs (USA). The amount of template used and the length of incubation were determined empirically, however the buffer used and incubation temperatures were set according to the manufacturer's instructions. In accordance with common practice in this laboratory, approximately 10U of enzyme were used in digestion experiments. After independent enzyme titration experiments the number of units used for digestions were reduced, in line with the manufacturer's guidelines. The conditions for digests are shown in Table 2.9. All digests were performed in a final volume of 20µl except for *Rsa I*, *Taq I* and *Ksp I*, which were performed in a final volume of 25µl.

Restriction fragment length analysis was performed by electrophoresis of the total volume of digest products, together with 5µl agarose loading dye on 2% - 3.5% agarose gels containing 0.4µg/ml ethidium bromide (Appendix 2). Gels were visualised by UV transillumination, and 0.5µg of DNA size standard was included on each gel (Appendix 2). When a higher resolution was required, 3µl of digest products were loaded together with 3µl agarose loading dye onto an 8% polyacrylamide gel (Appendix 2), and electrophoresis was performed at 355V for 1.5 hours on the Multiphor™ II electrophoresis unit, using 1XTBE electrophoresis buffer (Appendix 2). Visualisation was by silver staining (Appendix 2).

**Table 2.9** Summary of the protocols used for the restriction enzyme digests.

| Enzyme | Manufacturer | Protocol |
|---|---|---|
| *Acs I* | Roche | 10µl PCR, 10U enzyme, Buffer B @ 50°C overnight. |
| *Alw26 I* | Promega | 15µl PCR, 5U enzyme, Buffer C @ 37°C for 1.5 hours, thereafter an extra 5U enzyme added, 37°C overnight. |
| *Ban I* | Promega | 17µl PCR, 12U enzyme, Buffer G @ 50°C for 4 hours. |
| *BstE II* | Promega | 16.8µl PCR, 12U enzyme, Buffer D @ 60°C for 1.5 hours. |
| *Bst X I* | Roche | 15µl PCR, 1U enzyme, Buffer H @ 45°C overnight. |
| *Hsp92 II* | Promega | 10µl PCR, 10U enzyme, Buffer K @ 37°C overnight. |
| *Ksp I* | Boehringer | 10µl PCR, 0.5U enzyme, Buffer L @ 37°C for 0.5 hours, thereafter an extra 1U enzyme added, 37°C 1 hour. |
| *Mse I* | NE Biolabs | 15µl PCR, 2.5U enzyme, Buffer 2 @ 37°C for 3 hours, thereafter an extra 2.5U enzyme added, 37°C overnight. |
| *Mnl I* | NE Biolabs | 15µl PCR, 2.5U enzyme, Buffer 2 @ 37°C for 3 hours, thereafter an extra 2.5U enzyme added, 37°C overnight. |
| *Nla IV* | NE Biolabs | 15µl PCR, 2.5U enzyme, Buffer 4 @ 37°C for 3 hours, thereafter an extra 2.5U enzyme added, 37°C overnight. |
| *Rsa I* | Promega | 10µl PCR, 0.5U enzyme, Buffer L @ 37°C for 0.5 hours, thereafter an extra 1U enzyme added, 37°C 1 hour. |
| *Taq I* | Roche | 10µl PCR, 5U enzyme, Buffer B @ 65°C for 3 hours, thereafter an extra 5U enzyme added, 65°C overnight. |

### 2.3.6 Multiplex ligation-dependent probe amplification

There are no reports as to the frequency of large gene deletions in RDDs, and these can go undetected by conventional gene screens when only the one full length allele is amplified and analysed. In order to investigate the contribution of these mutations, it was decided to perform multiplex ligation-dependent probe amplification (MLPA), which detects the copy number of target genomic DNA. The manufacturers of MLPA kits, MRC-Holland (Amsterdam, The Netherlands), were contacted and asked whether development of a kit to analyse RDD genes could be considered. The manufacturers requested a list of candidate genes and *RHO*, *RP1* and *RPE65* were suggested. It was agreed that the incorporation of these candidates into a MLPA kit was justifiable, and the manufacturers requested SNP and mutation information for each of the genes. This information was supplied to MRC-Holland, who then designed the MLPA probes and kindly donated one MLPA kit for our use. The probe sequences were aligned onto each of the gene sequences, and the locations can be viewed in Appendix 1.

The MLPA kit used, SALSA MLPA kit P115 Retina, contained the probes designed to flank the specific target sequences in *RP1*, *RHO*, and *RPE65*. The probes flanking a target sequence ligate, after which they are amplified using a primer pair. Probes to different target sequences can be used in a multiplex PCR reaction, as all probes are recognised by the same primer pair. Amplification products are separated on the ABI Prism™ 3100 system and the peak area of each amplification product is compared to that of a control, thereby reflecting the relative copy number of the target sequence.

DNA from a cohort of 50 individuals was screened for deletions or insertions in *RP1*, *RHO* and *RPE65* using the SALSA MLPA kit P115 Retina. Prior to MLPA, the quality of all samples was determined by electrophoresis of 1µl of genomic DNA on a 1% agarose gel, using agarose loading dye (Appendix 2). Intact DNA samples were selected, and were quantified using the NanoDrop® ND-1000 spectrophotometer (NanoDrop technologies, USA). Samples were diluted to a final concentration of 100ng/µl and MLPA was

performed on 200ng according to the manufacturer's instructions. MLPA PCR products were diluted 1 in 10, and 1µl of this was mixed with 0.3µl ROX™ 500 size standard (Applied Biosystems, USA) and 8.5µl of deionised formamide. The samples were then heat denatured at 95°C for 5 minutes and placed immediately on ice. Samples were run on the ABI Prism™ 3100 and analysed using GeneMapper™ version 3.0 (Applied Biosystems, USA). Data was normalised using an Excel data sheet downloaded from the MRC-Holland website (http://www.mrc-holland.com) and altered by Ms. Alvera Vorster to show the normalised data as line charts. The normalised copy number of each amplicon in each sample was assessed for the presence of deletions or insertions (observed as a sudden skewing of the copy number of a probe).

## 2.4 Variant analysis

Variants were classified as being novel or previously reported. Novel variants were further analysed to determine whether they were pathogenic (i.e. disease-causing mutations) or nonpathogenic (i.e. polymorphisms) according to the following criteria [80] :

- The type of mutation and its likelihood of affecting the protein and thereby causing disease.
- Whether the variant co-segregated with the disease phenotype – the presence of the variant in an asymptomatic individual could indicate nonpenetrance, however the absence of a variant in an affected individual proves that variant to be nonpathogenic.
- The frequency of the variant – a variant occurring in more than 1% of the alleles in a population is unlikely to be pathogenic

Variants of the form g.123A>G were described according to the following convention; wild type – A/A, heterozygous – A/G, and homozygous – G/G.

The methods of assessment and analysis of variants are described in more detail below.

### 2.4.1 Assessing the effect on the protein

If a variation was detected in a sequence, the first step in characterising the variant was to determine whether the nucleotide affected was intronic or exonic. This was performed by aligning the variant onto the relevant gene annotation described in section 2.2. The nomenclature of the noncoding variants (intronic, 5'UTR or 3'UTR) was based on the genomic sequences with the following NCBI accession numbers: U49742 (*RHO*); NT_032977 (*RPE65*).

Exonic variations were analysed by performing an NCBI BLAST [68] of the translated sequence vs. the protein database. This allowed each variation to be characterised as a substitution, truncation, frameshift or silent variant. Silent variants were analysed for their effects on splicing (section 2.4.5), but were otherwise considered polymorphisms and not investigated further. Each variant was described in terms of the accepted nomenclature system [81]. The nomenclature of the coding variants was based on their position after the start codon in the mRNA sequences with the following accession numbers: NM_000539 (*RHO*); NM_006269 (*RP1*); NM_000329 (*RPE65*). In addition, the nomenclature of each of the coding variants was reported in terms of the amino acid change of the protein.

### 2.4.2 Literature search

An important characterisation of each variant was to determine whether the variant was novel or had been reported previously. To this end, the following online databases were searched:

- Retina International Mutation Database (http://www.retina-international.org/sci-news/mutation.htm)
- Online Mendelian Inheritance in Man™ (http://www.ncbi.nlm.nih.gov)
- The Human Gene Mutation Database at the Institute of Medical Genetics in Cardiff [82] (http://www.hgmd.cf.ac.uk/)
- NCBI PUBMED (http://www.ncbi.nlm.nih.gov)

If a variant was not listed in any of the above sources, it was considered to be novel. If the variant was previously reported, those reports were analysed for any relevant information such as effect on protein, association with disease, and molecular mechanism of disease.

### 2.4.3 Segregation analysis

To determine whether a novel variant was associated with disease, DNA from family members of the individual in whom the variant had originally been identified, was tested. These family members had been recruited for the research and their DNA samples had already been archived. These individuals were tested to determine whether there was co-segregation of the variant with the disease phenotype. The presence of the variant in each sample was tested by means of restriction enzyme digest, or (if the variant did not alter a restriction enzyme site) by the previously described WAVE mixing experiment. In the case of a deletion, size-based separation on the WAVE® was used to determine co-segregation of the variant with the phenotype.

### 2.4.4 Population frequency analysis

Population frequency analysis was performed to determine whether a novel variant occurred in less than 1% of the alleles in a population (i.e. was a disease-causing mutation), or to compare population frequencies of known polymorphisms with reported statistics. In these analyses, DNA from a cohort of 50 individuals was screened for the variant of interest, using a restriction enzyme digest or the WAVE mixing experiment. The control cohort tested was matched for ethnic group to the test sample and had not been assessed specifically for the presence of retinal degenerative disease.

### 2.4.5 Splice site prediction

Intronic variants and silent polymorphisms were analysed using version NNSPLICE 0.9 of the Berkeley Drosophila Genome Project's splice site prediction by neural network [83, 84]. This software is 95% accurate and is available at http://www.fruitfly.org/seq_tools/splice.html. Splice prediction was initially performed using each wild type sequence to ensure that the program was accurately predicting the known splice sites. If the correct splice sites were predicted using the wild type sequence, the sequence containing the variant was analysed to predict any effect on splicing.

### 2.4.6 Additional analysis

#### 2.4.6.1 Determining the evolutionary conservation of an amino acid

In order to add additional weight to the interpretation of a specific amino acid change, an examination of the evolutionary conservation of that amino acid was undertaken. This was achieved by manually aligning the protein sequences of *Homo sapiens* (human), *Mus musculus* (mouse), *Rattus norvegicus* (rat), *Drosophila melanogaster* (fruit fly), *Takafugu rubripes* (blowfish), *Danio rerio* (zebra fish), *Canis familiaris* (dog), *Gallus gallus* (chicken), *Tetraodon nigroviridis* (puffer fish), *Xenopus tropicalis* (frog) and *Bos taurus* (cow). The conservation of the amino acid in question was then assessed across these 11 species.

#### 2.4.6.2 Disproving uniparental disomy

In order to prove that homozygosity of a reported dominant *RHO* mutation was not due to uniparental disomy, genotyping was performed. The ABI Prism™ Linkage Mapping Set version 2 was used to this end (Applied Biosystems, USA). Panels 5, 6 and 7 of this linkage mapping set contain 23 markers spanning chromosome 3, and these were used for amplification (together with three markers selected prior to the start of this project) with the

True Allele® PCR Premix (Applied Biosystems, USA) according to manufacturer's instructions. Products were diluted 1 in 10; 1µl of the diluted products was mixed with 1.5µl loading dye (Appendix 2) containing ROX™ 500 size standard prior to heat denaturation (95°C for 5 minutes). Separation was performed on the ABI Prism™ 377 sequencer, using a polyacrylamide gel (Appendix 2). Analysis was performed using the Genotyper® software version 2.1 (Applied Biosystems, USA).

#### 2.4.6.3 Determining the presence of a deletion

When a potential low MLPA copy number was detected, PCR amplification was performed with primers flanking the probe of interest. A 5.6kb fragment was amplified using the Expand Long Template PCR system (Roche diagnostics, SA) with 0.3µM each primer, 0.5mM dNTPs, 1X buffer 2, 500ng genomic DNA and 3.75U Expand Long Template Taq. Cycling conditions were as follows: 94°C for 2 minutes, followed by 10 cycles of {94°C for 10 seconds, 58°C for 30 seconds, 68°C for 4 minutes}, followed by 20 cycles of the same, but with increasing elongation time by 20 seconds per cycle, and finally 68°C for 7 minutes. An aliquot of 10µl of PCR product was subjected to electrophoresis on a 0.8% agarose gel, using agarose loading dye (Appendix 2) to determine whether two products of different lengths were obtained, thereby proving the presence of a deletion.

When this showed no deletion greater than 2kb, primers were redesigned (according to the primer design method discussed previously) 2.6kb apart. The product was amplified using the same conditions as above, except for 0.35mM dNTPs and a cycling condition of: 94°C for 2 minutes, 10 cycles of touchdown PCR {94°C for 10 seconds, 63°C→58°C for 30 seconds, 68°C for 2 minutes}, 20 cycles of {94°C for 15 seconds, 58°C for 30 seconds, 68°C for 2 minutes+20 sec/cycle minutes}, and finally 68°C for 7 minutes. An aliquot of 25µl of PCR product was subjected to electrophoresis on a 2% agarose gel, using agarose loading dye (Appendix 2) to determine whether two products of different lengths were obtained, thereby proving the presence of a deletion.

### 2.4.7 Diagnostic assay development

#### 2.4.7.1 Validation

In order to achieve the level of accuracy and reliability desired of a diagnostic assay, either restriction enzyme digestion, or sequencing were performed. Normally restriction enzyme digestion was the preferred method of analysis and sequencing was used only where no restriction enzyme digest was possible. Positive controls and uncut samples were included in every restriction enzyme experiment. Each diagnostic test was performed on two separate occasions, preferably on two samples of DNA isolated on different days, to guard against sample mix-ups.

#### 2.4.7.2 Protocol for diagnostic testing

The following protocol for diagnostic testing was established in consultation with Sr. Lecia Bartmann, the clinical co-ordinator on the RDD project, Professor Jacquie Greenberg, a genetic counsellor (who is also a medical scientist and the co-principal investigator of the broader RDD research project), and Ms. Frieda Basson, the intern genetic counsellor on the RDD project.

1. Patients (individuals and/or families) are recruited for research, and DNA from the proband is screened for mutations
2. If a mutation is detected in the proband, DNA from the family is tested for that mutation (at this point the proband will have been tested several times, and the family members tested once).
3. The proband is contacted, counselled and given the molecular results to take back to the rest of the family.
4. If a family member requests an individual diagnostic result, and that individual has tested mutation positive (in the research programme) and is clinically affected, they are re-tested once so that they will have a total of two tests performed on two separate days (ideally on two

DNA samples isolated on different days, if however this is not possible then two tests on a single DNA sample are acceptable).

5. If an asymptomatic family member requests an individual diagnostic result they should be retested on a fresh DNA sample, from blood taken after appropriate pre-test counselling. This individual will have a total of two tests, performed on two separate days, on two DNA samples isolated on different days. This should be assessed on a case by case basis, depending on consent and pre-test counselling.
6. If a new family member (not recruited for the research project) requests a diagnostic test, two tests should be performed after counselling. Ideally two DNA isolations should be used. If this is not possible, tests on a single isolation are acceptable.
7. A report is written describing the PCR primers used, the type of test performed, the interpretation of the test result and references to relevant literature.

The DNA consent forms used for recruiting families and the protocol established in this Division for the delivery of molecular results can be viewed in Appendix 3.

# 3 Results

In order to facilitate the flow of the presentation of the results, the data have been grouped according to the genes screened. This does not fully reflect the chronological order in which the experiments were performed. All results are summarised in Table 3.4 (page 91).

## *3.1 RHO*

The *RHO* gene was screened for mutations in DNA from 61 individuals (47 with ADRP and 14 with ARRP). None of these 61 individuals had an RDD mutation identified prior to this screening. The mutation screening technique used was SSCP analysis: some samples were partially screened prior to the onset of this project using traditional SSCP and all these results were re-analysed; 17 samples were screened using traditional SSCP, and the remainder of the cohort was screened using SSCP on the Multiphor™ II electrophoresis system.

### 3.1.1 *RHO* exon 1A

Three common mobility patterns were observed by SSCP analysis of exon 1A using the traditional system (Figure 3.1). Direct sequencing of one sample representative of each pattern revealed that the three patterns corresponded to the wild type sequence and the g.269A>G variation in the homozygous and heterozygous state. The g.269A>G change is reported in a known SNP database (dbSNP) at the NCBI, has a database ID (dbSNP:rs7984), and is nonpathogenic [85]. Of the 17 samples screened using traditional SSCP, six exhibited the heterozygous g.269A>G SSCP pattern and two the G/G homozygous pattern. These results were all confirmed by restriction enzyme analysis as the g.269A>G variant creates a *Ksp I* site. Of the remaining nine samples, eight exhibited the wild type patterns on both gel conditions, while one appeared to have a possible mobility shift on the urea-containing gel. This sample was sequenced and no sequence variant was obtained, indicating a false positive SSCP mobility shift.

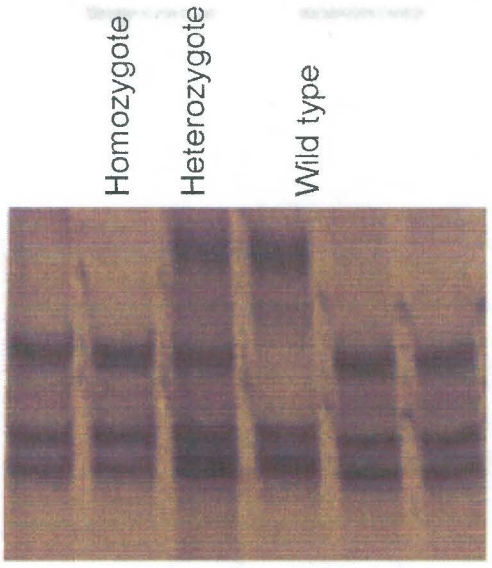



**Figure 3.1** Digital photograph of a 10% polyacrylamide gel showing the three common SSCP patterns of *RHO* exon 1A: the wild type with upper and lower bands, the heterozygous variant with upper, middle and lower bands, and the homozygous variant with middle and lower bands. This gel contained 7.5% urea and was visualised by silver staining.

Prior to the start of this project, 23 samples had been screened for exon 1A using traditional SSCP and no mobility shifts had been observed. When these samples were tested with the *Ksp I* digest, however, 14 of the samples were found to be wild type, five were found to be heterozygous for g.269A>G; and four were homozygous for g.269A>G. These nine samples therefore represented false negative SSCP results.

The remaining 21 samples were screened using the Multiphor™ II electrophoresis system and based on the SSCP banding patterns observed, 12 samples were identified as being wild type, four as heterozygous for g.269A>G, and four as homozygotes. One sample (from a Caucasian individual) was identified as having a unique mobility shift due to the presence of both the heterozygous polymorphism and another mutation, and is discussed separately below. After the *Ksp I* digests, four samples had conflicting SSCP and digest results. Sequencing was performed and revealed that one sample exhibited a false positive SSCP shift, while three partial digests were responsible for causing homozygous variants to appear

heterozygous, or heterozygous variants to appear wild type. The final results including the ethnic groups from which the samples were derived are summarised in Table 3.1.

**Table 3.1** Ethnic breakdown of RP samples in which g.269A>G was detected.

| Cohort | Wild type (A/A) | Heterozygous (A/G) | Homozygous (G/G) | Total |
|---|---|---|---|---|
| **Traditional SSCP** | | | | 17 |
| Caucasian | 9 | 4 | - | |
| Mixed Ancestry | - | 2 | - | |
| Black | - | - | 1 | |
| Indian | - | - | 1 | |
| | | | | |
| **Prior Screen** | | | | 23 |
| Caucasian | 12 | 3 | 1 | |
| Mixed Ancestry | 2 | 1 | 1 | |
| Black | - | 1 | 2 | |
| | | | | |
| **Multiphor™ Screen** | | | | 20 |
| Caucasian | 11 | 4 | - | |
| Mixed Ancestry | 1 | - | - | |
| Black | - | 1 | 4 | |
| | | | | |

The population frequency of g.269A>G was examined in order to ascertain the population-group based incidences of the change, and this was achieved by screening DNA from 54 Black control individuals and 53 Caucasian control individuals using the traditional SSCP method. The inferred genotypes obtained as a result of this population screen are summarised in Table 3.2.

**Table 3.2** The results of the frequency study of g.269A>G in Caucasian and Black control populations.

| **Cohort** | **Total** | **Number (and percentage) detected** | | |
|---|---|---|---|---|
| | | **Wild type (A/A)** | **Heterozygotes (A/G)** | **Homozygotes (G/G)** |
| **Caucasian Controls** | 53 | 35 (66%) | 16 (30%) | 2 (4%) |
| **Black Controls** | 54 | 3 (5%) | 8 (15%) | 43 (80%) |

The variant was found to exist in 34% of the SA Caucasian population (18.9% of the alleles) and 95% of the indigenous Black population (87.03% of the alleles).

A unique mobility shift was observed in the exon 1A SSCP profile of the sample 63.2 (from a Caucasian individual), using the Multiphor™ system. The sample from the proband affected with ADRP was sequenced and the c.50C>T variation was identified (Figure 3.2). The c.50C>T mutation causes a Thr17Met amino acid change, which is a reported pathogenic mutation [86-89].

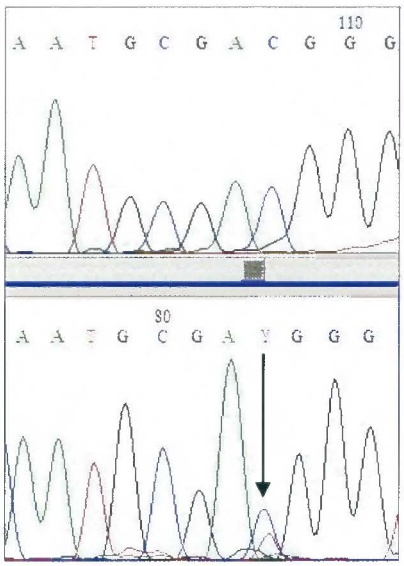



**Figure 3.2** Sequencing electropherogram of *RHO* exon 1A, showing the wild type sequence (top) and the c.50C>T mutation (bottom, indicated by an arrow).

The Thr17Met mutation creates a *BstX I* restriction enzyme site, and the restriction enzyme digest was performed on all available samples from family 63. The variation was found to co-segregate with the disease, as it was present in two affected individuals, 63.2 and 63.4, and absent in a spouse, 63.1 (Figure 3.3). No DNA was available from individual 63.3. The family pedigree and clinical information of individuals in family 63 is presented in Appendix 4.

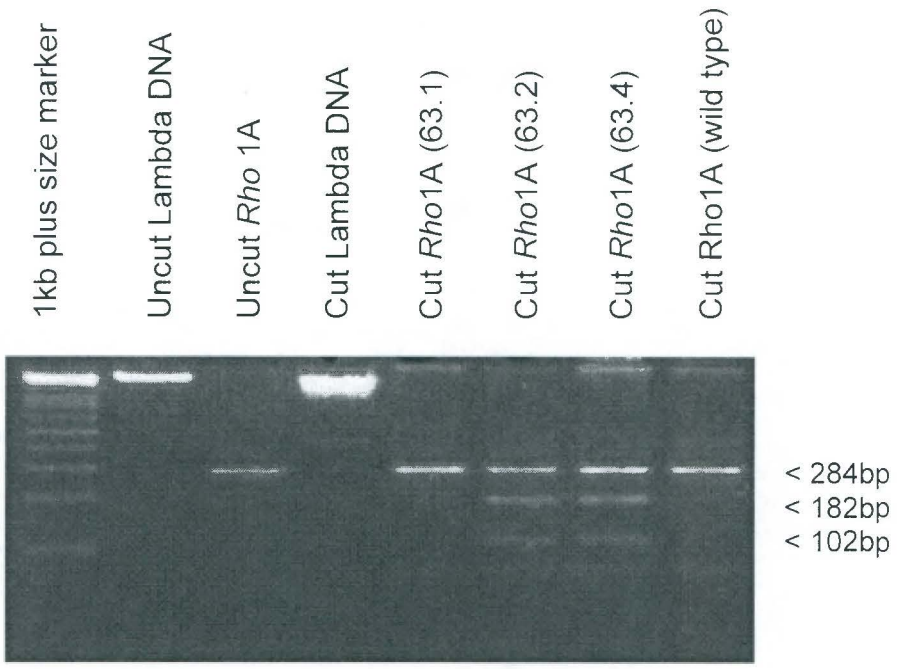



**Figure 3.3** Digital photograph of a 3% agarose gel showing the products of the *BstX I* restriction enzyme digest of samples from family 63. The PCR product of 284bp is cut into fragments of 284, 182 and 102bp when the heterozygous Thr17Met mutation is present. This gel was stained with ethidium bromide and visualised by UV transillumination.

Two variations were detected after complete analysis of exon 1A in 61 samples: the nonpathogenic g.269A>G (26/61); and the pathogenic Thr17Met (1/61).

### 3.1.2 *RHO* exon 1B

Prior to the start of this project, 31 samples had been screened for exon 1B. Re-analysis of these results revealed that none of these samples exhibited SSCP mobility shifts. Similarly, the 17 samples screened using traditional SSCP exhibited wild type banding patterns on both SSCP conditions used (urea and glycerol). The final 13 samples were screened using the Multiphor™ II system, and while seven samples exhibited no changes, the remaining six samples exhibited a subtle (inconclusive) variation in mobility. These variations were not reproducible and were not apparent on a second electrophoresis run.

No variations were detected after complete analysis of exon 1B in 61 samples.

### 3.1.3 *RHO* exon 2

Prior to the start of this project, 29 samples had been screened for exon 2 of *RHO* and re-analysis of these SSCP results revealed only wild type banding patterns. Of the 17 samples screened using traditional SSCP, 12 exhibited wild type patterns, and three exhibited a possible variation in mobility on the urea-containing gel only. These subtle variations were not apparent on a second electrophoresis run. A further two samples exhibited possible variations – one on the urea-containing gel and one on the glycerol-containing gel. These variations could not be resolved so the samples were re-screened using the Multiphor™ II system and no changes were observed. The remaining 15 samples were screened using the Multiphor™ II system. Nine of these exhibited wild type banding patterns, five samples exhibited bands that ran slightly higher on the gel than the wild type, and one sample exhibited a subtle variation. All six of these potential mobility shifts were not apparent in a second electrophoresis run.

No variations were detected after complete analysis of exon 2 in 61 samples.

### 3.1.4 *RHO* exon 3

Prior to the start of this project, 29 samples were screened and re-analysis of these results indicated 27 wild type samples and two variants. One sample carried the c.579G>T variation which is a silent polymorphism (the threonine at codon 193 remains unchanged). The second sample carried a variation in intron 3, IVS3+4C>T. This variation has previously been reported as a polymorphism found in 10% of the normal population, and is apparently not associated with disease [90]. The splice prediction program correctly predicted the intron-exon boundaries of exon 3 and these were not affected by the presence of the IVS3+4C>T change or the Thr193Thr polymorphism.

Of the 17 samples screened using traditional SSCP, 13 exhibited no variations, and four exhibited variations on both gel conditions. One of the four mobility shifts was not reproducible, and was not apparent on a second electrophoresis run. The three remaining samples exhibited similar mobility shifts to each other and were therefore analysed by sequencing one sample as a representative. This sample was found to have the IVS3+4C>T variation mentioned above. This variation destroys an *Rsa I* restriction enzyme site, and a subsequent digest revealed that all three samples were heterozygous for this polymorphism. The SSCP mobility shift caused by IVS3+4C>T is shown in Figure 3.4.

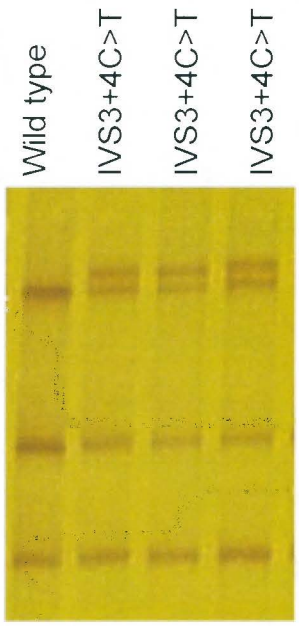



**Figure 3.4** Digital photograph showing the SSCP profile of a wild type sample and three samples with the heterozygous IVS3+4C>T variation. This 10% polyacrylamide gel containing 5% glycerol was visualised by silver staining.

The final 15 samples were screened using SSCP on the Multiphor™ II system. Ten of these samples exhibited wild type banding patterns, while three exhibited similar mobility shifts and two exhibited unique mobility shifts. One representative of each mobility shift was sequenced. The common mobility shift was found to be caused by IVS3+4C>T and an *Rsa I* restriction enzyme digest confirmed this in the three samples with the same banding

pattern. The two unique banding patterns were found to be due to the presence of the same two variations in both cases. These changes were: IVS3+4C>T and the silent variant c.624C>T (which has no effect on the phenylalanine it encodes at position 208, and was not predicted to alter splicing).

Three polymorphisms were detected after complete analysis of exon 3 in 61 samples: Thr193Thr (1/61), IVS3+4C>T (9/61) and Phe208Phe (1/61).

### 3.1.5 *RHO* exon 4

None of the samples had been screened for exon 4 prior to the start of this project. In order to screen the 17 samples using traditional SSCP, the PCR fragment was subjected to digestion using *BstE II* restriction enzyme, which generated a small (143bp) and a large (259bp) fragment. Fourteen of the samples screened in this way showed wild type banding patterns of both the small and large fragments. Two samples showed subtle mobility shifts of the smaller fragment on the urea-containing gel, which could not be resolved with a second electrophoresis run. Direct sequencing showed no variations present in these samples.

One sample exhibited a mobility shift of the large fragment on both gel conditions (Figure 3.5). Direct sequencing revealed the presence of a homozygous IVS4+1G>T variation in this Caucasian individual with ARRP (Figure 3.6).

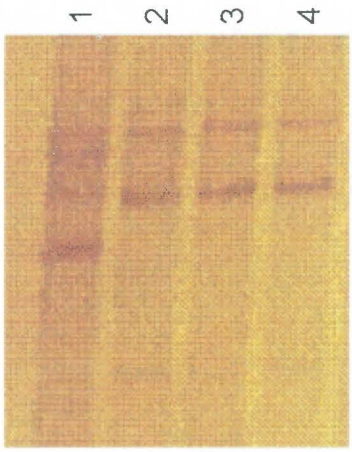



**Figure 3.5** Digital photograph of an SSCP gel that shows the mobility shift in sample 185.1 (lane 1) compared to the normal banding pattern (lanes 2-4). This 10% polyacrylamide gel contained 7.5% urea and was visualised by silver staining.

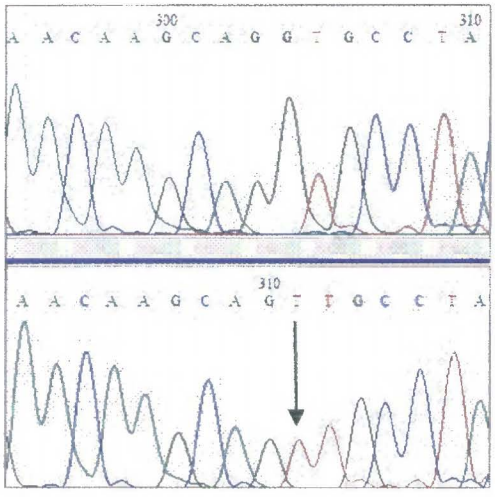



**Figure 3.6** Sequencing electropherogram of *RHO* exon 4, showing the wild type sequence (top) and the homozygous IVS4+1G>T mutation (bottom, indicated by an arrow).

The IVS4+1G>T variant disrupts the intronic splice site and has been reported as causing disease in heterozygous carriers [91-93]. There has also been a report of asymptomatic mutation carriers [94], which has resulted in discussion as to whether this mutation acts in a dominant or recessive manner. There have been no reports of this variant in the homozygous state. In order to increase the number of samples available for analysis in this family another 10 individuals from this family were recruited onto the

research project. The IVS4+1G>T mutation destroys a *Ban I* restriction enzyme site, and the subsequent restriction enzyme digestion analysis confirmed the presence of the homozygous IVS4+1G>T mutation in individual 185.1, the heterozygous mutation in eight family members, and the absence of the mutation in two family members of family 185. The family pedigree and clinical information of individuals in family 185 is presented in Appendix 4.

In order to exclude uniparental disomy as the cause of this homozygosity, genotyping was performed on DNA from the homozygous proband as well as a heterozygous sibling. Twenty-three markers spanning chromosome 3 from the ABI Prism™ Linkage Mapping Set version 2 were analysed, and in order to improve the density of the map around *RHO*, an additional three markers were examined that had been established for research prior to the start of this project (D3S1610, D3S3606 and D3S1576). The result of this experiment and the physical and genetic distances between these markers (including the position of *RHO*) are summarised in Table 3.3.

**Table 3.3** The markers used to determine heterozygosity of chromosome 3, the distances between them, and their peak sizes. The heterozygous results are indicated in green and homozygous results in yellow. *RHO* is indicated in red for perspective on marker positions.

| Marker | D number | Distance (Kosambi cM) | Distance (bp) | Individual 185.1 | | Individual 185.2 | |
|---|---|---|---|---|---|---|---|
| | | | | Allele 1 | Allele 2 | Allele 1 | Allele 2 |
| AFM217xd2 | D3S1297 | | | | | | |
| | | 14.02 | 4880823 | | | | |
| AFM234tf4 | D3S1304 | | | 261.05 | 268.77 | 261.29 | 268.93 |
| | | 13.77 | 4597982 | | | | |
| AFM079yg5 | D3S1263 | | | 200.52 | 208.44 | | |
| | | 6 | 5332158 | | | | |
| AFMa037zf5 | D3S2338 | | | 103.15 | 103.15 | 99.39 | 103.15 |
| | | 10.5 | 11107982 | | | | |
| AFM095xc1 | D3S1266 | | | | | | |
| | | 8.92 | 6698446 | | | | |
| AFM164we1 | D3S1277 | | | | | | |
| | | 9.89 | 19823682 | | | | |
| AFM198yf2 | D3S1289 | | | | | | |
| | | 8.91 | 6030427 | | | | |

**Table 3.3 continued**

| Marker | D number | Distance (Kosambi cM) | Distance (bp) | Individual 185.1 | | Individual 185.2 | |
|---|---|---|---|---|---|---|---|
| | | | | Allele 1 | Allele 2 | Allele 1 | Allele 2 |
| AFM220yh4 | D3S1300 | | | 238.39 | 258 | 238.54 | 250.58 |
| | | 10.86 | 4429252 | | | | |
| AFM191yg5 | D3S1285 | | | 241.25 | 247.3 | 241.11 | 247.04 |
| | | 6.57 | 5467881 | | | | |
| AFM234tb8 | D3S1566 | | | 161.54 | 171.19 | 161.56 | 184.72 |
| | | 11.47 | 9512338 | | | | |
| AFMc003zg5 | D3S3681 | | | | | 143.08 | 155.35 |
| | | 8.54 | 22323002 | | | | |
| AFM126zc5 | D3S1271 | | | 99.08 | 99.08 | 98.93 | 98.93 |
| | | 10.13 | 12337524 | | | | |
| AFM321xf5 | D3S1610 | | | | | | |
| | | 1.84 | 2051985 | | | | |
| AFM164xc5 | D3S1278 | | | | | | |
| | | 9.39 | 7918990 | | | | |
| AFM116xh2 | D3S1267 | | | 111.15 | 120.8 | 111.25 | 120.98 |
| | | 4.82 | 4156981 | | | | |
| AFMb020zb9 | D3S3606 | | | 174.51 | 174.51 | 174.51 | 180.43 |
| | | | 2047272 | | | | |
| RHODOPSIN | | | | | | | |
| | | | 2376178 | | | | |
| AFM199xd6 | D3S1292 | | | 123.83 | 123.83 | 123.88 | 123.88 |
| | | 4.89 | 5789644 | | | | |
| AFM267xd9 | D3S1576 | | | 190.68 | 190.68 | 190.68 | 192.58 |
| | | 6.89 | 5951585 | | | | |
| AFM240xb2 | D3S1569 | | | 170.74 | 170.74 | 161.16 | 172.71 |
| | | 11.22 | 7653788 | | | | |
| AFM164yg9 | D3S1279 | | | | | | |
| | | 8.15 | 17184747 | | | | |
| AFM345th5 | D3S1614 | | | 114.11 | 117.97 | 114.01 | 117.87 |
| | | 8.29 | 5272693 | | | | |
| AFM224zb12 | D3S1565 | | | 184.7 | 186.62 | 184.68 | 186.69 |
| | | 15.1 | 12740663 | | | | |
| AFM059xa9 | D3S1262 | | | 127.67 | 133.63 | 127.67 | 133.7 |
| | | 6.59 | 2319500 | | | | |
| AFM270zg9 | D3S1580 | | | 225.26 | 229.26 | 225.26 | 225.26 |
| | | 6.72 | 3134418 | | | | |
| AFM308yf1 | D3S1601 | | | 311.7 | 323.61 | 299.89 | 327.32 |
| | | 10.43 | 5342411 | | | | |
| AFM254ve1 | D3S1311 | | | 154.89 | 158.74 | 151.09 | 152.96 |

Eighteen of the genotyping PCRs were successful and the DNA of the proband was heterozygous for 12 of the markers and homozygous for only six of them. An asymptomatic sibling was used as a control to show heterozygosity exists in the family, and the DNA from this person was heterozygous for 15 markers and homozygous for three. Heterozygosity of the markers was observed along the length of chromosome 3, and as close as 6.2Mb from *RHO* (marker D3S1267, shown in Figure 3.7). The results of this experiment prove that uniparental disomy is not the cause of the homozygous IVS4+1G>T in the proband of family 185.

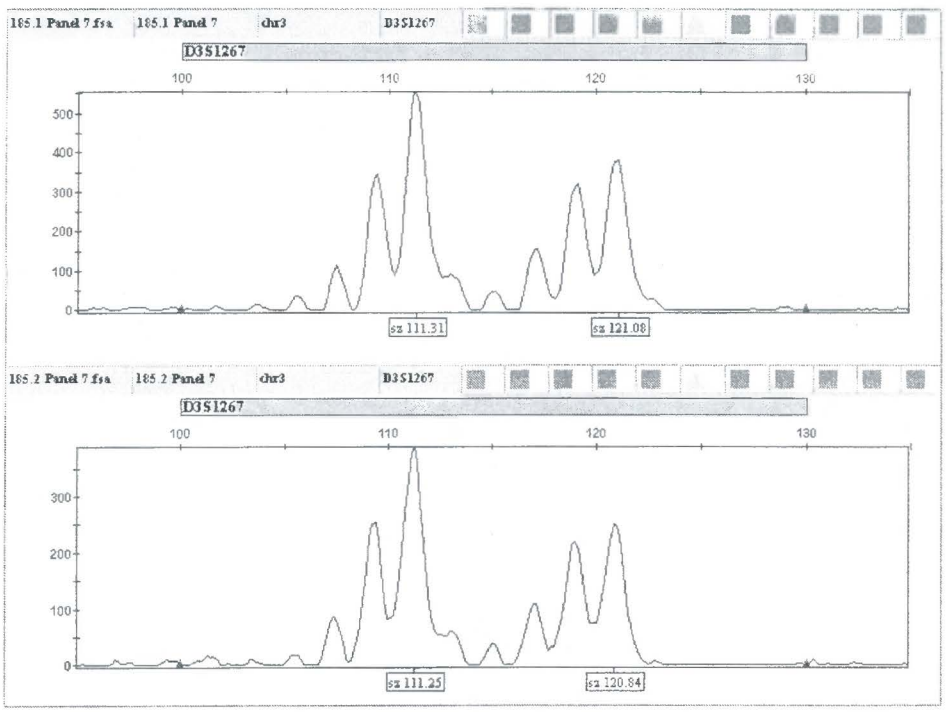



**Figure 3.7** Genotyping results of marker D3S1267 showing two alleles in individuals 185.1 (top) and 185.2 (bottom).

The remaining 44 samples were screened for mutations in exon 4 using the Multiphor™ II system, and although one inconclusive variation was observed in one sample, it was not apparent on a second electrophoresis run. The other 43 samples all exhibited the wild type SSCP pattern.

One pathogenic variant was detected after complete analysis of exon 4 in 61 samples: IVS4+1G>T (1/61).

### 3.1.6 *RHO* exon 5

Prior to the start of this project, 31 of the samples had been screened for *RHO* exon 5, and re-analysis of these results revealed 27 samples of the wild type and four samples carrying the IVS4-23G>A variation. This variation has been reported as an infrequent polymorphism (dbSNP:rs2071092) and is not pathogenic [85]. Splice prediction analysis of IVS4-23G>A revealed that this variant had no effect on splicing.

The traditional SSCP screen revealed no variations in the 17 samples screened, although two subtle mobility shifts were observed on the urea-containing gel which were not apparent on a second electrophoresis run. The Multiphor™ screen also revealed no variations in the 13 samples screened; although one sample exhibited a band running slightly higher than usual, this was not apparent on a second electrophoresis run.

One polymorphism was detected after complete analysis of exon 5 in 61 samples: IVS4-23G>A (4/61).

### 3.1.7 *RHO* MLPA

For the MLPA screen to analyse *RHO*, 48 individuals with ADRP were initially selected. Results are only available for 34 of these, as the reactions failed in 14 samples. The reaction was repeated in three of the failed samples and a second failure indicated the DNA quality of these 14 samples may have been the limiting factor, despite being checked prior to MLPA.

No deletions or insertions of *RHO* were detected in samples from 34 individuals with ADRP.

## 3.2 *RP1*

For the *RP1* mutation screen, DNA from a cohort of 70 individuals with ADRP was subjected to dHPLC analysis. Of these individuals, 13 had mutations previously identified in other RDD genes including five with *RHO* mutations. These individuals were included to investigate the effect of possible modifier alleles.

### 3.2.1 *RP1* exon 4F

Upon dHPLC analysis four samples were found to exhibit variant chromatogram profiles, when compared to the wild type sample. Three of these samples showed subtle changes - peak shifts - at 54°C and normal peaks at 56.1°C, however direct sequencing of the fragment revealed no sequence variations in any of these samples. The fourth sample showed a subtle broadening of the peak at 54°C and an obvious heteroduplex peak at 56.1°C (Figure 3.8). Direct sequencing of the fragment in this sample from a Caucasian individual (524.2) revealed the presence of a heterozygous c.2029C>T (Arg677X) variation. This variation has been reported as pathogenic and truncates the protein by 1480 amino acids [43]. Arg677X is the most common mutation in *RP1* and is responsible for 3% of ADRP in the US.

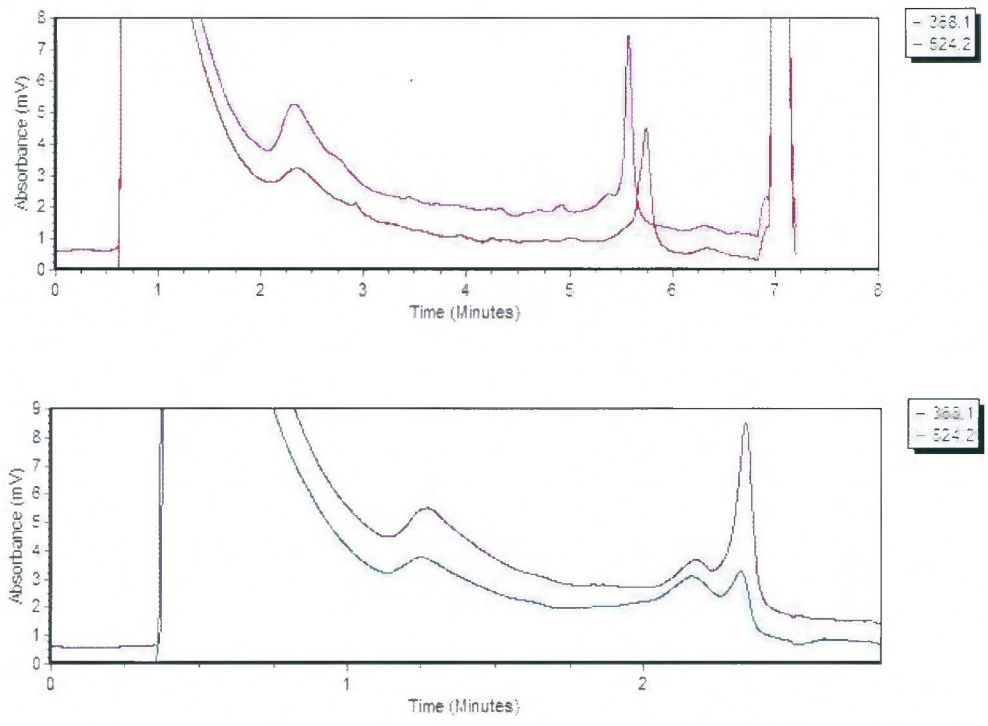



**Figure 3.8** dHPLC chromatograms of the test sample 524.2 and the wild type sample 368.1 at 54°C (top) and 56.1°C (bottom). Differences between the two samples are subtle at 54°C and more obvious at 56.1°C.

The Arg677X variant disrupts a *Taq I* site, and the restriction enzyme digest was performed on DNA from family 524. Samples were only available from two affected individuals in this family, and the digest revealed the mutation was present in both individuals in the heterozygous state (Figure 3.9). The family pedigree and clinical information of individuals in family 524 is presented in Appendix 4.

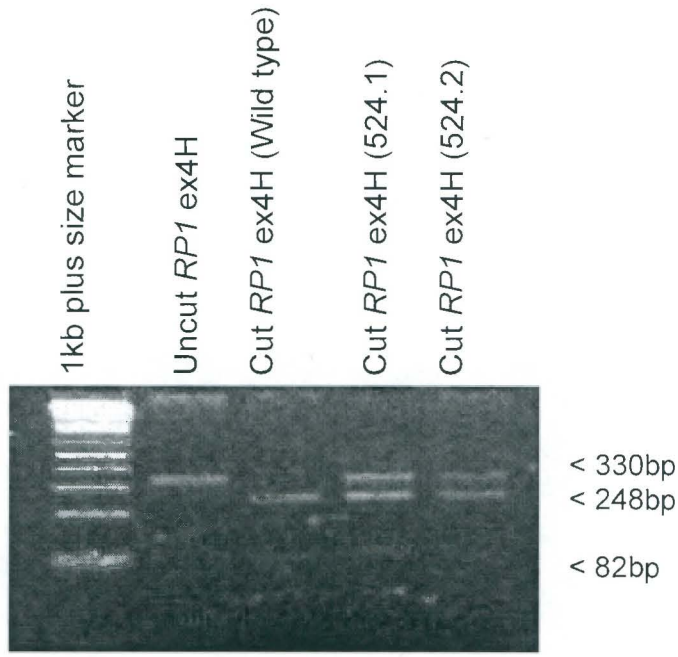



**Figure 3.9** Digital photograph of a 2.5% agarose gel showing the products after *Taq I* restriction enzyme digest of samples from family 524. The PCR product of 330bp is cut into fragments of 248, and 82bp in the wild type and 330, 248 and 82bp when the heterozygous c.2029C>T mutation is present. This gel was stained with ethidium bromide and visualised by UV transillumination.

One pathogenic mutation was detected after complete analysis of exon 4F in 70 samples: Arg677X (1/70).

### 3.2.2 *RP1* exon 4G

Upon dHPLC analysis of exon 4G, five samples were identified as having identical peak profiles different to the wild type at 54°C and 54.5°. No variations were observed in these samples at 57.5°C. One of the five samples was selected as a representative and sequenced. Direct sequencing revealed the presence of a novel heterozygous c.2255C>T variation. This variant encoding a Thr752Met amino acid change creates an *Hsp92 II* restriction enzyme site. An additional sample was identified with a similar but not identical aberrant dHPLC profile, and upon sequencing was found to

carry the same Thr752Met variant (Figure 3.10). A restriction enzyme digest revealed this variant was present in the heterozygous state in all six samples with identical aberrant peak profiles.

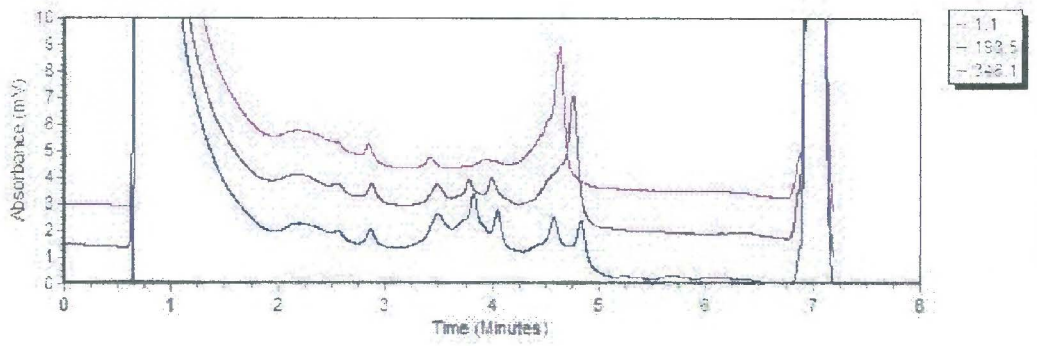



**Figure 3.10** dHPLC chromatograms at 54°C showing the wild type peak profile (1.1), and the two profiles caused by the heterozygous Thr752Met variant: the common profile (346.1) and similar aberrant profile (193.5).

Two of the six probands carrying this variant already had known pathogenic *RHO* mutations identified, indicating that the Thr752Met change in *RP1* is not pathogenic in these families. Samples from the extended family members of one of these individuals were screened using the *Hsp92 II* digest for the *RP1* Thr752Met variant. The *RP1* variant did not co-segregate with disease in this family, nor did affected individuals with this variant have an earlier age of onset than affected individuals without this variation.

In order to determine the population frequency of Thr752Met, samples from 51 Caucasian and 52 Black control individuals were assessed for the presence of this variant using a WAVE mixing experiment at 54°C. The Thr752Met change was detected in one Caucasian control individual (1.89% of control individuals; 0.98% of control alleles) and three Black control individuals (5.77% of control individuals; 2.9% of control alleles), indicating it is a rare variant in the South African population. This information was submitted to dbSNP and a reference number was assigned to this variant (dbSNP:rs28399531).

DNA from two probands with ADRP exhibited peak shifts and another two showed subtle peak changes on dHPLC, however sequencing of these samples revealed they carried the wild type exon 4G sequence.

One polymorphism was detected after complete analysis of exon 4G in 70 samples: Thr752Met (6/70).

### 3.2.3 *RP1* exon 4H

After dHPLC analysis of exon 4H, 19 samples were identified as having the same aberrant chromatogram profile at all three temperatures, and a further two samples had similar but not identical profiles to this pattern (Figure 3.11). Three of these samples were sequenced and all were found to carry the heterozygous c.2615G>A variant which encodes an Arg872His change. This variant was previously reported as a nonpathogenic polymorphism present in 25% of the population [44], and there has recently been a report suggesting that this variant is associated with a decreased risk of RP [52].

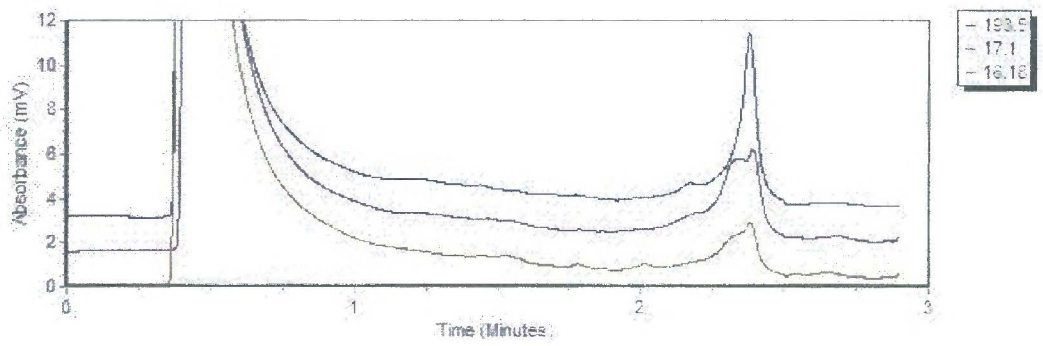



**Figure 3.11** dHPLC chromatograms at 55°C, showing the wild type profile (193.5) and the two profiles caused by the heterozygous Arg872His – the common profile (17.1) and the similar profile (16.18).

The remaining 18 samples were tested for the presence of the Arg872His change using the WAVE mixing experiment and all 18 samples were confirmed as carrying the heterozygous polymorphism. The ethnic origins of the 21 individuals with this variant were classified as follows: 15 Caucasian,

four Black, one Indian and one Mixed Ancestry. One sample (from a Caucasian individual) with a unique dHPLC profile was sequenced and found to carry the Arg872His polymorphism in the heterozygous state, with no other apparent variations. One other sample (also from a Caucasian individual) was found to carry the variant in the homozygous state, together with a pathogenic mutation (Ser911X, described below). The Arg872His variation was therefore detected in 23 of 70 (33%) individuals with ADRP and 24 of 114 (21%) alleles.

In order to determine the frequency of the Arg872His variant in the general population, DNA from 51 Caucasian and 52 Black control individuals was screened using the WAVE mixing experiment at 56°C. Using this experiment the polymorphism was detected in 24 Caucasian control individuals (47% of individuals; 23.53% of alleles) and 12 Black control individuals (23% of individuals, 11.5% of alleles). This information was submitted to dbSNP and was added to an existing reference assigned to this variant (dbSNP:rs444772). A chi-square test was performed and there is a significant ($p = 0.037$) association between ethnicity and the prevalence of this polymorphism in SA.

One of the samples screened for exon 4H had a subtle dHPLC change, and sequencing revealed no variations in the sequence of that sample. A further two samples (both from Caucasian individuals) presented with unique changes at all three temperatures (Figure 3.12), and these two samples were both found to be carrying novel pathogenic mutations after sequencing.

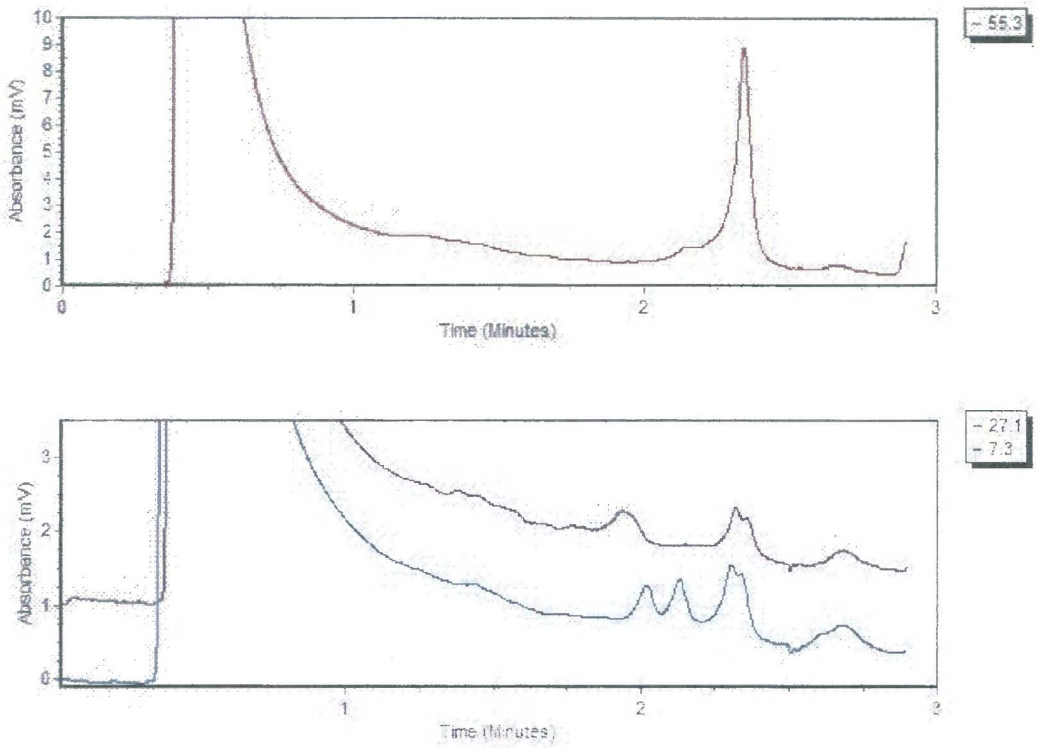



**Figure 3.12** dHPLC chromatograms showing the profiles of the wild type (top) and pathogenic mutations (bottom) of fragment 4H at 55°C

Sample 7.3 had (in addition to the homozygous Arg872His polymorphism) a novel c.2732C>A variation which causes the protein to be truncated by 1246 amino acids (Figure 3.13).

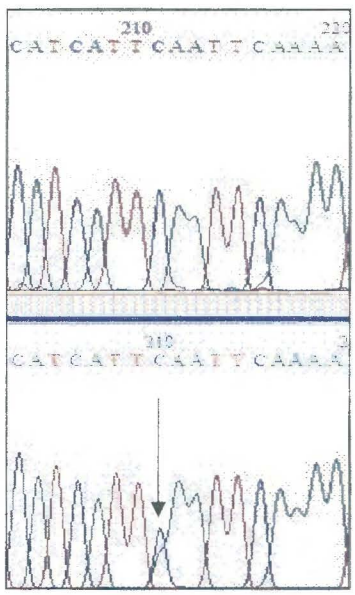


**Figure 3.13** Sequencing electropherogram showing the wild type *RP1* exon 4H sequence (top) and the heterozygous c.2732C>A mutation in sample 7.3 (bottom, indicated by the arrow).

Population frequency analysis was performed using dHPLC, and the Ser911X mutation was not detected in 102 chromosomes examined in Caucasian control individuals nor 104 chromosomes examined in Black control individuals. Ser911X creates a site for *Acs I* restriction enzyme and its co-segregation with disease in family 7 was confirmed by a restriction enzyme digest. The mutation was detected in two affected individuals and two at-risk individuals and was absent in one at-risk individual. The family pedigree and clinical information of individuals in family 7 is presented in Appendix 4.

Sample 27.1 was found to carry a novel 10bp deletion, c.2590-2599delATAACTTTAA (Figure 3.14). The mutation is predicted to cause a frameshift at codon 864, truncating the protein by 1280 amino acids. Due to these dramatic effects on RP1, it was not necessary to perform population frequency analysis.

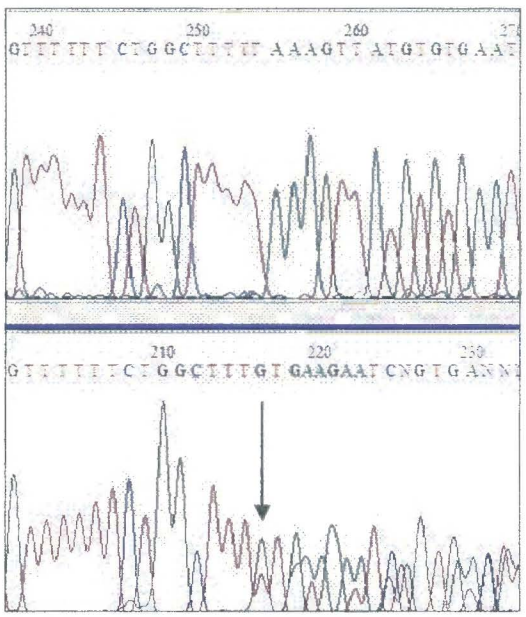


**Figure 3.14** Sequencing electropherograms showing the wild type *RP1* exon 4H sequence (top) and the c.2590-2599delATAACTTTAA mutation (bottom). The sequence shown here was generated from the reverse primer and is the reverse-complement of the sequence on the forward strand. The arrow indicates the point at which the deletion occurs and leads to double sequence after this point.

The co-segregation of the 10bp deletion with disease in family 27 was confirmed using nondenaturing, sized-based separation on the WAVE (Figure 3.15). The deletion was present in four affected members, in four successive generations of the family and in one at-risk individual. The family pedigree and clinical information of individuals in family 27 is presented in Appendix 4.

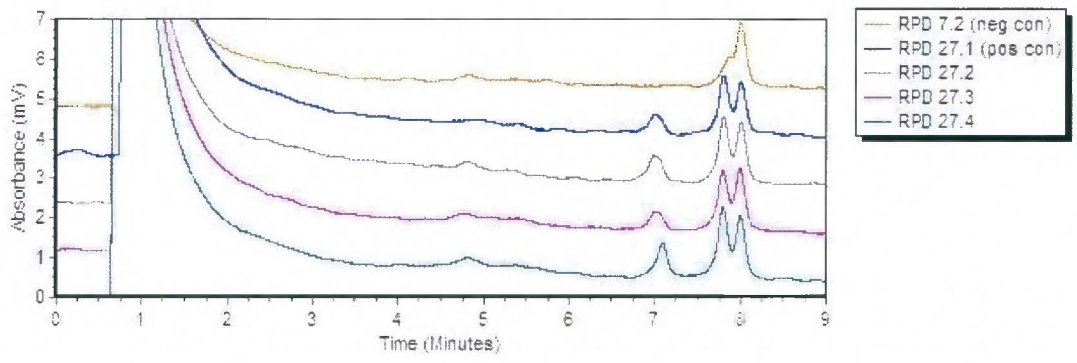



**Figure 3.15** Chromatograms showing the size-based separation of the wild type and deleted alleles in samples from family 27.

Three variations were detected after complete analysis of exon 4G: the Arg872His polymorphism (23/70); the Ser911X mutation (1/70) and the c.2590-2599delATAACTTTAA mutation (1/70).

### 3.2.4 *RP1* MLPA

As described for the *RHO* MLPA, results are available for 34 individuals, although 48 individuals with ADRP were initially selected. One sample with a 10bp deletion in *RP1* was included to determine whether a deletion of this small size could be identified by MLPA, although it is unlikely that such a deletion would be detected unless it occurred at the hybridisation position of a probe. No deletions or insertions of *RP1* were detected in samples from 34 individuals with ADRP and the MLPA failed to detect the known 10bp deletion.

## 3.3 *RPE65*

For the *RPE65* mutation screen, a cohort of 87 individuals was selected. Of these individuals, 18 were classified as having LCA and 69 as having early onset ARRP (with an age of onset younger than 15 years). None of the individuals selected for *RPE65* screening had causative mutations identified in other genes, although one of the individuals with LCA had a single heterozygous mutation previously identified in *RPE65*. This sample was included as the other mutation causing disease in this individual was unknown.

### 3.3.1 *RPE65* exon 1

No mutations were detected in exon 1 of the 18 samples from LCA patients. One sample from an individual with ARRP had a subtle change at one temperature, which was not apparent on repeat analysis.

One sample, 568.4, had a different chromatogram profile to the wild type at multiple temperatures. This sample was sequenced and found to carry a heterozygous IVS1+1G>T mutation, a novel variant. The splice prediction program correctly predicted the intron-exon boundaries of exon 1 and the donor splice site was abolished by the presence of IVS1+1G>T. This variant creates an *Mse I* restriction enzyme site, and a restriction enzyme digest was performed to test whether the variant co-segregated with disease in family 568 (Figure 3.16). IVS1+1G>T was detected in one parent and three siblings (two of which were affected) in the heterozygous state. The family pedigree and clinical information of individuals in family 568 is presented in Appendix 4.

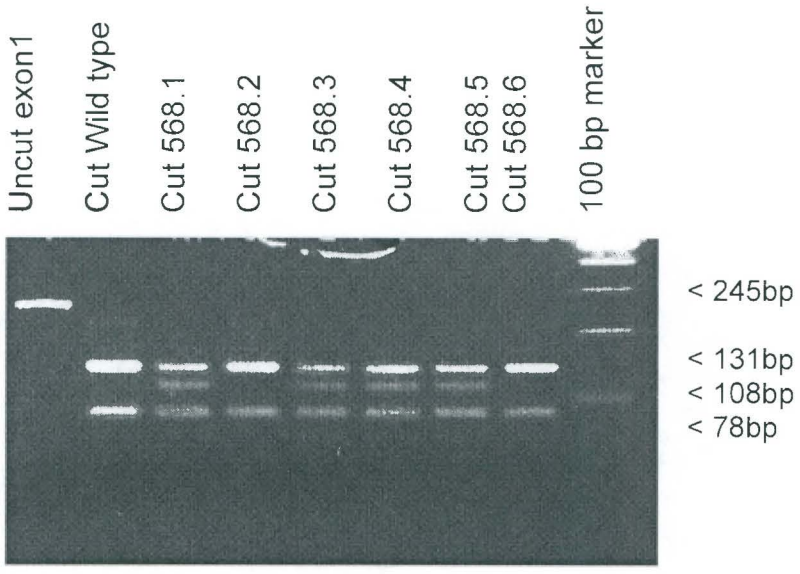



**Figure 3.16** Digital photograph of a 3% agarose gel showing the products of *Mse I* digestion of samples from family 568. The wild type sequence produces fragments of 131, 78 and 36bp after cutting, while the heterozygous variant sequence produces fragments of 131, 108, 78, 36 and 23bp. This gel was stained with ethidium bromide and visualised by UV transillumination. Fragments smaller than 78bp are not visible on this image.

In order to determine the population frequency of IVS1+1G>T in a cohort matched for ethnicity to family 568, DNA from 50 unaffected Indian control individuals was tested for the presence of this variant using the *Mse I* digest. The variation was not detected in the 100 chromosomes tested, indicating that IVS1+1G>T is a pathogenic mutation and is causative of disease in this family.

One pathogenic mutation was detected after complete analysis of exon 1 in 87 samples: IVS1+1G>T (1/87).

### 3.3.2 *RPE65* exon 2

No variations were detected in exon 2 of the 18 samples from LCA patients. A subtle variation was observed in one sample from an individual with ARRP, but was not apparent on a second dHPLC analysis. Two samples from individuals with ARRP had unexplained peaks which raised the baseline and could have masked potential variations in their chromatograms. Direct sequencing revealed no variations in either of these samples.

A variation was observed in one sample, 373.1, at both temperatures analysed. Sequencing revealed the presence of two heterozygous variations in exon 2 of this individual with ARRP: c.61G>A encodes a Glu21Lys variation and c.65T>C encodes a Leu22Pro variation. The Leu22Pro variant has been previously reported as a pathogenic mutation [95] while the Glu21Lys variation is novel.

Glu21Lys destroys a *MnlI* restriction enzyme site, and Leu22Pro creates an *NlaIV* site so these restriction enzyme digests were performed on samples from two individuals in the family (the affected individual and her mother). Both variations were present in the affected individual (373.1) and only the Glu21Lys variation was present in the mother (373.2) (Figure 3.17). The family pedigree and clinical information of the affected individual in family 373 is presented in Appendix 4.

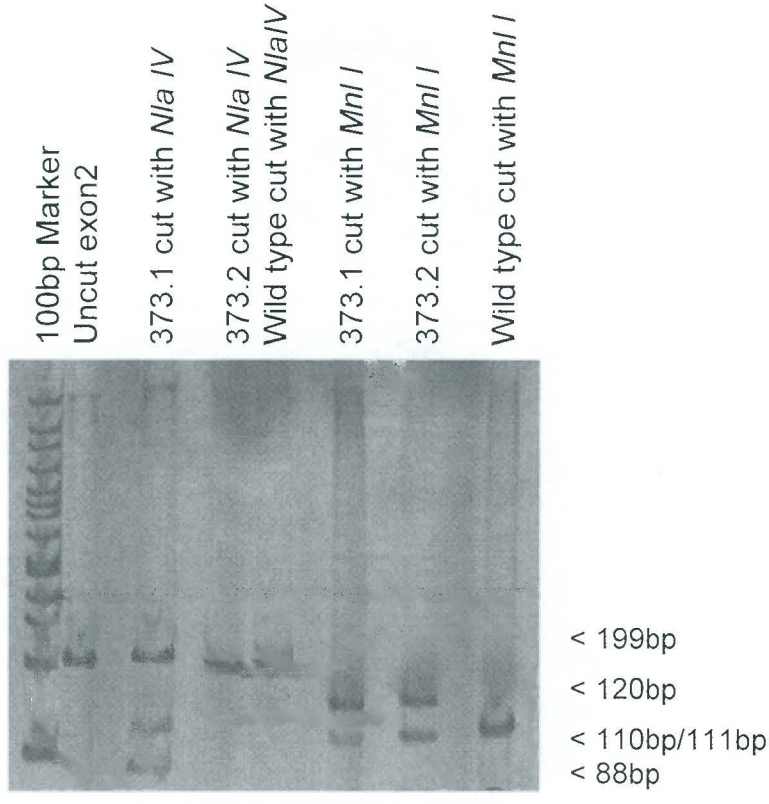



**Figure 3.17** Digital image of a Multiphor™ gel showing the segregation of Leu22Pro and Glu21Lys in samples from family 373. When Leu22Pro is cut with *Nla IV*, the wild type band of 199bp is cut into 111bp and 88bp fragments. When Glu21Lys is cut with *Mnl I*, the wild type profile of 110, 53, 26 and 10bp fragments gains an additional band at 120bp. Fragments smaller than 88bp are not visible on this gel image. This 8% polyacrylamide gel was visualised by silver staining.

In order to determine whether Glu21Lys was causative of disease, DNA from 50 unaffected Caucasian control individuals was tested using the *Mnl I* digest and none of these 100 chromosomes were found to carry the variation. The amino acid change results in an uncharged, polar, hydrophilic amino acid being replaced by a positively charged, polar, hydrophilic amino acid. This evidence indicates that Glu21Lys is pathogenic and together with Leu22Pro is causative of disease in individual 373.1.

Two pathogenic mutations were detected after complete analysis of exon 2 in 87 samples: Glu21Lys (1/87) and Leu22Pro (1/87).

### 3.3.3 *RPE65* exon 3

Of the 18 samples from LCA patients screened for exon 3, one sample exhibited a peak shift and one sample exhibited a change at both temperatures analysed. Both samples were sequenced and the sample with the peak shift was found to have no discernable variations, while the other sample was found to carry an intronic change, IVS2-18G>A. The splice prediction program correctly predicted the intron-exon boundaries of exon 3, and could therefore be used to analyse the effect of IVS2-18G>A. This novel variant was not predicted to have an effect on splicing. This variation was also detected in two samples from ARRP patients which exhibited aberrant chromatograms at both temperatures. Sequencing of these two samples revealed both carried the IVS2-18G>A change and another novel intronic change, IVS3+80T>A. Once again, the splice prediction program correctly predicted the intron-exon boundaries of exon 3 and did not detect any change in splicing as a result of IVS3+80T>A. Four samples from individuals with ARRP exhibited peak shifts at 60.5°C, but these were not apparent on a second dHPLC run.

Two polymorphisms were detected after complete analysis of exon 3 in 87 samples: IVS2-18G>A (3/87) and IVS3+80T>A (2/87).

### 3.3.4 *RPE65* exon 4/5

No variations were detected in exon 4 or 5 of the 18 samples from LCA patients, but three samples from ARRP patients were found to have aberrant dHPLC profiles. Direct sequencing of all three samples revealed the presence of a heterozygous c.394G>A variation in exon 5 of each (Figure 3.18).

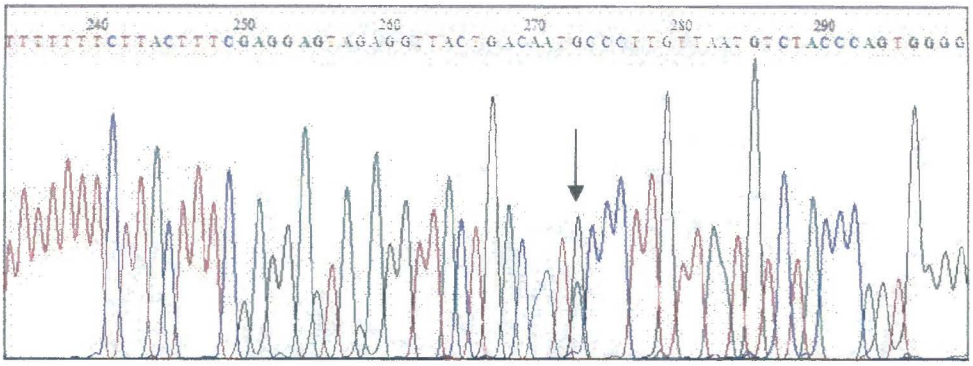


**Figure 3.18** Sequencing electropherogram of *RPE65* exon 5. The arrow indicates the position of the c.394G>A mutation.

This variation encodes an Ala132Thr change to the protein that has previously been reported as causing ARRP [55, 96]. This mutation does not affect a restriction enzyme site, so family studies were performed using the WAVE mixing experiments. In family 568 (the Indian family in which IVS1+1G>T was found in the father and two affected children) the Ala132Thr mutation was detected in the mother and both affected siblings. The mutation was absent in two asymptomatic siblings. The family pedigree and clinical information of individuals in family 568 is presented in Appendix 4.

In family 394 (also an Indian family), Ala132Thr was detected in two affected siblings, and was absent in a spouse. The family pedigree and clinical information of individuals in family 394 is presented in Appendix 4.

In family 387 (a Caucasian family), Ala132Thr was detected in one parent, one affected child and one asymptomatic child (Figure 3.19). The family pedigree and clinical information of individuals in family 387 is presented in Appendix 4.

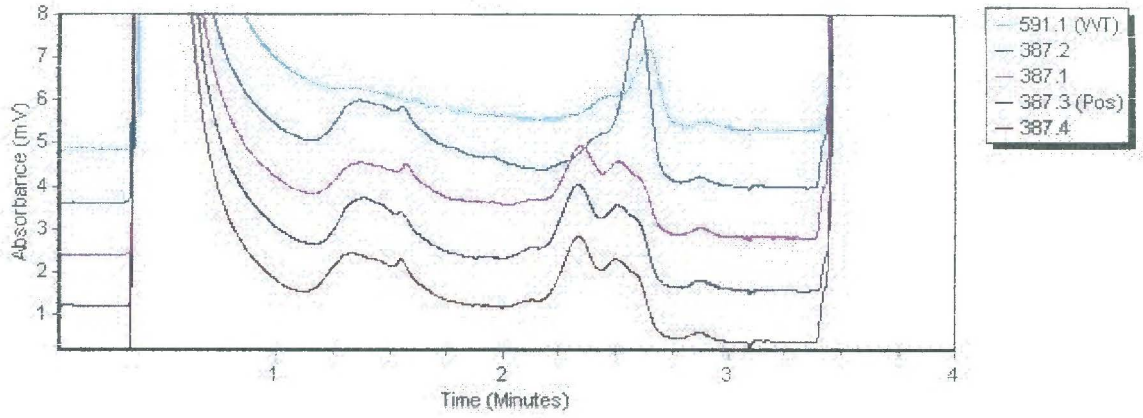



**Figure 3.19**. dHPLC chromatograms showing the profile of the Ala132Thr variation present in samples 387.1, 387.3 and 387.4, and absent in 387.2 and the wild type sample (591.1).

One pathogenic mutation was detected after complete analysis of exons 4 and 5 in 87 samples: Ala132Thr (3/87).

### 3.3.5 *RPE65* exon 6

Two similar but non-identical aberrant dHPLC profiles were detected in exon 6 of the samples from individuals with LCA. Direct sequencing revealed that both samples carried a heterozygous intronic variant, IVS6+22C>T. A further seven samples from individuals with ARRP carried similar dHPLC profile changes and sequencing of one of these samples revealed the same intronic variation was present. The IVS6+22C>T variation destroys a *MnlI* restriction enzyme site and a digest was performed on the nine samples with similar dHPLC profiles. IVS6+22C>T was detected in the heterozygous state in all these samples. This variation is a reported SNP (dbSNP: rs2274321) and was not predicted to have an effect on splicing.

Three samples with subtle dHPLC profile variations were sequenced and found to have wild type sequences.

One polymorphism was detected after complete analysis of exon 6 in 87 samples: IVS6+22C>T (9/87).

### 3.3.6 *RPE65* exon 7/8

Of the 18 samples from LCA patients screened for exon 7 and 8, only one exhibited a variant dHPLC chromatogram. This sample was sequenced and found to carry the heterozygous c.750C>T which is a silent polymorphism, as it does not alter the amino acid isoleucine at position 250 of the protein. This isocoding change was not predicted to have any effect on splicing.

Samples from two ARRP patients exhibited variant dHPLC chromatograms. Both of these samples were sequenced and found to carry a variation, however one of these (IVS7+98C>T) was intronic and not predicted to affect splicing. The other variation, in sample 568.4 (from the individual with the IVS1+1G>T and Ala132Thr mutations), was a c.746A>G change in exon 8 which encodes a Tyr249Cys amino acid change. This is a novel variation which results in a polar amino acid with an aromatic ring being replaced by a nonpolar amino acid which forms disulphide bridges. The family study was performed using the WAVE mixing experiment, and the variation was detected in one parent (individual 568.2) and two affected children, and absent in two asymptomatic children. The Tyr249Cys change was inherited together with the known mutation Ala132Thr in all cases. In order to determine whether the amino acid at position 249 is evolutionary conserved, the protein sequence of 11 species was aligned and all were found to have a Tyrosine at this position. It is therefore unclear as to whether Tyr249Cys is pathogenic, but it would appear that IVS1+1G>T and Ala132Thr are the two causative mutations in family 568. The family pedigree and clinical information of individuals in family 568 is presented in Appendix 4.

Three variations were detected after complete analysis of exons 7 and 8 in 87 samples: Ile250Ile (1/87), IVS7+98C>T (1/87) and Tyr249Cys (1/87).

### 3.3.7 *RPE65* exon 9

Of the 18 samples from LCA patients screened for exon 9, five samples had an unexplained peak which raised the baseline and could have masked potential variations in their chromatograms. Sequencing of these five samples revealed no variations. One sample from an LCA patient had an altered profile at three temperatures and this was the sample known to have an exon 9 variation (c.963T>G or Asn321Lys). This heterozygous variation was identified in sample 218.4 prior to this project but the sample was included in order to determine the second pathogenic variant. The sample was sequenced and the Asn321Lys variation was verified.

Of the samples from individuals with ARRP, two samples exhibited slight deviations from the wild type dHPLC profile, but these variations were not apparent during a second dHPLC analysis.

No new variations were detected after complete analysis of exon 9 in 87 samples and the expected Asn321Lys (1/87) mutation was verified.

### 3.3.8 *RPE65* exon 10

Two variants were detected in exon 10 of the 18 samples from LCA patients, and direct sequencing revealed that both samples had the silent polymorphism c.1056G>A (Glu352Glu; dbSNP:rs12145904). The variant was present in the homozygous state in one individual and the heterozygous state in the other. This polymorphism has been previously reported as nonpathogenic, and occurring at a frequency of 14% of controls and 15% of patients [55]. Of the samples from individuals with ARRP, 18 samples had identical aberrant profiles at one or more temperatures, one sample had a similar profile to this common profile and two samples had subtle changes. One sample exhibiting the common profile was sequenced and found to carry the Glu352Glu polymorphism. Exon 10 has a single *Mnl I* restriction enzyme site, which is destroyed when Glu352Glu is present. The restriction enzyme

digest was performed on all samples from individuals with ARRP showing deviations from the wild type profile and 18 samples were confirmed as having the heterozygous variant and one as being homozygous for the variant. Glu352Glu was not predicted to affect splicing.

The remaining two samples were found not to have the Glu352Glu change. One of these samples had the subtle dHPLC peak deviation, and sequencing revealed that this sample had the wild type exon 10 sequence. The final sample, 52.1, had a similar dHPLC profile to the one caused by Glu352Glu however sequencing revealed the presence of a homozygous c.1102T>C, which encodes a Tyr368His change (Figure 3.20).

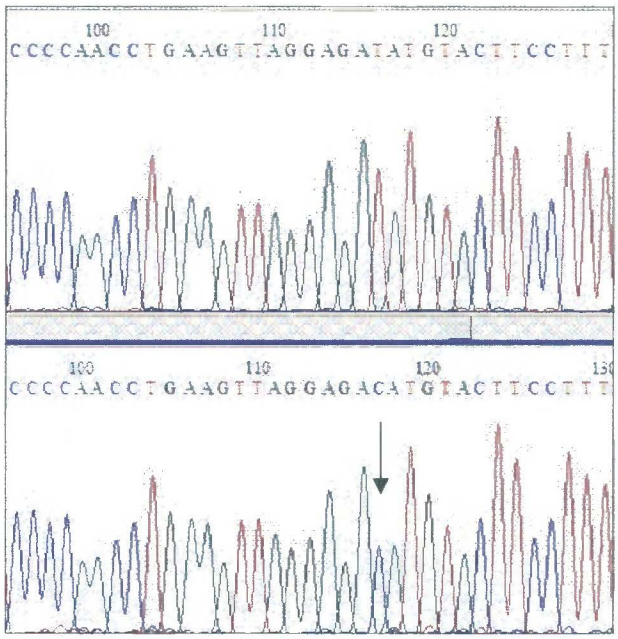



**Figure 3.20** Sequencing electropherogram of the *RPE65* exon 10 wild type sequence (top) and exon 10 of sample 52.1 (bottom). The arrow indicates the position of the homozygous T>C change.

The Tyr368His mutation has been reported as being pathogenic, due to the significant change caused to the protein structure by replacement of a negatively charged tyrosine by a positively charged histidine [97]. This mutation creates an *Alw26 I* restriction enzyme site in exon 10, so a restriction enzyme digest was performed in order to study the segregation of the mutation in family 52, a Mixed Ancestry family (Figure 3.21). The mutation was detected in the homozygous state in 52.1, in the heterozygous

state in the parent (52.2) and was absent in the stepsibling 52.3. No sample was available from the other parent. The family pedigree and clinical information of individuals in family 52 is presented in Appendix 4.

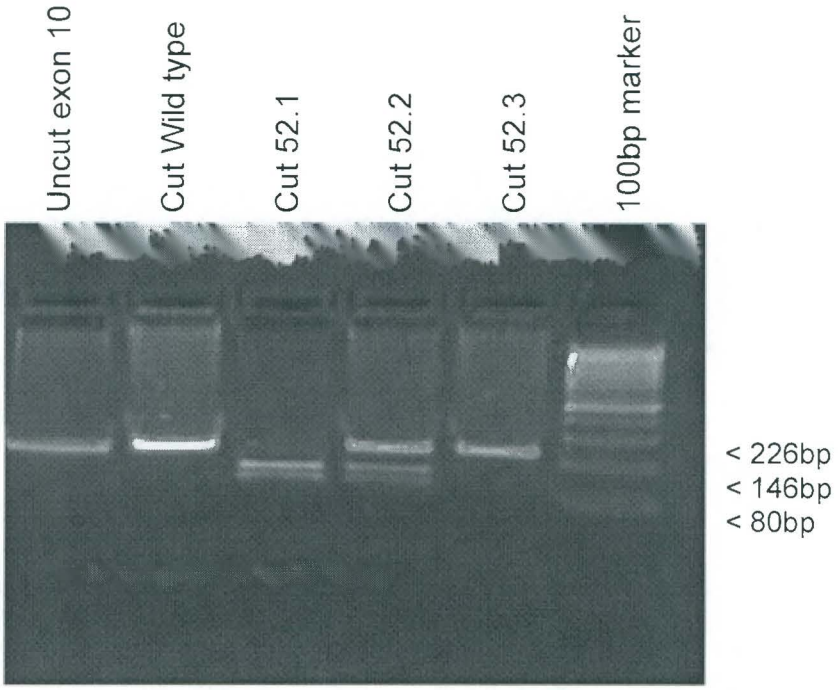



**Figure 3.21** Digital photograph of the products after *Alw26 I* restriction enzyme digest of samples from family 52. The 226bp fragment in the wild type is cut into 146 and 80bp fragments when the homozygous Tyr368His mutation is present. All three bands are present in heterozygous carriers. This 3.5% agarose gel was stained with ethidium bromide and visualised by UV transillumination.

Two variations were detected after complete analysis of exon 10 in 87 samples: the nonpathogenic Glu352Glu (21/87) and the pathogenic Tyr368His (1/87).

### 3.3.9 *RPE65* exon 11/12/13

Of the 18 samples from LCA patients screened for exons 11-13, three samples showed variations and these were identified by sequencing as; IVS12-39T>C (heterozygous in one sample), IVS11+29G>A (heterozygous in one sample) and IVS12+20A>C (homozygous in one sample). The first change is a reported SNP (dbSNP:rs13375676) while the second is novel,

and both changes were not predicted to have any effect on splicing (the prediction software correctly predicted the appropriate intron-exon boundaries). The IVS12+20A>C change was previously reported as nonpathogenic (dbSNP:rs12564647) and occurring at a frequency of 4% in control individuals and 3% in patients [55]. This intronic change was analysed using the splice prediction program, and was not found to affect splicing.

Of the samples from individuals with ARRP, nine samples were identified with similar aberrant dHPLC profiles and one sample with a unique profile. Sequencing of the unique change identified it as a c.1155G>A variation which encodes a silent polymorphism, Thr385Thr. This nonpathogenic polymorphism has been reported previously [55], and was not predicted to have an effect on splicing.

The nine samples with similar dHPLC profiles were sequenced and the following changes were found: three samples had the heterozygous IVS12+20A>C polymorphism; four samples had the heterozygous IVS12-39T>C polymorphism; one sample had both IVS11+29G>A and IVS12+20A>C in the heterozygous state and one sample was heterozygous for both Thr385Thr and IVS12-39T>C.

Four polymorphisms were detected after complete analysis of exons 11, 12 and 13 in 87 samples: IVS11+29G>A (2/87), IVS12-39T>C (6/87), IVS12+20A>C (5/87) and Thr385Thr (2/87).

### 3.3.10 *RPE65* exon 14

No variations were detected after complete analysis of exon 14 in 87 samples.

### 3.3.11 *RPE65* MLPA

For MLPA analysis of *RPE65*, 16 individuals with LCA and two with early onset ARRP were selected. The two individuals with ARRP were included as only one mutant allele had been identified in *RPE65* in each of these individuals. In two of the samples from LCA patients the reactions failed on two ocassions, indicating that the DNA quality of these samples may have been the limiting factor despite being checked prior to MLPA. Results are therefore available for 14 samples from LCA patients and two samples from ARRP patients. All but one of these 16 samples clearly showed no insertions or deletions of *RPE65*. One sample, 733.1, showed a possible deletion, which manifested as a drop in normalised copy number of the probe rpe65ex3 (Figure. 3.22).

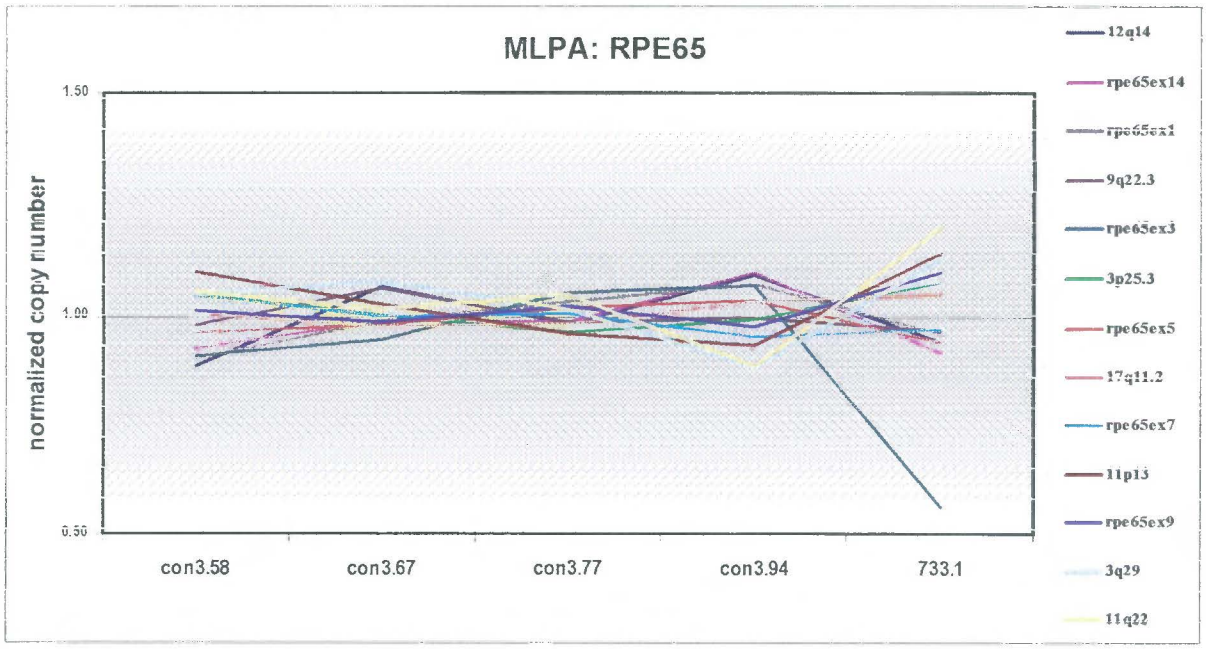



**Figure 3.22** Normalised copy numbers of the MLPA probes for four control samples and sample 733.1. The copy number of probe rpe65ex3 shows a decrease tending towards 0.5, whilst all other probes have copy numbers approximating 1.

The flanking probes (rpe65ex1 and rpe65ex5) showed no skewing in sample 733.1 and the maximum possible size of the deletion was thus calculated to be 5.3kb (the positions of the probes are annotated in Appendix 1). The drop in copy number of the rpe65ex3 probe was proven to be a false positive result, as PCR amplification of a 5.6kb fragment surrounding this probe in sample 733.1 failed two show two alleles of different sizes. This indicated that if a deletion was present, it could be no greater than 2kb. PCR amplification of a 2.6kb fragment surrounding this probe allowed greater resolution, and still only one band was present, indicating no deletion in sample 733.1 (Figure 3.23). MLPA was performed on a sample from an affected relative of individual 733.1 and no variations in copy number were observed. The drop in copy number of probe rpe65ex3 in sample 733.1 is thus possibly due to a SNP under the probe.

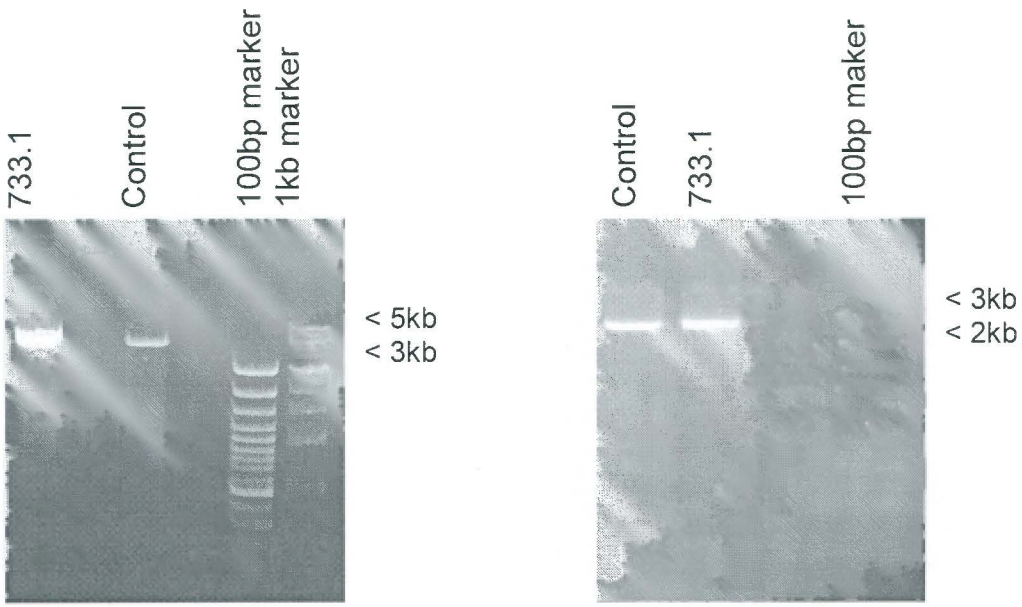



**Figure 3.23** Digital image of PCR amplification products of the 5kb fragment (left) and the 2kb fragment (right) spanning the MLPA probe rpe65ex3. Only one PCR product is visible in the test sample and the wild type control in both cases. These 0.8% agarose gels were stained with ethidium bromide and visualised by UV transillumination.

**Table 3.4** Summary of variations detected in *RHO*, *RP1* and *RPE65*. The proven pathogenic mutations are highlighted in bold text.

| Gene | Variant | Pathogenic | Novel | Frequency in affected cohort |
|---|---|---|---|---|
| *RHO* | g.269A>G | No | No | 26/61 |
| | **Thr17Met** | Yes | No | 1/61 |
| | Thr193Thr | No | Yes | 1/61 |
| | IVS3+4C>T | No | No | 9/61 |
| | Phe208Phe | No | Yes | 1/61 |
| | **IVS4+1G>T** | Yes | No | 1/61 |
| | IVS4-23G>A | No | No | 4/61 |
| *RP1* | **Arg677X** | Yes | No | 1/70 |
| | Thr752Met | No | No | 6/70 |
| | Arg872His | No | No | 23/70 |
| | **Ser911X** | Yes | Yes | 1/70 |
| | **c.2590-2599delATAACTTTAA** | Yes | Yes | 1/70 |
| *RPE65* | **IVS1+1G>T** | Yes | Yes | 1/87 |
| | **Glu21Lys** | Yes | Yes | 1/87 |
| | **Leu22Pro** | Yes | No | 1/87 |
| | IVS2-18G>A | No | Yes | 3/87 |
| | IVS3+80T>A | No | Yes | 2/87 |
| | **Ala132Thr** | Yes | No | 3/87 |
| | IVS6+22C>T | No | No | 9/87 |
| | Ile250Ile | No | Yes | 1/87 |
| | IVS7+98C>T | No | Yes | 1/87 |
| | Tyr249Cys | Unknown | Yes | 1/87 |
| | Glu352Glu | No | No | 21/87 |
| | **Tyr368His** | Yes | No | 1/87 |
| | IVS11+29G>A | No | Yes | 2/87 |
| | IVS12-39T>C | No | No | 6/87 |
| | IVS12+20A>C | No | No | 5/87 |
| | Thr385Thr | No | No | 2/87 |

# 4 Discussion

The primary purpose of genetic research is the advancement of knowledge. Within the Division of Human Genetics at UCT, the added goal is to perform research that can be applied to the benefit of the patients affected by the disease being studied. The aim of this particular project was to determine the distribution and clinical utility of mutations in three important RDD candidate genes in a subset of South African RDD patients. The patient cohorts were selected for screening based on the patterns of disease inheritance and clinical diagnoses.

A large body of data was accumulated during the course of this research project, generating four peer-reviewed publications [77, 98-100] (Appendix 5). These data can be classified in two ways:

- Data which is of interest for RDD research with a view to understanding more about this group of diseases.
- Data which is of immediate benefit to patients affected with RDDs.

The data accumulated during the course of this project will be reviewed briefly, followed by an assessment of the information obtained in terms of these two categories.

## 4.1 Outcomes

### *4.1.1 RHO*

DNA from 61 individuals was screened for mutations in the *RHO* gene. Mutations in *RHO* are the most common cause of RP, accounting for approximately 15% of all inherited retinal disease, 30% of cases of ADRP and for rare cases of ARRP [4, 7, 8, 101]. Of the 61 probands selected, 47 were classified as having ADRP and 14 as having ARRP. Two pathogenic mutations were identified in these 61 individuals (3.3%). The Thr17Met mutation was identified in family 63, a Caucasian family classified as having ADRP. The IVS4+1G>T mutation was identified in family 185, a Caucasian

family classified as having ARRP. Five SNPs were also identified in *RHO*. The silent variations Thr193Thr and Phe208Phe were identified in one individual each, while the intronic variations IVS3+4C>T and IVS4-23G>A were identified in nine (14.8%) and four (6.6%) of the individuals respectively. The g.269A>G variant in the 5' UTR was the most frequently detected change, identified in 26 of the samples screened (42.6%).

### *4.1.2 RP1*

DNA from 70 individuals was screened for variations in the reported hotspot of the *RP1* gene. Mutations in this region are responsible for approximately 4-10% of ADRP [43, 46]. Only three cases of ARRP have been reportedly caused by mutations in *RP1* [45] therefore no samples from individuals classified as having ARRP were included in the cohort for the mutation screen. Of the 70 probands selected (all of whom were classified as having ADRP), 13 had mutations previously identified in other RDD genes including five with *RHO* mutations. These samples were included in order to investigate the effect of possible modifier alleles. Three pathogenic mutations were identified in the 57 individuals where no causative mutation had been identified prior to this project (5.3%). Each mutation was identified in one family only (1.8%), and all three mutations were identified in Caucasian families with origins in the British Isles. The common Arg677X mutation, which has been reported to account for 2-3% of ADRP [43, 46], was identified in family 524. It has been suggested that the high frequency of this mutation is because it occurs in a hypermutable CpG dinucleotide [46]. The novel truncating mutation, Ser911X, was identified in family 7 and the novel 10bp deletion (c.2590-2599delATAACTTTAA) was identified in family 27. Two SNPs were identified in the total cohort of 70 samples screened: the novel Thr752Met was present in six individuals (8.6%), and Arg872His was identified in 23 individuals (32.9%).

### *4.1.3 RPE65*

DNA from 87 individuals was screened for mutations in the *RPE65* gene. Mutations in *RPE65* are reported to be responsible for approximately 2% of ARRP and 16% of LCA [55]. Of the 87 individuals, 18 were classified as having LCA and 69 as having early onset ARRP (with an age of onset younger than 15 years). No pathogenic mutations were identified in any of the individuals with LCA. Five different pathogenic mutations were identified in the 69 individuals with early onset ARRP (7.3%) – in three families both pathogenic mutations were identified, and in two families a single mutation was identified. Family 568, an Indian family, was found to carry the pathogenic mutations IVS1+1G>T and Ala132Thr, as well as Tyr249Cys, a variant of unknown pathogenicity. Family 373, a Caucasian family of Greek origin, was found to carry the pathogenic mutations Glu21Lys and Leu22Pro. Family 52, a Mixed Ancestry family, was the only one in which a homozygous mutation (Tyr368His) was identified. The Ala132Thr mutation was identified in another Indian family (394) and in a Caucasian family (387), although the second mutation in these families was not identified. The Ala132Thr mutation was therefore the single most common variation detected – it was identified in three of the 69 individuals classified as having ARRP (4.4%). Ten SNPs were identified in the total cohort, of which seven were intronic variations and three were isocoding changes. The intronic variations IVS7+98C>T, IVS3+80T>A, IVS11+29G>A and IVS2-18G>A were infrequent, as were the silent changes Ile250Ile and Thr385Thr. The more common variations were IVS12+20A>C (5.8%), IVS12-39T>C (6.9%), IVS6+22C>T (10.3%) and Glu352Glu (24%).

## 4.2 Research

During the course of this project, information was obtained that is valuable in the research context although not immediately translatable to service delivery. This information includes the distribution and frequency of RDD-causing mutations in South African patients and the identification of genetic modifiers of disease. The research into the molecular pathology of RDDs is

necessary for understanding the disease process, which in turn is required for the development of therapeutic interventions. Research is the first step in identifying families that are eligible for possible future therapeutic trials if, and when, they do become available. This type of information therefore has the potential to make an impact on RDD families in the long term, and should not be trivialised.

### 4.2.1 Genetic modifiers and frequencies of variations

Considering the fact that mutations in *RHO* have been reported to cause up to 30% of ADRP, one would have expected as many as 14 families with ADRP to be identified with mutations in this gene during the course of this project. In fact, only one ADRP and one ARRP mutation was detected in the cohort screened. Neither of the two most common RDD mutations (Pro23His and Pro347Leu) was detected. These two mutations are at relatively low frequencies in the SA ADRP population [99, 100]. This implies the "molecular demography' for RDDs in SA may be different from other international study sites. This hypothesis is supported by the allele frequencies of two SNPS, namely g.269A>G in *RHO* and Arg872His in *RP1*. The *RHO* variant g.269A>G was detected in 43% of the affected individuals screened, a frequency much greater than the 14% reported in a similar cohort in the USA [85]. Furthermore this variant was present in 34% of the SA Caucasian control individuals screened and in 95% of the indigenous Black control individuals screened. The *RP1* variant Arg872His was detected in 33% of affected individuals screened, a frequency greater than the 25% reported in an undefined population [44]. The Arg872His variant was present in 47% of the Caucasian and 23% of the indigenous Black control individuals screened. There is therefore a significant association between ethnicity and the frequency of the alleles of these two SNPs in SA.

The finding that the G allele of g.269A>G is significantly more frequent and that the Arg872His variant is significantly less frequent in the SA Black population is especially interesting given the recent report that g.269A>G is associated with an increased risk of RP, while R872H is associated with a

decreased risk of RP [52]. Unfortunately, although the abstract of that report is available in English, personal communication with the contributing author, Dr. Wang, determined that the full length article is only available in Chinese. The report was translated by colleagues at UCT, however it is still uncertain as to whether the altered "risk of RP" actually implies a modification to the phenotype. This should be investigated further as it may prove to be useful in predicting the clinical course of disease in patients with RDDs.

The Wang *et al.* publication (2005) has proposed an interaction between one variant in *RHO* (IVS4-23G>A) and two in *RP1* (N985Y and C2033Y) that act together to increase the risk of developing RP [52]. Although IVS4-23G>A was identified in nine samples, no samples carrying the two *RP1* variants were identified during the course of this project. Given the possibility of genetic or environmental factors modifying the phenotype of *RP1* mutations, the novel *RP1* variant, Thr752Met was investigated. This variant was detected in six probands, two of which had known pathogenic *RHO* mutations. Analysis of the clinical information of the larger of these two families showed that affected individuals with the *RHO* mutation (Asp190Asn) and this Thr752Met variant did not have a different age of onset than affected individuals without this variation. The second family had a single affected individual and was therefore uninformative. The Thr752Met variation was detected in 1.89% of the Caucasian and 5.77% of the indigenous Black control individuals screened, indicating it is a rare variant in the South African population.

*RPE65* mutations were not detected in any individuals with LCA, which was unexpected: given the reported frequency of 16%, one would have expected two or three individuals to be identified with mutations in this gene during the course of this project. The frequency of *RPE65* mutations identified in individuals with early onset ARRP (7.3%) was also higher than the 2% reported in the literature, although the published data included sporadic individuals with RP which may have reduced the frequency [55]. The cohort selected for this project was enriched by including only individuals with an age of onset of younger than 15 years, as it has been recently suggested that

LCA and early onset ARRP may represent a continuum of phenotypes of the same disease [16].

Of the families selected for mutation analysis of the *RPE65* gene, three were Mixed Ancestry, 10 were Indian and 66 were Caucasian. Five families were identified with mutations in *RPE65*: two Caucasian, two Indian and one Mixed Ancestry. Interestingly, the most common mutation (Ala132Thr) was identified in the two Indian families. Also, the only homozygous mutation detected, Tyr368His identified in a Mixed Ancestry family, has been reported as a founder mutation which causes an early onset severe retinal dystrophy in an isolated Dutch population [63]. The presence of this particular mutation in a Mixed Ancestry family is interesting given the founder effects reported in South Africans descended from Dutch settlers for other diseases [64, 65]. The *RPE65* modifier, Leu450Met, which has been reported to protect against light induced damage [60-62] has not been detected in this cohort.

### 4.2.2 Nonpathogenic SNPs identified

Silent variations have on occasion been proven to be pathogenic, for example the c.768G>T mutation is an isocoding change (V256V) in the ATP-binding cassette transporter gene (*ABCA4*) that has been shown to affect splicing and cause cone-rod dystrophy [102]. Five silent variants were identified in the three candidate genes screened, of which three were novel. Splice prediction analysis of the isocoding changes detected does not suggest that these changes will affect splicing. It is therefore assumed that these variations are nonpathogenic.

Similarly, nine intronic variations were identified of which four were novel. All intronic changes were assessed using the splice prediction program and found to have no effect on the splicing of the closest exon(s). However empirical proof of accurate splicing would be preferable to bioinformatics predictions for all intronic and silent changes, both novel and reported.

One of the most difficult aspects of gene mutation analysis is determining which changes are pathogenic and further tests to prove or disprove the disease-causing nature of changes are required. These silent and intronic changes could affect mRNA splicing, which can be assessed by studying RNA species. Alternatively, they could affect the levels of transcription, which can be assessed by performing quantitative PCR.

### 4.2.3 Technical issues

The techniques for mutation analysis are constantly improving and evolving, and this was evident during the course of this research project. Technical developments impact on the rate and sensitivity of mutation detection – more mutations are detected in a shorter time period, which means the time between initiation of research and being able to offer a molecular diagnosis to patients is reduced.

Initially, traditional SSCP was used in this project, as this was the most widely preferred method of mutation analysis at the time. The sensitivity of traditional SSCP ranges from 70 - 95% and depends on the electrophoretic conditions used and the size of the DNA fragment being analysed [70-73]. Much optimisation and troubleshooting was required, the technique was labour-intensive as multiple electrophoretic conditions were required for each fragment, and the results obtained were often not reproducible. Both false positives and false negatives were a factor while using this technique. An alternative SSCP approach, using the Multiphor™ II system (Amersham Pharmacia Biotech, UK), was thus employed. This system was reported to increase the mutation detection rate of SSCP to 97.5%, and extend analysis to larger fragments due to the incorporation of a temperature control [74]. The Multiphor™ II electrophoresis system was initially reported to use a single defined condition, however this was not found to be the case for every fragment analysed during the course of this project. Although optimisation of electrophoresis conditions was still required using the new system, the reproducibility and sensitivity was much improved compared to traditional SSCP, even though false positive results still occurred. Analysis on the new

system was also found to be less time-consuming, as fewer fragments required multiple electrophoretic conditions.

The development of denaturing high performance liquid chromatography (dHPLC) as a mutation detection technique made a great impact on the rate and accuracy of screening in this project. The mutation detection application of the WAVE® has a specificity and sensitivity exceeding 96%, [75, 76]. dHPLC is less labour intensive; no gels and no visualisation steps are required and the time spent troubleshooting is greatly reduced. There is still a problem with false positives, when borderline dHPLC changes (subtle differences in the WAVE profiles) are observed. These borderline changes must always be investigated, although they rarely represent sequence variations. Nevertheless, analysis by dHPLC was found to be more efficient and genes were screened more rapidly using this method than by both of the SSCP techniques used. It must be noted that for all three mutation screening techniques utilised in this project, the promoter regions and deep intronic regions of the three genes were not screened, and there is therefore a possibility that some variations were not detected.

The development and use of the MLPA technique was important, despite the fact that no positive results were obtained during this project. The contribution of gross deletions and insertions to the RDD disease burden has not been investigated internationally, and these mutations may go undetected using conventional PCR-based mutation analysis techniques. It is important to consider the possibility of a deletion of part of the candidate gene, especially when only a single mutation is detected in a patient with an autosomal recessive disease. DNA from a cohort of 50 individuals was screened using MLPA and no deletions or insertions were detected, indicating that these mutations are not a major cause of RDDs, however a larger cohort should be investigated before a definitive conclusion can be made.

The development of microarrays for mutation detection will further enhance the "research to diagnostic" process. These disease chips have been designed for several RDDs including LCA [12], and contain known pathogenic mutations as well as some nonpathogenic SNPs. The yield of returnable results and the turnaround time will be improved if samples are initially sent for testing using such microarray chips. Full-length gene mutation analysis will then be required only for samples for which no known mutation was detected.

Finally, the level of stringency required when moving from research to a diagnostic service must be emphasised. Although high-throughput methods are preferred for large scale gene screening performed during research, more specific methods such as restriction enzyme digestion or direct sequencing are required before a molecular diagnostic result can be given to a family or individual. Novel variations must be investigated fully to confirm their pathogenicity, and both novel and reported mutations must be shown to co-segregate with disease in the family concerned. Appropriate controls must be used for every test and two tests on two distinct DNA isolations are preferred to ensure a high level of confidence in the result.

## 4.3 Diagnostics

One of the aims of this research project was to determine the clinical utility of identifying mutations that cause RDDs. During the course of this study, information was obtained that is immediately translatable to service delivery. Ten families with RDD-causing mutations were identified, the clinical data and pedigrees of which are presented in Appendix 4. In order to further assess clinical utility, it should be considered to what extent these families can be advised regarding their risks, disease prognoses and potential therapies or treatments.

The identification of a gene mutation in a family with RDD currently means that more accurate recurrence risks can be given during counselling. Molecular characterisation of a family also means that predictive and prenatal

testing can be provided for members of that family. The amount of information which can be given, however, regarding prognosis of disease, potential therapies and eligibility for treatment trials very much depends on how well that specific gene and/or mutation has been characterised (through research).

Two of the families (394 and 387, which have early onset ARRP) cannot be given results, as only one causative mutation (Ala132Thr) was identified in the *RPE65* gene. Samples from individuals in these two families were also screened for gross deletions or insertions of *RPE65* and no mutations of this kind were identified. There is a possibility that the second mutation is located in the portions of *RPE65* not analysed (the promoter region or deep intronic regions), however variations in these regions are difficult to asses in terms of pathogenicity. There is also the possibility that the second mutation exists in a different gene. Although digenic effects have not been reported in LCA, modifier effects have been described [12, 103], and microarray analysis has indicated that 7% of LCA cases have potential pathogenic variants in more than one gene [12]. For these two families (and families in which no pathogenic mutations were identified) the research has, as yet, proven to be of little benefit.

The benefits of research are obvious for family 63, in which a well-characterised *RHO* mutation was identified. The Thr17Met is a class II mutation [40] causing relatively mild ADRP [86, 104]. Patients with this mutation generally have a good prognosis: with later onset and a milder phenotype of regional or type 2 ADRP [105, 106].

The N-terminus of *RHO* is thought to enable correct folding and therefore stabilise the protein [107]. Thr17Met prevents glycosylation, resulting in an abnormally folded protein that is retained in the endoplasmic reticulum (ER), fails to translocate to the outer segment plasma membrane, and cannot reconstitute with 11-*cis* retinal [28, 106]. The accumulation of misfolded protein in the cell results in impairment of the ubiquitin-proteosome system

which is vital for cellular regulation, and this leads to cytotoxicity and apoptosis [40]. Histopathological findings include rod and cone abnormalities, RPE abnormalities and a greater loss of rods than cones in the macula and midperiphery [108]. A potential therapy in this case is the use of retinoids as chaperones to improve folding of the protein [40]. Vitamin A supplementation has been shown to increase the availability of 11-*cis* retinal, binding of which increases the stability of mutant RHO, allows correct localisation of the protein and slows the rate of visual decline in transgenic mice [109]. Oral vitamin A supplementation in patients with RP was also shown to reduce the rate of retinal degeneration [110].

In family 63 the identification of this mutation means that the risks of disease can be given for individuals in this family, together with a more accurate disease prognosis, and possible therapeutic intervention. Individual 63.4 has been on vitamin A therapy for 7 years, which has slowed the progression of disease. This family has been informed that individual molecular testing is now available, however individual 63.5 has refused testing and none of the younger generation have shown interest in receiving molecular results or counselling. This highlights a concern regarding the responsibility of the researcher/counsellor: should individual 63.5 and the younger members of the family be actively pursued or should the onus be on them to initiate molecular testing? It has been suggested that an ethical duty to return individual results exists, particularly when there is evidence that it may benefit others [111], furthermore individuals not wishing to learn their results should not donate their DNA samples for research [112]. This situation also serves to highlight the role which interpersonal dynamics can play when assessing the utility of testing for RDDs in families.

The second family identified with a *RHO* mutation was family 185. The only affected individual in this family was found to carry a homozygous splice site mutation, IVS4+1G>T. This mutation has not been reported in the homozygous form elsewhere, and a low incidence of RP amongst heterozygotes has been found, causing debate as to whether this mutation causes dominant or recessive disease [91-94]. The identification of the dual

copy mutation, and confirmation that it did not occur due to uniparental disomy clarified this issue somewhat. This is a consanguineous family and the homozygous *RHO* change is most likely a direct consequence of this consanguinity. A gene conversion event in the proband remains a possible reason for the homozygosity, but neither of the parents or other siblings were available to investigate this further. The assumption is therefore that IVS4+1G>T is a mutation causing recessive disease. The uncertain genotype-phenotype correlation led to the question whether presymptomatic testing should be made available to this family [98]. The proband in this family was given the molecular results, and no other individuals from family 185 have requested testing thus far. It has recently been suggested that changes in the levels of splicing factors can modulate the level of transcription from wild type gene alleles, resulting in disease. Mutations in the splicing factor gene *PRPF31* for example, have been found to inhibit splicing of *RHO* [113]. There is thus a possibility that a mutation in a splicing gene may cause the IVS4+1G>T allele to be expressed preferentially in some heterozygous individuals, which may explain the low incidence of disease in these carriers. This needs to be researched further in order to add value to the information which can be given to younger generations in family 185.

Little information regarding disease prognosis can be given to members of the three families identified with *RP1* mutations. A wide range of disease severity is caused by *RP1* mutations, from patients only 11 years old experiencing nightblindness, to asymptomatic carriers at age 66 [49, 50]. Heterozygous mutation carriers generally exhibit type 2 ADRP (mild, late onset RP with regional loss of rods and cones) while homozygous carriers are more severely affected and have an earlier age of onset [45, 50, 51]. This observation is true for family 524, which carries the common Arg677X mutation, but variable expressivity is clearly observed in the families with novel mutations (families 7 and 27 which have asymptomatic mutation carriers and individuals showing symptoms at an early age, respectively). This clinical heterogeneity suggests that genetic or environmental factors may modify the phenotype of *RP1* mutations. For families 7, 27 and 524, individuals may be counselled as to their mutation status. Counselling of

mutation positive individuals should include the potential variability of their prognosis compared to that of other affected family members. The probands of all three families have been made aware of the individual testing now available, however no other individuals from these families have requested molecular results or counselling to date.

Patients with *RPE65* mutations generally exhibit an early onset and rapid progression of disease, although this disease can manifest as LCA, juvenile ARRP or cone-rod dystrophy [16, 96, 97]. LCA caused by *RPE65* mutations has been described as mild in comparison to other forms of LCA [114], and LCA patients with *RPE65* mutations exhibit a wide range of visual acuity although cataracts, drusen-like deposits, diffuse pigmentary changes and chorioretinal atrophy are common features [115]. Interestingly, heterozygous carriers of *RPE65* mutations can also exhibit drusen-like deposits, pigmentary changes and chorioretinal atrophy without significant visual impairment [116]. It appears that disease severity is largely independent of the type of mutation(s) inherited, and is affected by modifier genes [12, 96]. This makes it difficult to provide information regarding disease prognosis for families 568 and 373. In both of these families a novel mutation was identified together with a previously reported one; however in family 568 a third variation of unknown pathogenicity was identified, further complicating the issue. One would assume that more detailed information could be provided to family 52 as the causative mutation and its associated clinical manifestations have been described previously, however the homozygous Tyr368His has been associated with a large variability in visual acuity [63]. Family 52 is consanguineous, and the presence of a homozygous Tyr368His in this family is probably a direct consequence of this. The risk of disease in future generations is thus reduced to the population frequency of other *RPE65* mutations.

An accurate prediction of disease progression and future visual ability may not be possible for the three families in which both *RPE65* mutations were identified, however these families stand to benefit the most from this research. *RPE65* therapy is likely to be the first RDD gene-based therapy

and clinical trials will soon start [22]. It is possible that therapy will not cure blindness in these affected individuals (the retinal degeneration may be too advanced) but participation in the clinical trials will certainly benefit future generations in these families.

## 4.4 Future prospects

Certain information arising from this research project should be investigated further, as it may prove to be of use when providing a molecular diagnostic service to families with RDDs in the future. The high and low-risk alleles of the two SNPs; *RHO* g.269A>G and *RP1* Arg872His were detected in higher and lower frequencies respectively in the SA Black population. These two SNPS should be assessed to determine whether they alter "risk of RP" as reported by Wang *et al.* (2005), or whether they act as modifiers of the RP phenotype. The presence of these two SNPs should be determined in each individual in whom an RDD mutation has been identified and the clinical information of individuals with and without these SNPs should be compared. This information may assist when providing a prognosis to patients, including the three families in whom clinically heterogeneous *RP1* mutations were identified. A modifier study of this type may go some way to clarifying the high levels of phenotypic heterogeneity observed in many RDD families.

The silent variations and intronic changes detected during mutation analysis of *RHO* and *RPE65* should be investigated further to ensure their nonpathogenic nature, and the non-coding regions of these genes should be screened. It is possible that some mutations may occur outside the region of *RP1* analysed. The region selected for analysis of *RP1* was based on the original description of the hotspot in exon 4 by Bowne *et al.* (1999) [67]. According to the Human Gene Mutation Database at the Institute of Medical Genetics in Cardiff [82] (http://www.hgmd.cf.ac.uk), 39 mutations have been now been described in *RP1*, 15 of which exist outside of the originally described hotspot. The region of analysis should therefore be extended to cover at least the entire hotspot region, and preferably the entire coding region of the gene.

To further substantiate the findings of this research that gross deletions and insertions are not a major cause of RDDs, MLPA or some other quantitative method (for example real-time PCR) should be performed on a larger cohort of patients. All samples that can be screened using the new microarray chips should be sent for such testing. This includes samples from all individuals with LCA and early onset ARRP in which single heterozygous mutations or no mutations have been detected in *RPE65*. This would greatly improve the rate at which mutations are detected and hence improve the molecular diagnostic service arising from research. This approach would also reduce the number of samples requiring full-length gene screening.

To improve on the information which can be given to carriers of the *RHO* IVS4+1G>T mutation, the possibility of mutations in other genes encoding splice machinery that may cause the mutant *RHO* allele to be expressed should be investigated. Candidate genes for this investigation include pre-mRNA processing factor 8 (*PRPF8*), pre-mRNA processing factor 3 (*PRPF3*) and pre-mRNA processing factor 31 (*PRPF31*), all of which have been identified as causing retinal disease. This is only possible if heterozygous carriers of the *RHO* splice mutation present with clinical symptoms.

The third *RPE65* variant in family 568 should be investigated as to whether it is pathogenic. Although this mutation is co-inherited with a known pathogenic mutation, the scientific question arises as to whether it too can cause or modify disease.

Three families were found in which both *RPE65* mutations were identified. Contact should be made with the research group performing clinical trials for *RPE65* gene replacement therapy to establish whether these families are eligible to participate.

## 4.5 Conclusion

South African families with RDDs have entrusted their samples to the Division of Human Genetics at UCT in the hope that research will lead to the development of a treatment or cure for this group of disorders. One means of measurement of success of the RDD programme in this Division is the number of families for whom a molecular diagnosis can be given. Another measurement of success is the utility of the information that can be provided together with the molecular diagnosis. The objectives of this project were therefore to:

- Identify families in the UCT RDD database for which a molecular service could be offered
- Assess the information which could be provided to the families with regards to their relevant mutation(s).

By screening three important retinal candidate genes, ten families were identified for whom a molecular service can now be provided. Accurate diagnostic assays with a high level of stringency were developed for each of the pathogenic mutations identified, and the amount of available data corresponding to each of these mutations was assessed.

A large amount of laboratory time and resources were required to identify these ten families - molecular analysis of samples from 183 probands was performed, and 19 amplicons were screened for mutations. It must be noted that although the positive results obtained in this project are certainly important for the ten families concerned, the negative results for the other families should not be ignored, as the exclusion of candidate genes is also an important outcome. The advances in mutation-screening methodologies (from SSCP to dHPLC) have already improved the rate and sensitivity of mutation screening, and continued evolution of technologies (for example the development of microarrays) will ensure an efficient "research to diagnostic" pipeline.

Evidence is accumulating regarding specific genetic variations that modify the phenotype of disease. This information may play an important role in the future in providing diagnostic or prognostic indicators, and the data obtained in this project may contribute to the understanding of this aspect of RDDs. The importance of such information should not be underestimated when assessing the utility of RDD research. Another factor to be considered is the level of uptake of the molecular service, once it is available: results have been given to five of the ten families, and none of the younger generations have made use of the molecular services available to them, even when a therapeutic intervention may be possible.

For most of the families identified through this research, detection of a genetic mutation currently allows accurate risk assessment, and the option for presymptomatic or prenatal testing. The information which can be provided regarding prognosis of disease and potential therapies is dependent on how thoroughly that specific gene and/or mutation has been characterised, which in turn requires more research. For four families identified in this project, information regarding potential therapies is available. Vitamin A therapy could delay disease progression in family 63, and families 568, 373 and 52 may be eligible for *RPE65* gene replacement therapy trials. For these four families the benefits and utility of this research is clear.

Finally, in terms of the two previously mentioned measures of success, ten families were identified during the course of this project, eight of which can be offered a comprehensive molecular diagnostic service. Four families are in a position to directly benefit by therapeutic intervention based on the knowledge of their genetic information. Further research will identify other families, and increase the amount of useful information available to these families. With evolving technologies the translation of research to diagnostic service is sure to improve in the future.

# 5 References


1. Clarke, G., E. Heon, and R.R. McInnes, *Recent advances in the molecular basis of inherited photoreceptor degeneration.* Clin Genet, 2000. **57**(5): p. 313-29.
2. van Soest, S., A. Westerveld, P.T. de Jong, E.M. Bleeker-Wagemakers, and A.A. Bergen, *Retinitis pigmentosa: defined from a molecular point of view.* Surv Ophthalmol, 1999. **43**(4): p. 321-34.
3. Hims, M.M., S.P. Diager, and C.F. Inglehearn, *Retinitis pigmentosa: genes, proteins and prospects.* Dev Ophthalmol, 2003. **37**: p. 109-25.
4. Daiger, S.P., *Retinal Information Network.* p. RetNet, is a service of the Laboratory for the Molecular Diagnosis of Inherited Eye Diseases, a joint program of the Hermann Eye Center, Dept. of Ophthalmology and Visual Science, and the Human Genetics Center, School of Public Health, at the University of Texas-Houston Health Science Center.
5. Daiger, S.P., *Identifying retinal disease genes: how far have we come, how far do we have to go?* Novartis Found Symp, 2004. **255**: p. 17-27; discussion 27-36, 177-8.
6. Rivolta, C., D. Sharon, M.M. DeAngelis, and T.P. Dryja, *Retinitis pigmentosa and allied diseases: numerous diseases, genes, and inheritance patterns.* Hum Mol Genet, 2002. **11**(10): p. 1219-27.
7. Wang, Q., Q. Chen, K. Zhao, L. Wang, and E.I. Traboulsi, *Update on the molecular genetics of retinitis pigmentosa.* Ophthalmic Genet, 2001. **22**(3): p. 133-54.
8. Phelan, J.K. and D. Bok, *A brief review of retinitis pigmentosa and the identified retinitis pigmentosa genes.* Mol Vis, 2000. **6**: p. 116-24.
9. Inglehearn, C.F., T.J. Keen, R. Bashir, M. Jay, F. Fitzke, A.C. Bird, A. Crombie, and S. Bhattacharya, *A completed screen for mutations of the rhodopsin gene in a panel of patients with autosomal dominant retinitis pigmentosa.* Hum Mol Genet, 1992. **1**(1): p. 41-5.
10. Mandal, M.N., J.R. Heckenlively, T. Burch, L. Chen, V. Vasireddy, R.K. Koenekoop, P.A. Sieving, and R. Ayyagari, *Sequencing arrays for screening multiple genes associated with early-onset human retinal degenerations on a high-throughput platform.* Invest Ophthalmol Vis Sci, 2005. **46**(9): p. 3355-62.
11. Cremers, F.P., J.A. van den Hurk, and A.I. den Hollander, *Molecular genetics of Leber congenital amaurosis.* Hum Mol Genet, 2002. **11**(10): p. 1169-76.
12. Zernant, J., M. Kulm, S. Dharmaraj, A.I. den Hollander, I. Perrault, M.N. Preising, B. Lorenz, J. Kaplan, F.P. Cremers, I. Maumenee, R.K. Koenekoop, and R. Allikmets, *Genotyping microarray (disease chip) for Leber congenital amaurosis: detection of modifier alleles.* Invest Ophthalmol Vis Sci, 2005. **46**(9): p. 3052-9.
13. Scholl, H.P., N.H. Chong, A.G. Robson, G.E. Holder, A.T. Moore, and A.C. Bird, *Fundus autofluorescence in patients with leber congenital amaurosis.* Invest Ophthalmol Vis Sci, 2004. **45**(8): p. 2747-52.
14. Allikmets, R., *Leber congenital amaurosis: a genetic paradigm.* Ophthalmic Genet, 2004. **25**(2): p. 67-79.

15. Koenekoop, R.K., *An overview of Leber congenital amaurosis: a model to understand human retinal development.* Surv Ophthalmol, 2004. **49**(4): p. 379-98.
16. Booij, J.C., R.J. Florijn, J.B. ten Brink, W. Loves, F. Meire, M.J. van Schooneveld, P.T. de Jong, and A.A. Bergen, *Identification of mutations in the AIPL1, CRB1, GUCY2D, RPE65, and RPGRIP1 genes in patients with juvenile retinitis pigmentosa.* J Med Genet, 2005. **42**(11): p. e67.
17. Campochiaro, P.A., *Gene therapy for retinal and choroidal diseases.* Expert Opin Biol Ther, 2002. **2**(5): p. 537-44.
18. Acland, G.M., G.D. Aguirre, J. Ray, Q. Zhang, T.S. Aleman, A.V. Cideciyan, S.E. Pearce-Kelling, V. Anand, Y. Zeng, A.M. Maguire, S.G. Jacobson, W.W. Hauswirth, and J. Bennett, *Gene therapy restores vision in a canine model of childhood blindness.* Nat Genet, 2001. **28**(1): p. 92-5.
19. Acland, G.M., G.D. Aguirre, J. Bennett, T.S. Aleman, A.V. Cideciyan, J. Bennicelli, N.S. Dejneka, S.E. Pearce-Kelling, A.M. Maguire, K. Palczewski, W.W. Hauswirth, and S.G. Jacobson, *Long-term restoration of rod and cone vision by single dose rAAV-mediated gene transfer to the retina in a canine model of childhood blindness.* Mol Ther, 2005. **12**(6): p. 1072-82.
20. Pang, J.J., B. Chang, A. Kumar, S. Nusinowitz, S.M. Noorwez, J. Li, A. Rani, T.C. Foster, V.A. Chiodo, T. Doyle, H. Li, R. Malhotra, J.T. Teusner, J.H. McDowell, S.H. Min, Q. Li, S. Kaushal, and W.W. Hauswirth, *Gene therapy restores vision-dependent behavior as well as retinal structure and function in a mouse model of RPE65 Leber congenital amaurosis.* Mol Ther, 2006. **13**(3): p. 565-72.
21. Chen, Y., G. Moiseyev, Y. Takahashi, and J.X. Ma, *RPE65 gene delivery restores isomerohydrolase activity and prevents early cone loss in Rpe65-/- mice.* Invest Ophthalmol Vis Sci, 2006. **47**(3): p. 1177-84.
22. Hauswirth, W.W., *The consortium project to treat RPE65 deficiency in humans.* Retina, 2005. **25**(8 Suppl): p. S60.
23. Bessant, D.A., R.R. Ali, and S.S. Bhattacharya, *Molecular genetics and prospects for therapy of the inherited retinal dystrophies.* Curr Opin Genet Dev, 2001. **11**(3): p. 307-16.
24. Lewin, A.S., K.A. Drenser, W.W. Hauswirth, S. Nishikawa, D. Yasumura, J.G. Flannery, and M.M. LaVail, *Ribozyme rescue of photoreceptor cells in a transgenic rat model of autosomal dominant retinitis pigmentosa.* Nat Med, 1998. **4**(8): p. 967-71.
25. Dejneka, N.S. and J. Bennett, *Gene therapy and retinitis pigmentosa: advances and future challenges.* Bioessays, 2001. **23**(7): p. 662-8.
26. Ahmad, I., *Stem cells: new opportunities to treat eye diseases.* Invest Ophthalmol Vis Sci, 2001. **42**(12): p. 2743-8.
27. Kniep, E.M., C. Roehlecke, N. Ozkucur, A. Steinberg, F. Reber, L. Knels, and R.H. Funk, *Inhibition of apoptosis and reduction of intracellular pH decrease in retinal neural cell cultures by a blocker of carbonic anhydrase.* Invest Ophthalmol Vis Sci, 2006. **47**(3): p. 1185-92.
28. Chapple, J.P., C. Grayson, A.J. Hardcastle, R.S. Saliba, J. van der Spuy, and M.E. Cheetham, *Unfolding retinal dystrophies: a role for molecular chaperones?* Trends Mol Med, 2001. **7**(9): p. 414-21.

29. Oswald, A.H., J. Goldblatt, G. Sampson, R. Clokie, and P. Beighton, *Retinitis pigmentosa in South Africa.* S Afr Med J, 1985. **68**(12): p. 863-6.
30. Greenberg, J., L. Bartmann, R. Ramesar, and P. Beighton, *Retinitis pigmentosa in southern Africa.* Clin Genet, 1993. **44**(5): p. 232-5.
31. McKie, A.B., J.C. McHale, T.J. Keen, E.E. Tarttelin, R. Goliath, J.J. van Lith-Verhoeven, J. Greenberg, R.S. Ramesar, C.B. Hoyng, F.P. Cremers, D.A. Mackey, S.S. Bhattacharya, A.C. Bird, A.F. Markham, and C.F. Inglehearn, *Mutations in the pre-mRNA splicing factor gene PRPC8 in autosomal dominant retinitis pigmentosa (RP13).* Hum Mol Genet, 2001. **10**(15): p. 1555-62.
32. Rebello, G., R. Ramesar, A. Vorster, L. Roberts, L. Ehrenreich, E. Oppon, D. Gama, S. Bardien, J. Greenberg, G. Bonapace, A. Waheed, G.N. Shah, and W.S. Sly, *Apoptosis-inducing signal sequence mutation in carbonic anhydrase IV identified in patients with the RP17 form of retinitis pigmentosa.* Proc Natl Acad Sci U S A, 2004. **101**(17): p. 6617-22.
33. McWilliam, P., G.J. Farrar, P. Kenna, D.G. Bradley, M.M. Humphries, E.M. Sharp, D.J. McConnell, M. Lawler, D. Sheils, C. Ryan, and et al., *Autosomal dominant retinitis pigmentosa (ADRP): localization of an ADRP gene to the long arm of chromosome 3.* Genomics, 1989. **5**(3): p. 619-22.
34. Dryja, T.P., T.L. McGee, E. Reichel, L.B. Hahn, G.S. Cowley, D.W. Yandell, M.A. Sandberg, and E.L. Berson, *A point mutation of the rhodopsin gene in one form of retinitis pigmentosa.* Nature, 1990. **343**(6256): p. 364-6.
35. Tarttelin, E., M. Al-Maghtheh, J. Keen, S. Bhattacharya, and C. Inglehearn, *Simple tests for rhodopsin involvement in retinitis pigmentosa.* J Med Genet, 1996. **33**(3): p. 262-3.
36. Hargrave, P.A., *Rhodopsin structure, function, and topography the Friedenwald lecture.* Invest Ophthalmol Vis Sci, 2001. **42**(1): p. 3-9.
37. Garriga, P. and J. Manyosa, *The eye photoreceptor protein rhodopsin. Structural implications for retinal disease.* FEBS Lett, 2002. **528**(1-3): p. 17-22.
38. Andres, A., P. Garriga, and J. Manyosa, *Altered functionality in rhodopsin point mutants associated with retinitis pigmentosa.* Biochem Biophys Res Commun, 2003. **303**(1): p. 294-301.
39. Wang, D.Y., W.M. Chan, P.O. Tam, L. Baum, D.S. Lam, K.K. Chong, B.J. Fan, and C.P. Pang, *Gene mutations in retinitis pigmentosa and their clinical implications.* Clin Chim Acta, 2005. **351**(1-2): p. 5-16.
40. Mendes, H.F., J. van der Spuy, J.P. Chapple, and M.E. Cheetham, *Mechanisms of cell death in rhodopsin retinitis pigmentosa: implications for therapy.* Trends Mol Med, 2005. **11**(4): p. 177-85.
41. Spence, M.A., R.S. Sparkes, J.R. Heckenlively, J.T. Pearlman, D. Zedalis, M. Sparkes, M. Crist, and S. Tideman, *Probable genetic linkage between autosomal dominant retinitis pigmentosa (RP) and amylase (AMY2): evidence of an RP locus on chromosome 1.* Am J Hum Genet, 1977. **29**(4): p. 397-404.
42. Blanton, S.H., J.R. Heckenlively, A.W. Cottingham, J. Friedman, L.A. Sadler, M. Wagner, L.H. Friedman, and S.P. Daiger, *Linkage mapping of autosomal dominant retinitis pigmentosa (RP1) to the pericentric region of human chromosome 8.* Genomics, 1991. **11**(4): p. 857-69.

43. Pierce, E.A., T. Quinn, T. Meehan, T.L. McGee, E.L. Berson, and T.P. Dryja, *Mutations in a gene encoding a new oxygen-regulated photoreceptor protein cause dominant retinitis pigmentosa.* Nat Genet, 1999. **22**(3): p. 248-54.
44. Sullivan, L.S., J.R. Heckenlively, S.J. Bowne, J. Zuo, W.A. Hide, A. Gal, M. Denton, C.F. Inglehearn, S.H. Blanton, and S.P. Daiger, *Mutations in a novel retina-specific gene cause autosomal dominant retinitis pigmentosa.* Nat Genet, 1999. **22**(3): p. 255-9.
45. Khaliq, S., A. Abid, M. Ismail, A. Hameed, A. Mohyuddin, P. Lall, A. Aziz, K. Anwar, and S.Q. Mehdi, *Novel association of RP1 gene mutations with autosomal recessive retinitis pigmentosa.* J Med Genet, 2005. **42**(5): p. 436-8.
46. Schwartz, S.B., T.S. Aleman, A.V. Cideciyan, A. Swaroop, S.G. Jacobson, and E.M. Stone, *De novo mutation in the RP1 gene (Arg677ter) associated with retinitis pigmentosa.* Invest Ophthalmol Vis Sci, 2003. **44**(8): p. 3593-7.
47. Liu, Q., A. Lyubarsky, J.H. Skalet, E.N. Pugh, Jr., and E.A. Pierce, *RP1 is required for the correct stacking of outer segment discs.* Invest Ophthalmol Vis Sci, 2003. **44**(10): p. 4171-83.
48. Liu, Q., J. Zuo, and E.A. Pierce, *The retinitis pigmentosa 1 protein is a photoreceptor microtubule-associated protein.* J Neurosci, 2004. **24**(29): p. 6427-36.
49. Berson, E.L., J.L. Grimsby, S.M. Adams, T.L. McGee, E. Sweklo, E.A. Pierce, M.A. Sandberg, and T.P. Dryja, *Clinical features and mutations in patients with dominant retinitis pigmentosa-1 (RP1).* Invest Ophthalmol Vis Sci, 2001. **42**(10): p. 2217-24.
50. Jacobson, S.G., A.V. Cideciyan, A. Iannaccone, R.G. Weleber, G.A. Fishman, A.M. Maguire, L.M. Affatigato, J. Bennett, E.A. Pierce, M. Danciger, D.B. Farber, and E.M. Stone, *Disease expression of RP1 mutations causing autosomal dominant retinitis pigmentosa.* Invest Ophthalmol Vis Sci, 2000. **41**(7): p. 1898-908.
51. Gao, J., K. Cheon, S. Nusinowitz, Q. Liu, D. Bei, K. Atkins, A. Azimi, S.P. Daiger, D.B. Farber, J.R. Heckenlively, E.A. Pierce, L.S. Sullivan, and J. Zuo, *Progressive photoreceptor degeneration, outer segment dysplasia, and rhodopsin mislocalization in mice with targeted disruption of the retinitis pigmentosa-1 (Rp1) gene.* Proc Natl Acad Sci U S A, 2002. **99**(8): p. 5698-703.
52. Wang, D.Y., B.J. Fan, W.M. Chan, O.S. Tam, W.Y. Chiang, S.C. Lam, and C.P. Pang, *[Digenic association of RHO and RP1 genes with retinitis pigmentosa among Chinese population in Hong Kong].* Zhonghua Yi Xue Za Zhi, 2005. **85**(23): p. 1613-7.
53. Hamel, C.P., E. Tsilou, B.A. Pfeffer, J.J. Hooks, B. Detrick, and T.M. Redmond, *Molecular cloning and expression of RPE65, a novel retinal pigment epithelium-specific microsomal protein that is post-transcriptionally regulated in vitro.* J Biol Chem, 1993. **268**(21): p. 15751-7.
54. Nicoletti, A., D.J. Wong, K. Kawase, L.H. Gibson, T.L. Yang-Feng, J.E. Richards, and D.A. Thompson, *Molecular characterization of the human gene encoding an abundant 61 kDa protein specific to the retinal pigment epithelium.* Hum Mol Genet, 1995. **4**(4): p. 641-9.
55. Morimura, H., G.A. Fishman, S.A. Grover, A.B. Fulton, E.L. Berson, and T.P. Dryja, *Mutations in the RPE65 gene in patients with*

*autosomal recessive retinitis pigmentosa or leber congenital amaurosis.* Proc Natl Acad Sci U S A, 1998. **95**(6): p. 3088-93.
56. Moiseyev, G., Y. Chen, Y. Takahashi, B.X. Wu, and J.X. Ma, *RPE65 is the isomerohydrolase in the retinoid visual cycle.* Proc Natl Acad Sci U S A, 2005. **102**(35): p. 12413-8.
57. Redmond, T.M., S. Yu, E. Lee, D. Bok, D. Hamasaki, N. Chen, P. Goletz, J.X. Ma, R.K. Crouch, and K. Pfeifer, *Rpe65 is necessary for production of 11-cis-vitamin A in the retinal visual cycle.* Nat Genet, 1998. **20**(4): p. 344-51.
58. Pang, J.J., B. Chang, N.L. Hawes, R.E. Hurd, M.T. Davisson, J. Li, S.M. Noorwez, R. Malhotra, J.H. McDowell, S. Kaushal, W.W. Hauswirth, S. Nusinowitz, D.A. Thompson, and J.R. Heckenlively, *Retinal degeneration 12 (rd12): a new, spontaneously arising mouse model for human Leber congenital amaurosis (LCA).* Mol Vis, 2005. **11**: p. 152-62.
59. Woodruff, M.L., Z. Wang, H.Y. Chung, T.M. Redmond, G.L. Fain, and J. Lem, *Spontaneous activity of opsin apoprotein is a cause of Leber congenital amaurosis.* Nat Genet, 2003. **35**(2): p. 158-64.
60. Wenzel, A., C.E. Reme, T.P. Williams, F. Hafezi, and C. Grimm, *The Rpe65 Leu450Met variation increases retinal resistance against light-induced degeneration by slowing rhodopsin regeneration.* J Neurosci, 2001. **21**(1): p. 53-8.
61. Samardzija, M., A. Wenzel, M. Naash, C.E. Reme, and C. Grimm, *Rpe65 as a modifier gene for inherited retinal degeneration.* Eur J Neurosci, 2006. **23**(4): p. 1028-34.
62. Wenzel, A., C. Grimm, M. Samardzija, and C.E. Reme, *The genetic modifier Rpe65Leu(450): effect on light damage susceptibility in c-Fos-deficient mice.* Invest Ophthalmol Vis Sci, 2003. **44**(6): p. 2798-802.
63. Yzer, S., L.I. van den Born, J. Schuil, H.Y. Kroes, M.M. van Genderen, F.N. Boonstra, B. van den Helm, H.G. Brunner, R.K. Koenekoop, and F.P. Cremers, *A Tyr368His RPE65 founder mutation is associated with variable expression and progression of early onset retinal dystrophy in 10 families of a genetically isolated population.* J Med Genet, 2003. **40**(9): p. 709-13.
64. Groenewald, J.Z., J. Liebenberg, I.M. Groenewald, and L. Warnich, *Linkage disequilibrium analysis in a recently founded population: evaluation of the variegate porphyria founder in South African Afrikaners.* Am J Hum Genet, 1998. **62**(5): p. 1254-8.
65. Defesche, J.C., D.E. van Diermen, P.J. Lansberg, R.J. Lamping, P.W. Reymer, M.R. Hayden, and J.J. Kastelein, *South African founder mutations in the low-density lipoprotein receptor gene causing familial hypercholesterolemia in the Dutch population.* Hum Genet, 1993. **92**(6): p. 567-70.
66. Ramesar, R.S., L. Roberts, G. Rebello, R. Goliath, A. Vorster, A. September, L. Ehrenreich, D. Gama, and J. Greenberg, *Retinal degenerative disorders in Southern Africa: a molecular genetic approach.* Adv Exp Med Biol, 2003. **533**: p. 35-40.
67. Bowne, S.J., S.P. Daiger, M.M. Hims, M.M. Sohocki, K.A. Malone, A.B. McKie, J.R. Heckenlively, D.G. Birch, C.F. Inglehearn, S.S. Bhattacharya, A. Bird, and L.S. Sullivan, *Mutations in the RP1 gene*

*causing autosomal dominant retinitis pigmentosa.* Hum Mol Genet, 1999. **8**(11): p. 2121-8.
68. Altschul, S.F., W. Gish, W. Miller, E.W. Myers, and D.J. Lipman, *Basic local alignment search tool.* J Mol Biol, 1990. **215**(3): p. 403-10.
69. Rozen, S. and H. Skaletsky, *Primer3 on the WWW for general users and for biologist programmers.* Methods Mol Biol, 2000. **132**: p. 365-86.
70. Orita, M., Y. Suzuki, T. Sekiya, and K. Hayashi, *Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction.* Genomics, 1989. **5**(4): p. 874-9.
71. Orita, M., H. Iwahana, H. Kanazawa, K. Hayashi, and T. Sekiya, *Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms.* Proc Natl Acad Sci U S A, 1989. **86**(8): p. 2766-70.
72. Grompe, M., *The rapid detection of unknown mutations in nucleic acids.* Nat Genet, 1993. **5**(2): p. 111-7.
73. Sheffield, V.C., J.S. Beck, A.E. Kwitek, D.W. Sandstrom, and E.M. Stone, *The sensitivity of single-strand conformation polymorphism analysis for the detection of single base substitutions.* Genomics, 1993. **16**(2): p. 325-32.
74. Liechti-Gallati, S., V. Schneider, D. Neeser, and R. Kraemer, *Two buffer PAGE system-based SSCP/HD analysis: a general protocol for rapid and sensitive mutation screening in cystic fibrosis and any other human genetic disease.* Eur J Hum Genet, 1999. **7**(5): p. 590-8.
75. *WAVE System operator's guide.*
76. Xiao, W. and P.J. Oefner, *Denaturing high-performance liquid chromatography: A review.* Hum Mutat, 2001. **17**(6): p. 439-74.
77. Roberts, L., L. Bartmann, R. Ramesar, and J. Greenberg, *Novel variants in the hotspot region of RP1 in South African patients with retinitis pigmentosa.* Mol Vis, 2006. **12**: p. 177-83.
78. Thompson, J.D., D.G. Higgins, and T.J. Gibson, *CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice.* Nucleic Acids Res, 1994. **22**(22): p. 4673-80.
79. Hall, T., *BioEdit: a user friendly biological sequence alignment editor and analysis program for Windows 95/98/NT.* Nucl. Acids. Symp. Ser, 1999. **41**: p. 95-98.
80. Cotton, R.G. and C.R. Scriver, *Proof of "disease causing" mutation.* Hum Mutat, 1998. **12**(1): p. 1-3.
81. den Dunnen, J.T. and S.E. Antonarakis, *Nomenclature for the description of human sequence variations.* Hum Genet, 2001. **109**(1): p. 121-4.
82. Stenson, P.D., E.V. Ball, M. Mort, A.D. Phillips, J.A. Shiel, N.S. Thomas, S. Abeysinghe, M. Krawczak, and D.N. Cooper, *Human Gene Mutation Database (HGMD): 2003 update.* Hum Mutat, 2003. **21**(6): p. 577-81.
83. Reese, M.G., Eeckmann, F.H. *Splice Sites: A detailed neural network study.* in *Genome Mapping & Sequencing Meeting.* 1996. Cold Spring Harbour, New York.
84. Reese, M.G., F.H. Eeckman, D. Kulp, and D. Haussler, *Improved splice site detection in Genie.* J Comput Biol, 1997. **4**(3): p. 311-23.

85. Sung, C.H., C.M. Davenport, J.C. Hennessey, I.H. Maumenee, S.G. Jacobson, J.R. Heckenlively, R. Nowakowski, G. Fishman, P. Gouras, and J. Nathans, *Rhodopsin mutations in autosomal dominant retinitis pigmentosa.* Proc Natl Acad Sci U S A, 1991. **88**(15): p. 6481-5.
86. Sheffield, V.C., G.A. Fishman, J.S. Beck, A.E. Kimura, and E.M. Stone, *Identification of novel rhodopsin mutations associated with retinitis pigmentosa by GC-clamped denaturing gradient gel electrophoresis.* Am J Hum Genet, 1991. **49**(4): p. 699-706.
87. Dryja, T.P., L.B. Hahn, G.S. Cowley, T.L. McGee, and E.L. Berson, *Mutation spectrum of the rhodopsin gene among patients with autosomal dominant retinitis pigmentosa.* Proc Natl Acad Sci U S A, 1991. **88**(20): p. 9370-4.
88. Bunge, S., H. Wedemann, D. David, D.J. Terwilliger, L.I. van den Born, C. Aulehla-Scholz, C. Samanns, M. Horn, J. Ott, E. Schwinger, and et al., *Molecular analysis and genetic mapping of the rhodopsin gene in families with autosomal dominant retinitis pigmentosa.* Genomics, 1993. **17**(1): p. 230-3.
89. Jacobson, S.G., C.M. Kemp, C.H. Sung, and J. Nathans, *Retinal function and rhodopsin levels in autosomal dominant retinitis pigmentosa with rhodopsin mutations.* Am J Ophthalmol, 1991. **112**(3): p. 256-71.
90. Dryja, T.P., T.L. McGee, L.B. Hahn, G.S. Cowley, J.E. Olsson, E. Reichel, M.A. Sandberg, and E.L. Berson, *Mutations within the rhodopsin gene in patients with autosomal dominant retinitis pigmentosa.* N Engl J Med, 1990. **323**(19): p. 1302-7.
91. Rosenfeld, P.J., G.S. Cowley, T.L. McGee, M.A. Sandberg, E.L. Berson, and T.P. Dryja, *A null mutation in the rhodopsin gene causes rod photoreceptor dysfunction and autosomal recessive retinitis pigmentosa.* Nat Genet, 1992. **1**(3): p. 209-13.
92. Macke, J.P., C.M. Davenport, S.G. Jacobson, J.C. Hennessey, F. Gonzalez-Fernandez, B.P. Conway, J. Heckenlively, R. Palmer, I.H. Maumenee, P. Sieving, and et al., *Identification of novel rhodopsin mutations responsible for retinitis pigmentosa: implications for the structure and function of rhodopsin.* Am J Hum Genet, 1993. **53**(1): p. 80-9.
93. Jacobson, S.G., C.M. Kemp, A.V. Cideciyan, J.P. Macke, C.H. Sung, and J. Nathans, *Phenotypes of stop codon and splice site rhodopsin mutations causing retinitis pigmentosa.* Invest Ophthalmol Vis Sci, 1994. **35**(5): p. 2521-34.
94. Rosenfeld, P.J., L.B. Hahn, M.A. Sandberg, T.P. Dryja, and E.L. Berson, *Low incidence of retinitis pigmentosa among heterozygous carriers of a specific rhodopsin splice site mutation.* Invest Ophthalmol Vis Sci, 1995. **36**(11): p. 2186-92.
95. Marlhens, F., J.M. Griffoin, C. Bareil, B. Arnaud, M. Claustres, and C.P. Hamel, *Autosomal recessive retinal dystrophy associated with two novel mutations in the RPE65 gene.* Eur J Hum Genet, 1998. **6**(5): p. 527-31.
96. Thompson, D.A., P. Gyurus, L.L. Fleischer, E.L. Bingham, C.L. McHenry, E. Apfelstedt-Sylla, E. Zrenner, B. Lorenz, J.E. Richards, S.G. Jacobson, P.A. Sieving, and A. Gal, *Genetics and phenotypes of RPE65 mutations in inherited retinal degeneration.* Invest Ophthalmol Vis Sci, 2000. **41**(13): p. 4293-9.

97. Lorenz, B., P. Gyurus, M. Preising, D. Bremser, S. Gu, M. Andrassi, C. Gerth, and A. Gal, *Early-onset severe rod-cone dystrophy in young children with RPE65 mutations.* Invest Ophthalmol Vis Sci, 2000. **41**(9): p. 2735-42.
98. Greenberg, J., L. Roberts, and R. Ramesar, *A rare homozygous rhodopsin splice-site mutation: the issue of when and whether to offer presymptomatic testing.* Ophthalmic Genet, 2003. **24**(4): p. 225-32.
99. Greenberg, J., L. Roberts, and R. Ramesar. *Unusual frequencies of Rhodopsin mutations and polymorphisms in South African patients with Retinitis Pigmentosa.* in *New insights into retinal degenerative diseases. Proceedings of the 9th International Symposium on Retinal Degeneration.* 2000. Durango, CO: Kluwer Academic/Plenum Publishers.
100. Roberts, L., R. Ramesar, and J. Greenberg, *Low frequency of rhodopsin mutations in South African patients with autosomal dominant retinitis pigmentosa.* Clin Genet, 2000. **58**(1): p. 77-8.
101. Chapple, P., *Looking at protein misfolding neurodegenerative disease through retinitis pigmentosa.* Advances in Clinical Neuroscience & Rehabilitation, 2003. **3**(1): p. 12-13.
102. Klevering, B.J., A. Blankenagel, A. Maugeri, F.P. Cremers, C.B. Hoyng, and K. Rohrschneider, *Phenotypic spectrum of autosomal recessive cone-rod dystrophies caused by mutations in the ABCA4 (ABCR) gene.* Invest Ophthalmol Vis Sci, 2002. **43**(6): p. 1980-5.
103. Silva, E., S. Dharmaraj, Y.Y. Li, A.L. Pina, R.C. Carter, M. Loyer, E. Traboulsi, G. Theodossiadis, R. Koenekoop, O. Sundin, and I. Maumenee, *A missense mutation in GUCY2D acts as a genetic modifier in RPE65-related Leber Congenital Amaurosis.* Ophthalmic Genet, 2004. **25**(3): p. 205-17.
104. Sandberg, M.A., C. Weigel-DiFranco, T.P. Dryja, and E.L. Berson, *Clinical expression correlates with location of rhodopsin mutation in dominant retinitis pigmentosa.* Invest Ophthalmol Vis Sci, 1995. **36**(9): p. 1934-42.
105. Hayakawa, M., Y. Hotta, Y. Imai, K. Fujiki, A. Nakamura, K. Yanashima, and A. Kanai, *Clinical features of autosomal dominant retinitis pigmentosa with rhodopsin gene codon 17 mutation and retinal neovascularization in a Japanese patient.* Am J Ophthalmol, 1993. **115**(2): p. 168-73.
106. Bell, C., C.A. Converse, H.M. Hammer, A. Osborne, and N.E. Haites, *Rhodopsin mutations in a Scottish retinitis pigmentosa population, including a novel splice site mutation in intron four.* Br J Ophthalmol, 1994. **78**(12): p. 933-8.
107. Berson, E.L., B. Rosner, C. Weigel-DiFranco, T.P. Dryja, and M.A. Sandberg, *Disease progression in patients with dominant retinitis pigmentosa and rhodopsin mutations.* Invest Ophthalmol Vis Sci, 2002. **43**(9): p. 3027-36.
108. Ben-Arie-Weintrob, Y., E.L. Berson, and T.P. Dryja, *Histopathologic-genotypic correlations in retinitis pigmentosa and allied diseases.* Ophthalmic Genet, 2005. **26**(2): p. 91-100.
109. Li, T., M.A. Sandberg, B.S. Pawlyk, B. Rosner, K.C. Hayes, T.P. Dryja, and E.L. Berson, *Effect of vitamin A supplementation on rhodopsin mutants threonine-17 --> methionine and proline-347 --> serine in*

*transgenic mice and in cell cultures.* Proc Natl Acad Sci U S A, 1998. **95**(20): p. 11933-8.
110. Berson, E.L., B. Rosner, M.A. Sandberg, K.C. Hayes, B.W. Nicholson, C. Weigel-DiFranco, and W. Willett, *A randomized trial of vitamin A and vitamin E supplementation for retinitis pigmentosa.* Arch Ophthalmol, 1993. **111**(6): p. 761-72.
111. Knoppers, B.M., Y. Joly, J. Simard, and F. Durocher, *The emergence of an ethical duty to disclose genetic research results: international perspectives.* Eur J Hum Genet, 2006.
112. Pullman, D. and K. Hodgkinson, *Genetic knowledge and moral responsibility: ambiguity at the interface of genetic research and clinical practice.* Clin Genet, 2006. **69**(3): p. 199-203.
113. Nissim-Rafinia, M. and B. Kerem, *The splicing machinery is a genetic modifier of disease severity.* Trends Genet, 2005. **21**(9): p. 480-3.
114. Yzer, S., B.P. Leroy, E. De Baere, T.J. de Ravel, M.N. Zonneveld, K. Voesenek, U. Kellner, J.P. Ciriano, J.T. de Faber, K. Rohrschneider, R. Roepman, A.I. den Hollander, J.R. Cruysberg, F. Meire, I. Casteels, N.G. van Moll-Ramirez, R. Allikmets, L.I. van den Born, and F.P. Cremers, *Microarray-based mutation detection and phenotypic characterization of patients with Leber congenital amaurosis.* Invest Ophthalmol Vis Sci, 2006. **47**(3): p. 1167-76.
115. Galvin, J.A., G.A. Fishman, E.M. Stone, and R.K. Koenekoop, *Evaluation of genotype-phenotype associations in leber congenital amaurosis.* Retina, 2005. **25**(7): p. 919-29.
116. Galvin, J.A., G.A. Fishman, E.M. Stone, and R.K. Koenekoop, *Clinical phenotypes in carriers of Leber congenital amaurosis mutations.* Ophthalmology, 2005. **112**(2): p. 349-56.

# Appendix 1: Annotated gene sequences

All exons are shown in uppercase and introns in lowercase. Primers are indicated in **bold and underlined**, while MLPA probes are shown in blue text. Nonpathogenic variations are highlighted in green and pathogenic mutations are highlighted in red, while variations with unknown pathogenicity are highlighted in turquoise.

## Annotated *RHO* Sequence

```
Summary of Exons in sequence:

RHO-Exon1 (5001-5456 -> 456bp)
RHO-Exon2 (7238-7406 -> 169bp)
RHO-Exon3 (8613-8778 -> 166bp)
RHO-Exon4 (8895-9134 -> 240bp)
RHO-Exon5 (9970-11696 -> 1727bp)
Total size for RHO = 2758 bp

Summary of SNPs in sequence:

1 - (20)(a/c)(dbSNP:rs9812975)
2 - (474)(t/g)(dbSNP:rs12496603)
3 - (1057)(c/t)(dbSNP:rs2713630)
4 - (1057)(g/a)(dbSNP:rs6439185)
5 - (1837)(a/g)(dbSNP:rs9823319)
6 - (2520)(t/g)(dbSNP:rs2625952)
7 - (2566)(g/a)(dbSNP:rs2713629)
8 - (2605)(c/t)(dbSNP:rs9837743)
9 - (2988)(a/g)(dbSNP:rs3774785)
10 - (2998)(t/a)(dbSNP:rs2713628)
11 - (3347)(a/g)(dbSNP:rs2625953)
12 - (3613)(g/a)(dbSNP:rs12633814)
13 - (3933)(t/c)(dbSNP:rs2625954)
14 - (4318)(c/a)(dbSNP:rs2625955)
15 - (4400)(a/c)(dbSNP:rs3755837)
16 - (5045)(g/a)(dbSNP:rs2269736)
17 - (5070)(a/g)(dbSNP:rs7984)
18 - (5253)(c/g)(dbSNP:rs28933395)
19 - (5268)(c/g)(dbSNP:rs28933394)
20 - (5411)(g/a)(dbSNP:rs28933994)
21 - (6444)(t/g)(dbSNP:rs2855552)
22 - (6630)(g/a)(dbSNP:rs2855553)
23 - (7798)(g/t)(dbSNP:rs6803468)
24 - (7845)(g/a)(dbSNP:rs6803484)
25 - (7855)(a/t)(dbSNP:rs2855556)
26 - (8650)(g/a)(dbSNP:rs28933992)
27 - (8702)(t/g)(dbSNP:rs28933995)
28 - (8714)(a/c)(dbSNP:rs28933993)
29 - (9441)(a/t)(dbSNP:rs2855557)
30 - (9825)(t/a)(dbSNP:rs2625964)
31 - (9947)(g/a)(dbSNP:rs2071092)
32 - (10123)(c/a)(dbSNP:rs2071093)
33 - (10220)(t/-
)(dbSNP:rs11359208)
34 - (10312)(a/g)(dbSNP:rs2410)
35 - (10928)(a/g)(dbSNP:rs2625969)
36 - (10992)(a/g)(dbSNP:rs2855558)
37 - (11179)(g/a)(dbSNP:rs3733148)
38 - (11288)(g/a)(dbSNP:rs3733149)
39 - (14887)(c/t)(dbSNP:rs9874024)
40 - (15387)(g/a)(dbSNP:rs1905440)
41 - (16040)(c/a)(dbSNP:rs1905441)
42 - (16050)(g/a)(dbSNP:rs1905442)

Total of 42 SNPs in annotation

Summary of STSs in sequence:

1 - (4960-5216)(STS:G30927)
2 - (6784-6965)(STS:GDB:512865)
3 - (6842-7054)(STS:GDB:181225)
4 - (6845-6964)(STS:GDB:171129)
5 - (7197-7447)(STS:GDB:437730)
6 - (9960-10188)(STS:GDB:186557)
7 - (10237-10386)(STS:SHGC-31114)
8 - (10288-10437)(STS:SGC31296)
9 - (10419-10540)(STS:RH78627)
10 - (10532-10728)(STS:RH26341)
11 - (11360-11509)(STS:SHGC-30855)

Total of 11 STSs in annotation
```

Annotated sequence file:

```
LOCUS       3 16706 bp DNA HTG 26-MAY-2006
DEFINITION  Homo sapiens chromosome 3 NCBI36 partial sequence
            130725172..130741877 reannotated via EnsEMBL
ACCESSION   chromosome:NCBI36:3:130725172:130741877:1
VERSION     chromosome:NCBI36:3:130725172:130741877:1
KEYWORDS    .
SOURCE      human
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata;
Euteleostomi;
            Mammalia; Eutheria; Euarchontoglires; Primates; Catarrhini;
            Hominidae; Homo.
COMMENT     This sequence was annotated by the Ensembl system. Please visit
the
            Ensembl web site, http://www.ensembl.org/ for more information.
COMMENT     All feature locations are relative to the first (5') base of the
            sequence in this file.  The sequence presented is always the
            forward strand of the assembly. Features that lie outside of the
            sequence contained in this file have clonal location coordinates
in
            the format: .:..
COMMENT     The /gene indicates a unique id for a gene,
            /note="transcript_id=..." a unique id for a transcript,
/protein_id
            a unique id for a peptide and note="exon_id=..." a unique id for
an
            exon. These ids are maintained wherever possible between
versions.
COMMENT     All the exons and transcripts in Ensembl are confirmed by
            similarity to either protein or cDNA sequences.
FEATURES             Location/Qualifiers
     source          1..16706
                     /organism="Homo sapiens"
                     /db_xref="taxon:9606"
     gene            5001..11696
                     /gene=ENSG00000163914
     mRNA            join(5001..5456,7238..7406,8613..8778,8895..9134,
                     9970..11696)
                     /gene="ENSG00000163914"
                     /note="transcript_id=ENST00000296271"
     CDS             join(5096..5456,7238..7406,8613..8778,8895..9134,
                     9970..10080)


BASE COUNT 3922 a 4558 c 4254 g 3972 t
ORIGIN
     1 gtgggggacc aggagaaag^a aagccaagga agaggaggag gaggaggaga aggaggagaa
       (20)(a/c)(dbSNP:rs9812975)
    61 ggatgctgac ctagcagctc ctctcacagc agctcctctc ttgcagaggc tgaagagcga
   ...
  4921 cccaatctcc cagatgctga ttcagccagg agcttagga^g ggggaggtca ctttataagg
       (4960-5216)(STS:G30927)
RHO-Exon1 (5001-5456 -> 456bp)
  4981 gtctgggggg gtcagaaccc AGAGTCATCC AGCTGGAGCC CTGAGTGGCT GAGCTCAGGC
  5041 CTTC^GCAGCA TTCTTGGGTG GGAGCAGCC^A CGGGTCAGCC ACAAGGGCCA CAGCC^ATGAA
  Exon1A_fwd,(5045)(g/a)(dbSNP:rs2269736),g.269A>G)(5070)a/g)(dbSNP:rs7984),
start
  5101 TGGCACAGAA GGCCCTAACT TCTACGTGCC CTTCTCCAAT GCGACGGGTG TGGTACGCAG
                                                  Thr17Met
  5161 CCCCTTCGAG TACCCACAGT ACTACCTGGC TGAGCCATGG CAGTTCTCCA TGCTGGCCGC
  5221 CTACATGTTT CTGCTGATCG TGCTGGGCTT CC^CCATCAAC TTCCTCA^CGC TCTACGTCAC
       (5253)(c/g)(dbSNP:rs28933395),(5268)(c/g)(dbSNP:rs28933394)Exon1B_fwd
  5281 CGTCCAGCAC AAGAAGCTGC GCACGCCTCT CAACTACATC CTGCTCAACC TAGCCGTGGC
                                Exon1A_rev,          MLPA exon1
  5341 TGACCTCTTC ATGGTCCTAG GTGGCTTCAC CAGCACCCTC TACACCTCTC TGCATGGATA
```

```
  5401 CTTCGTCTTC ^GGGCCCACAG GATGCAATTT GGAGGGCTTC TTTGCCACCC TGGGCGgtat
       (5411)(g/a)(dbSNP:rs28933994)
  5461 gagccgggtg tgggtggggt gtgcaggagc ccgggagcat ggaggggtct gggagagtcc
  5521 cgggcttggc ggtggtggct gagaggcctt ctccCTTCTC CTGTCCTGTC AATGttatcc
                                          Exon1B_rev
  5581 aaagccctca tatattcagt caacaaacac cattcatggt gatagccggg ctgctgtttg
  5641 tgcagggctg gcactgaaca ctgccttgat cttatttgga gcaatatgcg cttgtctaat
  ...
  7141 ctggttgcct tcctagctac cctctccctg tctaggggggg agtgcaccct ccttag^gcag
       (7197-7447)(STS:GDB:437730)
RHO-Exon2 (7238-7406 -> 169bp)
  7201 tggggtctg^t gctgaccgcc tgctgactgc cttgcagGTG AAATTGCCCT GTGGTCCTTG
       (7210)(branch site - YNYYRAY), Exon2_fwd
  7261 GTGGTCCTGG CCATCGAGCG GTACGTGGTG GTGTGTAAGC CCATGAGCAA CTTCCGCTTC
  7321 GGGGAGAACC ATGCCATCAT GGGCGTTGCC TTCACCTGGG TCATGGCGCT GGCCTGCGCC
             MLPA exon2
  7381 GCACCCCCAC TCGCCGGCTG GTCCAGgtaa tggcactgag cagaagggaa gaagctccgg
                                        Exon2_rev
  7441 gggctctttg tagggtcctc cagtcaggac tcaaacccag tagtgtctgg ttccaggcac
  7501 tgaccttgta tgtctcctgg cccaaatgcc cactcagggt aggggtgtag ggcagaagaa
  ...
  8521 tgtgaagccc cagaaagggc cagcgctcgg cagccacctt ggctgttccc aagtccctca
RHO-Exon3 (8613-8778 -> 166bp)                    Exon3_fwd
  8581 caggcagggt ctccctacct gcctgtcctc agGTACATCC CCGAGGGCCT GCAGTGCTCG
  8641 TGTGGAATC^G ACTACTACAC GCTCAAGCCG GAGGTCAACA ACGAGTCTTT TGTCATCTAC
       (8650)(g/a)(dbSNP:rs28933992), Thr193Thr MLPA exon3
  8701 A^TGTTGGTGG TCC^ACTTCAC CATCCCCATG ATTATCATCT TTTTCTGCTA TGGGCAGCTC
      (8702)(t/g)(dbSNP:rs28933995),Phe208Phe, (8714)(a/c)(dbSNP:rs28933993)
  8761 GTCTTCACCG TCAAGGAGgt agggccgggg gggtgggcgg cctcacggct ctgagggtcc
                           IVS3+4C>T,                Exon4_fwd
  8821 agccccagc atgcatctgc ggctcctgct ccctggagga gccatggtct ggacccgggt
RHO-Exon4 (8895-9134 -> 240bp)          Exon3_rev
  8881 cccgtgtcct gcagGCCGCT GCCCAGCAGC AGGAGTCAGC CACCACACAG AAGGCAGAGA
                                                  MLPA exon4
  8941 AGGAGGTCAC CCGCATGGTC ATCATCATGG TCATCGCTTT CCTGATCTGC TGGGTGCCCT
  9001 ACGCCAGCGT GGCATTCTAC ATCTTCACCC ACCAGGGCTC CAACTTCGGT CCCATCTTCA
  9061 TGACCATCCC AGCGTTCTTT GCCAAGAGCG CCGCCATCTA CAACCCTGTC ATCTATATCA
  9121 TGATGAACAA GCAGgtgcct actgcgggtg ggagggcccc agtgccccag gccacaggcg
                     IVS4+1G>T
  9181 ctgcctgcca aggacaagct actccagg gcaggggagg gggctccatc agggttactg
         Exon4_rev
  9241 gcagcagtct tgggtcagca gtcccaatgg ggagtgtgtg agaaatgcag attcctggcc
  9301 ccactcagaa ctgctgaatc tcagggtggg cccaggaacc tgcatttcca gcaagccctc
  9361 cacaggtggc tcagatgctc actcaggtgg gagaagctcc agtcagctag ttctggaagc
  9421 ccaatgtcaa agtcagaagg ^acccaagtcg ggaatgggat gggccagtct ccataaagct
       (9441)(a/t)(dbSNP:rs2855557)
  9481 gaataaggag ctaaaaagtc ttattctgag gggtaaaggg gtaaagggtt cctcggagag
  9541 gtacctccga ggggtaaaca gttgggtaaa cagtctctga agtcagctct gccatttttct
  9601 agctgtatgg ccctgggcaa gtcaatttcc ttctctgtgc tttggtttcc tcatccatag
  9661 aaaggtagaa agggcaaaac accaaactct tggattacaa gagataattt acagaacacc
  9721 cttggcacac agagggcacc atgaaatgtc acgggtgaca cagccccctt gtgctcagtc
  9781 cctggcatct ctaggggtga ggagcgtctg cctagcaggt tccc^tccagg aagctggatt
       (9825)(t/a)(dbSNP:rs2625964)
  9841 tgagtggatg gggcgctgga atcgtgaggg gcagaagcag gcaaagggtc ggggcgaacc
  9901 tcactaacgt gccagttcca agcacactgt gggcagccct gg^ccct^Gact caagcctct^t
                                                    Exon5_fwd
 (9943)(branch site - YNYYRAY), IVS4-23G>A(9947)(g/a)(dbSNP:rs2071092),
(9960-10188)(STS:GDB:186557)
RHO-Exon5 (9970-11696 -> 1727bp)
  9961 gccttccagT TCCGGAACTG CATGCTCACC ACCATCTGCT GCGGCAAGAA CCCACTGGGT
                                    MLPA exon5
 10021 GACGATGAGG CCTCTGCTAC CGTGTCCAAG ACGGAGACGA GCCAGGTGGC CCCGGCC^TAA
                                                                  stop
 10081 GACCTGCCTA GGACTCTGTG GCCGACTATA GGCGTCTCCC AT^CCCCTACA CCTTCCCCCA
         Exon5_rev              (10123)(c/a)(dbSNP:rs2071093)
 10141 GCCACAGCCA TCCCACCAGG AGCAGCGCCT GTGCAGAATG AACGAAGTCA CATAGGCTCC...
```

## Annotated *RP1* Sequence

Summary of Exons in sequence:

RP1-Exon1 (5001-5136 -> 136bp)
RP1-Exon2 (9889-10515 -> 627bp)
RP1-Exon3 (11051-11222 -> 172bp)
RP1-Exon4 (13604-19768 -> 6165bp)

Summary of SNPs in sequence:

1 - (127)(t/c)(dbSNP:rs1102421)
2 - (127)(t/c)(dbSNP:rs369240)
3 - (474)(t/c)(dbSNP:rs7844339)
4 - (830)(c/t)(dbSNP:rs12682580)
5 - (1012)(a/c)(dbSNP:rs2375083)
6 - (1215)(g/a)(dbSNP:rs10112903)
7 - (1392)(c/g)(dbSNP:rs405998)
8 - (1405)(a/g)(dbSNP:rs4327841)
9 - (1859)(t/g)(dbSNP:rs421844)
10 - (2027)(g/a)(dbSNP:rs16920597)
11 - (2115)(g/a)(dbSNP:rs16920600)
12 - (2180)(t/a)(dbSNP:rs16920601)
13 - (2515)(c/t)(dbSNP:rs2888995)
14 - (2544)(c/a)(dbSNP:rs435326)
15 - (2609)(g/t)(dbSNP:rs396881)
16 - (2631)(g/t)(dbSNP:rs7002210)
17 - (2927)(c/t)(dbSNP:rs446102)
18 - (3292)(t/a)(dbSNP:rs420455)
19 - (3309)(g/a)(dbSNP:rs405545)
20 - (3314)(t/c)(dbSNP:rs402441)
21 - (3780)(a/c)(dbSNP:rs387287)
22 - (3786)(a/t)(dbSNP:rs416104)
23 - (4105)(c/g)(dbSNP:rs439532)
24 - (4834)(t/c)(dbSNP:rs3817658)
25 - (5327)(a/g)(dbSNP:rs702761)
26 - (5385)(t/c)(dbSNP:rs3857921)
27 - (5447)(c/t)(dbSNP:rs9643828)
28 - (5746)(-/a)(dbSNP:rs5891543)
29 - (5760)(t/-)(dbSNP:rs5891544)
30 - (5861)(t/a)(dbSNP:rs6473949)
31 - (5986)(a/g)(dbSNP:rs145290)
32 - (7672)(a/g)(dbSNP:rs7814894)
33 - (7699)(c/g)(dbSNP:rs7831624)
34 - (7888)(c/t)(dbSNP:rs12056382)
35 - (7891)(c/t)(dbSNP:rs436527)
36 - (8792)(c/a)(dbSNP:rs7463859)
37 - (8813)(c/a)(dbSNP:rs3906755)
38 - (9292)(t/-)(dbSNP:rs5891545)
39 - (9361)(a/g)(dbSNP:rs2375079)
40 - (9450)(t/-)(dbSNP:rs5891546)
41 - (9738)(a/c)(dbSNP:rs11780170)
42 - (9795)(c/t)(dbSNP:rs16920613)
43 - (11187)(a/g)(dbSNP:rs16920614)
44 - (12078)(g/a)(dbSNP:rs6988404)
45 - (12744)(g/a)(dbSNP:rs428854)
46 - (13293)(t/c)(dbSNP:rs424499)
47 - (13391)(a/-)(dbSNP:rs11332494)
48 - (13401)(a/g)(dbSNP:rs13280650)
49 - (13512)(t/c)(dbSNP:rs429668)
50 - (15071)(c/t)(dbSNP:rs28399531)
51 - (15431)(g/a)(dbSNP:rs444772)
52 - (15649)(g/t)(dbSNP:rs16920621)
53 - (15769)(a/t)(dbSNP:rs2293869)
54 - (17824)(g/a)(dbSNP:rs446227)
55 - (17887)(t/c)(dbSNP:rs414352)
56 - (17991)(a/g)(dbSNP:rs441800)
57 - (18754)(c/a)(dbSNP:rs4397384)
58 - (19301)(a/c)(dbSNP:rs11995606)
59 - (19535)(-/tt)(dbSNP:rs10654889)
60 - (20258)(g/a)(dbSNP:rs7003351)
61 - (21812)(c/a)(dbSNP:rs3891358)
62 - (22645)(-/ag)(dbSNP:rs3077384)
63 - (22671)(c/a)(dbSNP:rs7844330)
64 - (22673)(a/c)(dbSNP:rs28758293)
65 - (22679)(a/c)(dbSNP:rs536048)
66 - (23441)(c/-)(dbSNP:rs11300666)
67 - (23974)(c/t)(dbSNP:rs396859)

Total of 67 SNPs in annotation

Summary of STSs in sequence:

1 - (14614-14885)(STS:RH123325)
2 - (14640-14827)(STS:STS-AA018812)

Total of 2 STSs in annotation

Annotated sequence file:

```
LOCUS       8 24768 bp DNA HTG 4-JUL-2006
DEFINITION  Homo sapiens chromosome 8 NCBI36 partial sequence
            55686180..55710947 reannotated via EnsEMBL
ACCESSION   chromosome:NCBI36:8:55686180:55710947:1
VERSION     chromosome:NCBI36:8:55686180:55710947:1
KEYWORDS    .
SOURCE      human
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata;
Euteleostomi;
            Mammalia; Eutheria; Euarchontoglires; Primates; Haplorrhini;
            Catarrhini; Hominidae; Homo.
COMMENT     This sequence was annotated by the Ensembl system. Please visit
the
            Ensembl web site, http://www.ensembl.org/ for more information.
COMMENT     All feature locations are relative to the first (5') base of the
            sequence in this file.  The sequence presented is always the
            forward strand of the assembly. Features that lie outside of the
            sequence contained in this file have clonal location coordinates
in
            the format: .:..
COMMENT     The /gene indicates a unique id for a gene,
            /note="transcript_id=..." a unique id for a transcript,
/protein_id
            a unique id for a peptide and note="exon_id=..." a unique id for
an
            exon. These ids are maintained wherever possible between
versions.
COMMENT     All the exons and transcripts in Ensembl are confirmed by
            similarity to either protein or cDNA sequences.
FEATURES             Location/Qualifiers
     source          1..24768
                     /organism="Homo sapiens"
                     /db_xref="taxon:9606"
     gene            5001..19768
                     /gene=ENSG00000104237
     mRNA            join(5001..5136,9889..10515,11051..11222,13604..19768)
                     /gene="ENSG00000104237"
                     /note="transcript_id=ENST00000220676"
     CDS             join(9901..10515,11051..11222,13604..19287)


BASE COUNT  7558 a 4664 c 4816 g 7730 t
ORIGIN
     1 ttctggcctc caaggtttct aatgagaaac tggtgaataa tcatatagag gatcccttgt
    61 atctgatgaa ttgcttccct tttgatgctt taatgtattt cagtgcaggt ctcttgtagt
   ...
  4801 tactggtgaa gtacttttтc tggaggataa cac^taggcaa gaaagaagat gcaagggaaa
       (4834)(t/c)(dbSNP:rs3817658)
  4861 ccttcatgag gaagatttca caactgcaga gcatgctagg aactggtttg cttctggctg
  4921 ttgtctcctt agggtgagct ctgtctggtg attagcatca ccatggatta aattaattgg
RP1-Exon1(5001-5136 -> 136bp) MLPA exon1
  4981 ctgtactcaa tcccttgctg GACATACTGA GAATAAATCC AAAGACATTA GTTTCTTTGC
                                MLPA ~exon1
  5041 ACGAAATGAG GTTACATATC CAGTGACATT TATTTGAGCT ATTTAAACAA CTTAAACATC
  5101 TTTTTCTTTT CTTAATAAGG GACGTTTCAA GTTGTGgtaa gtacaaaact atttgaaatc
  5161 tttctttgga aaatattctg ttttgctagt tgtattttgt tttccatgtt atacttagat
...
  9781 acttctgtga atat^cctgga tgtctgcagc tatatttagc atgcattagt attaccatgt
       (9795)(c/t)(dbSNP:rs16920613)
RP1-Exon2 (9889-10515 -> 627bp)
  9841 attcgctatg gtgc^tgtgat tctggagata attttctttc ttctctagGT CTCAGCCAAA
       (9855)(branch site - YRYRAY)
  9901 ^ATGAGTGATA CCCCTTCTAC TGGTTTTTCC ATCATTCATC CTACGTCTTC TGAAGGTCAA
       start       MLPA exon2
  9961 GTTCCACCCC CTCGCCATTT GAGCCTCACT CATCCTGTTG TGGCCAAGCG AATCAGTTTC
 10021 TACAAGAGCG GAGACCCCCA ATTCGGCGGG GTCAGGGTGG TGGTCAACCC TCGCTCCTTT
 10081 AAGTCCTTTG ATGCTCTGCT GGATAACTTG TCCAGGAAGG TGCCCCTCCC TTTTGGAGTG
```


10141 AGGAACATCA GCACCCCTCG GGGCAGGCAC AGCATCACGC GCCTGGAGGA GCTGGAGGAC
10201 GGCGAGTCCT ACCTATGTTC CCACGGCAGG AAGGTGCAGC CTGTAGACCT GGACAAAGCC
10261 CGTCGGCGCC CGCGGCCCTG GCTCAGCAGC CGGGCCATTA GCGCGCACTC ACCGCCCCAC
10321 CCCGTAGCCG TCGCTGCTCC CGGCATGCCC CGCCCCCCAC GGAGCCTAGT GGTCTTCAGG
10381 AATGGCGACC CGAAGACGAG GCGTGCGGTT CTTCTGAGCA GGAGGGTCAC CCAGAGCTTC
10441 GAGGCATTTC TACAGCACCT GACAGAGGTC ATGCAGCGCC CTGTGGTCAA GCTGTACGCT
10501 ACGGACGGAA GGAGGgtgag cgttctgggg gctcctcgag cctgagctca ttttgagcac
10561 cctactaatt ggattcgtgt gggatatgaa tggtggcccc cgggaaggaa atcttccttc
...
10981 gcaaagttat aaaattacca cttagtataa aatgtgctca tctcaggata atgactctgg
RP1-Exon3 (11051-11222 -> 172bp)
11041 tctcttttag GTTCCCAGCC TCCAGGCAGT GATCCTGAGC TCTGGAGCTG TGGTGGCGGC
MLPA exon3
11101 AGGAAGGGAG CCATTTAAAC CAGGAAATTA TGACATCCAA AAATACTTGC TTCCTGCTAG
11161 ATTACCAGGG ATCTCTCAGC GTGTGT^ACCC CAAGGGAAAT GCAAAGTCAG AAAGCAGAAA
(11187)(a/g)(dbSNP:rs16920614)
11221 GAgtaagtca cttattaata tatagcccat atttttagcc ctgagatttt ttttgcctca
11281 aggacggcaa aatccatgct tcaatgacca gtttcttcca ccacagaaac gaaaaggaga
...
13501 tctctttctt t^ttttgctgc ctcttccttt ggatatttct aacttctctg ccttccatat
(13512)(t/c)(dbSNP:rs429668)
RP1-Exon4 (13604-19768 -> 6165bp)
13561 tata^ttttga tgtgggcacc ttttactctt aaaatcttta aagTAAGCAC ACATATGTCT
(13565)(branch site - YNYYRAY)
13621 TCAAGCTCAA GGTCCCAGAT TTATTCTGTT TCTTCTGAGA AAACACATAA TAATGATTGC
13681 TACTTAGACT ATTCTTTTGT TCCTGAAAAG TACTTGGCCT TAGAAAAGAA TGATTCTCAG
13741 AATTTACCAA TATATCCTTC TGAAGATGAT ATTGAGAAAT CAATTATTTT TAATCAAGAC
13801 GGCACTATGA CAGTTGAGAT GAAAGTTCGA TTCAGAATAA AAGAGGAAGA AACCATAAAA
13861 TGGACAACTA CTGTCAGTAA AACTGGTCCT TCTAATAATG ATGAAAAGAG TGAGATGAGT
13921 TTTCCAGGAA GAACAGAAAG TCGATCATCT GGTTTAAAGC TTGCAGCATG TTCATTCTCT
13981 GCAGATGTGT CACCTATGGA GCGAAGCAGT AATCAAGAGG GCAGTTTGGC AGAGGAGATA
MLPA exon4
14041 AACATTCAAA TGACAGATCA AGTGGCTGAA ACTTGCAGTT CTGCTAGTTG GGAGAATGCT
14101 ACTGTGGACA CAGATATCAT CCAGGGAACT CAAGACCAAG CAAAGCATCG TTTTTATAGG
14161 CCCCCTACAC CTGGACTAAG AAGAGTGAGA CAAAAGAAAT CTGTGATTGG CAGTGTGACC
14221 TTAGTATCTG AAACTGAGGT TCAAGAGAAA ATGATTGGAC AGTTTTCATA TAGTGAAGAA
14281 AGGGAAAGTG GGGAAAACAA GTCTGAGTAT CACATGTTTA CACATTCTTG CAGTAAAATG
14341 TCATCAGTAT CTAACAAACC AGTACTTGTT CAGATCAATA ACAATGATCA AATGGAGGAG
14401 TCATCATTAG AAAGAAAAAA GGAAAACAGT CTGCTTAAGT CAAGTGCAAT AAGTGCTGGT
14461 GTTATAGAAA TTACAAGTCA GAAGATGTTA GAGATGTCAC ATAATAATGG TTTGCCATCA
14521 ACTATATCAA ATAACTCAAT TGTGGAGGAA GATGTAGTTG ATTGTGTGGT ATTGGACAAC
14581 AAAACTGGTA TCAAGAACTT CAAAACTTAT GGT^AACACCA ATGATAGGTT CAGTCCTAT^T
(14614-14885)(STS:RH123325), (14640-14827)(STS:STS-AA018812)
14641 TCAGCAGATG CAACCCATTT TTCAAGTAAT AACTCTGGAA CTGACAAAAA TATTTCTGAG
14701 GCTCCAGCTT CAGAAGCATC CTCTACTGTC ACTGCAAGAA TTGACAGACT AATTAATGAA
14761 TTTGCTCAGT GTGGTTTAAC AAAACTTCCA AAAAATGAAA AGAAGATTTT GTCATCTGTT
exon4F_fwd
14821 GCCAGCAAAA AGAAGAAAAA ATCTGGACAG CAAGCAATAA ATTCCAGGTA TCAAGATGGA
Arg677X
14881 CAGCTTGCAA CCAAAGGAAT TCTTAATAAG AATGAGAGAA TAAACACAAA AGGTAGAATT
14941 ACAAAGGAAA TGATAGTGCA AGATTCAGAT AGTCCCCTTA AAGGAGGGAT ACTTTGTGAG
15001 GAAGACCTCC AGAAAAGTGA TACTGTAATT GAATCAAATA CTTTTTGTTC CAAAAGTAAT
exon4G_fwd
15061 CTCAATTCCA ^CGATTTCCAA GAATTTCCAT AGAAATAAAT TAAATACTAC TCAAAATTCC
Met1?X?Met(15071)(c/t)(dbSNP:rs28399531) exon4F_rev
15121 AAGGTTCAAG GACTTTTAAC CAAAAGAAAA TCTAGATCAC TAAATAAAAT AAGCTTAGGA
15181 GCACCTAAAA AAAGAGAAAT CGGTCAAAGA GATAAAGTGT TTCCTCACAA TGAATCTAAA
15241 TATTGCAAAA GTACTTTTGA AAACAAAAGT TTATTTCATG TATTTAACAT CCTTGAGCAA
15301 AAACCCAAAG ATTTTTATGC ACCGCAATCT CAAGCAGAAG TGGCATCTGG GTATTTGAGA
exon4H_fwd exon4G rev
15361 GGAATGGCAA AGAAGAGTTT AGTTTCAAAA GTTACTGATT CACATATAAG TTTAAAAAGC
c.2590-2599delATAACTTTAT
15421 CAGAAAAAAC ^CTAAAGGGGA TAAAGTGAAA GCAAGTGCTA TTTTAAGTAA ACAACATGCT
Arg872His(15431)(g/a)(dbSNP:rs4444772)
15481 ACAACCAGGG CAAATTCTTT AGCTTCTTTG AAAAAACCTG ATTTTCCTGA GGCTATTGCT
15541 CATCATTCAA TTCAAAATTA TATACAGAGT TGGTTGCAGA ACATAAATCC ATATCCAACT
Ser91X
15601 TTAAAGCCTA TAAAATCAGC TCCAGTATGT AGAAATGAAA CGAGTGTG^GT AAATTGTAGC
(15649)(g/t)(dbSNP:rs16920621) exon4H_rev
15661 AATAATAGTT TTTCAGGGAA TGATCCCCAT ACAAATTCTG GAAAAATAAG TAATTTTGTT
15721 ATGGAAAGTA ATAAGCACAT AACTAAAATT GCCGGTTTGA CAGGAGAT^AA TCTATGTAAA

```
      (15769)(a/t)(dbSNP:rs2293869)
15781 GAGGGAGATA AGTCTTTTAT TGCCAATGAC ACTGGTGAAG AAGATCTCCA TGAGACACAG
15841 GTTGGATCTC TGAATGATGC TTATTTGGTT CCCCTGCATG AACACTGTAC TTTGTCACAG
15901 TCAGCTATTA ATGATCATAA TACTAAAAGT CATATAGCTG CTGAAAAATC AGGACCAGAG
15961 AAAAAACTTG TTTACCAGGA AATAAACCTA GCTAGAAAAA GGCAAAGTGT AGAGGCTGCC
16021 ATTCAAGTAG ATCCTATAGA AGAGGAAACT CCAAAAGACC TCTTACCAGT CCTGATGCTT
...
19621 GTTGTTAGCT TGGTGTAAAA TGTATATTGA CTGTATTGGT GAATAAATTG AATAGACATA
19681 ACCTCAAAGT ACTTCACTTA TTCTTTTTAA CTACTGATTT GATAAAAAGT ATGATTATAA
19741 GATATCCACG ACAATCTCAT AGTTTCTTgt gccaaatatg ttgagtccag ttcctcagaa
19801 tatccagtga aattgcaagc atttccatgg gacaggattt agtgtttttc taatagacta
```

## Annotated *RPE65* Sequence

Note! the sequence in this output has been reversed w.r.t. the input data, the summary numbering will therefore not correspond to the entries in the original file

Summary of Exons in sequence:

RPE65-Exon1 (5001-5065 -> 65bp)
RPE65-Exon2 (6254-6336 -> 83bp)
RPE65-Exon3 (8100-8250 -> 151bp)
RPE65-Exon4 (10077-10184 -> 108bp)
RPE65-Exon5 (10288-10429 -> 142bp)
RPE65-Exon6 (13960-14107 -> 148bp)
RPE65-Exon7 (15318-15399 -> 82bp)
RPE65-Exon8 (15637-15769 -> 133bp)
RPE65-Exon9 (15879-16018 -> 140bp)
RPE65-Exon10 (16644-16773 -> 130bp)
RPE65-Exon11 (23375-23489 -> 115bp)
RPE65-Exon12 (23584-23678 -> 95bp)
RPE65-Exon13 (23784-23895 -> 112bp)
RPE65-Exon14(25033-26136 -> 1104bp)

Summary of SNPs in sequence:

1 - (30761)(c/a)(dbSNP:rs3125889)
2 - (30261)(a/g)(dbSNP:rs9436835)
3 - (30261)(a/g)(dbSNP:rs3118413)
4 - (28554)(c/t)(dbSNP:rs3125890)
5 - (27295)(t/a)(dbSNP:rs11209300)
6 - (26889)(g/a)(dbSNP:rs4264030)
7 - (26811)(a/g)(dbSNP:rs2419988)
8 - (26727)(t/c)(dbSNP:rs3118414)
9 - (26347)(t/c)(dbSNP:rs3118415)
10 - (26099)(g/c)(dbSNP:rs3118416)
11 - (26006)(t/c)(dbSNP:rs3118417)
12 - (25910)(-/ct)(dbSNP:rs10626313)
13 - (25795)(c/t)(dbSNP:rs2182315)
14 - (25715)(t/c)(dbSNP:rs3118418)
15 - (23999)(g/a)(dbSNP:rs932783)
16 - (23745)(a/g)(dbSNP:rs13375676)
17 - (23729)(c/t)(dbSNP:rs12046996)
18 - (23698)(t/g)(dbSNP:rs12564647)
19 - (23223)(c/t)(dbSNP:rs17130685)
20 - (22879)(g/a)(dbSNP:rs12124063)
21 - (21586)(g/a)(dbSNP:rs1886906)
22 - (21257)(a/g)(dbSNP:rs3125891)
23 - (20983)(g/a)(dbSNP:rs11581095)
24 - (20881)(g/a)(dbSNP:rs12030710)
25 - (20847)(t/c)(dbSNP:rs3125892)
26 - (20845)(t/c)(dbSNP:rs12034425)
27 - (20843)(c/t)(dbSNP:rs12140914)
28 - (20404)(t/c)(dbSNP:rs3118419)
29 - (20284)(-/t)(dbSNP:rs5774935)
30 - (20231)(g/t)(dbSNP:rs1555845)
31 - (20083)(c/t)(dbSNP:rs1555846)
32 - (19785)(ctcttgtttctttttctggctt/-)(dbSNP:rs11269074)
33 - (19417)(c/t)(dbSNP:rs3125893)
34 - (19358)(t/g)(dbSNP:rs1555847)
35 - (19211)(t/g)(dbSNP:rs3790469)
36 - (18972)(t/c)(dbSNP:rs3125894)
37 - (18908)(c/a)(dbSNP:rs3125895)
38 - (18807)(g/a)(dbSNP:rs3125896)
39 - (18788)(g/a)(dbSNP:rs3125897)
40 - (18732)(t/c)(dbSNP:rs3125898)
41 - (18651)(t/c)(dbSNP:rs17130688)
42 - (18406)(t/c)(dbSNP:rs3125899)
43 - (18400)(c/g)(dbSNP:rs12749393)
44 - (18245)(g/a)(dbSNP:rs3125900)
45 - (17478)(g/a)(dbSNP:rs3125901)
46 - (17447)(a/g)(dbSNP:rs17130689)

```
47 -
(16701)(c/t)(dbSNP:rs12145904)
48 -
(16455)(t/a)(dbSNP:rs12083082)
49 - (16377)(t/c)(dbSNP:rs3125902)
50 - (16288)(g/t)(dbSNP:rs3118420)
51 - (16225)(a/g)(dbSNP:rs3118421)
52 -
(16130)(a/g)(dbSNP:rs12138573)
53 - (15285)(g/c)(dbSNP:rs1925955)
54 -
(15097)(c/a)(dbSNP:rs12058579)
55 - (14859)(c/t)(dbSNP:rs3125903)
56 -
(14662)(t/a)(dbSNP:rs17130691)
57 - (14553)(g/a)(dbSNP:rs3125904)
58 - (14129)(g/a)(dbSNP:rs2274321)
59 -
(13602)(g/t)(dbSNP:rs12074337)
60 - (13116)(a/c)(dbSNP:rs3125905)
61 - (12160)(g/a)(dbSNP:rs2038900)
62 - (12142)(t/c)(dbSNP:rs2038901)
63 -
(11774)(t/c)(dbSNP:rs17130693)
64 -
(11530)(c/t)(dbSNP:rs17130694)
65 -
(11515)(a/g)(dbSNP:rs12408546)
66 -
(11140)(g/a)(dbSNP:rs12077372)
67 - (10031)(c/t)(dbSNP:rs3790471)
68 - (9644)(c/a)(dbSNP:rs3790472)
69 - (9557)(c/g)(dbSNP:rs11799958)
70 - (9530)(a/g)(dbSNP:rs3790473)
71 - (8624)(-
/ctag)(dbSNP:rs10685616)
72 - (8534)(a/c)(dbSNP:rs2148138)
73 - (8493)(g/a)(dbSNP:rs11209301)
74 - (7888)(t/c)(dbSNP:rs2012235)
75 - (7706)(t/g)(dbSNP:rs3118423)
76 - (7549)(g/t)(dbSNP:rs3118424)
77 - (7548)(c/t)(dbSNP:rs3118425)
78 - (7404)(g/a)(dbSNP:rs9436836)
79 - (7184)(a/c)(dbSNP:rs2986125)
80 - (7066)(g/t)(dbSNP:rs2986124)
81 - (5699)(a/g)(dbSNP:rs3125906)
82 - (5578)(c/a)(dbSNP:rs3118426)
83 - (5518)(g/a)(dbSNP:rs2419982)
84 - (5164)(a/t)(dbSNP:rs3125907)
85 - (4685)(a/g)(dbSNP:rs635213)
86 - (4520)(g/c)(dbSNP:rs382422)
87 - (4090)(t/c)(dbSNP:rs3118427)
88 - (3974)(g/a)(dbSNP:rs3125908)
89 - (3793)(g/t)(dbSNP:rs12759602)
90 - (3361)(a/g)(dbSNP:rs2477974)
91 - (3358)(c/a)(dbSNP:rs3125909)
92 - (3289)(t/c)(dbSNP:rs3118428)
93 - (2895)(g/a)(dbSNP:rs12407140)
94 - (2310)(c/t)(dbSNP:rs3125910)
95 - (2252)(tg/-
)(dbSNP:rs10533654)
96 - (1748)(c/t)(dbSNP:rs436070)
97 - (1735)(a/g)(dbSNP:rs28437112)
98 - (1729)(c/t)(dbSNP:rs11209302)
99 - (1609)(c/t)(dbSNP:rs414020)
100 -
(1599)(g/a)(dbSNP:rs13375666)
101 - (1386)(g/a)(dbSNP:rs3118429)
102 - (365)(g/a)(dbSNP:rs12040869)
103 - (344)(a/g)(dbSNP:rs411362)
104 - (200)(t/c)(dbSNP:rs3118430)
105 - (187)(c/t)(dbSNP:rs12121997)
106 - (62)(a/g)(dbSNP:rs429404)

Total of 106 SNPs in annotation

Summary of STSs in sequence:

1 - (25215-25461)(STS:RH80292)
2 - (25187-25410)(STS:SHGC-31528)
3 - (25033-25401)(STS:PMC19699P1)
4 - (25033-25241)(STS:PMC19699P15)
5 - (24900-25077)(STS:PMC19699P14)
6 - (23739-23937)(STS:PMC19699P13)
7 - (23552-23727)(STS:PMC19699P12)
8 - (16602-16799)(STS:PMC19699P11)
9 - (15798-16063)(STS:PMC19699P10)
10 - (15597-15810)(STS:PMC19699P9)
11 - (15282-15466)(STS:PMC19699P8)
12 - (13925-14135)(STS:PMC19699P7)
13 - (10246-10465)(STS:PMC19699P6)
14 - (10049-10210)(STS:PMC19699P5)
15 - (8058-8287)(STS:PMC19699P4)
16 - (6223-6378)(STS:PMC19699P3)
17 - (4985-5137)(STS:PMC19699P2)
18 - (2187-2457)(STS:G60461)
19 - (2179-2368)(STS:D1S2803)

Total of 19 STSs in annotation
```

Annotated sequence file:

```
LOCUS       1 31136 bp DNA HTG 4-JUL-2006
DEFINITION  Homo sapiens chromosome 1 NCBI36 partial sequence
            68662095..68693230 reannotated via EnsEMBL
ACCESSION   chromosome:NCBI36:1:68662095:68693230:1
VERSION     chromosome:NCBI36:1:68662095:68693230:1
KEYWORDS    .
SOURCE      human
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata;
Euteleostomi;
            Mammalia; Eutheria; Euarchontoglires; Primates; Haplorrhini;
            Catarrhini; Hominidae; Homo.
COMMENT     This sequence was annotated by the Ensembl system. Please visit
the
            Ensembl web site, http://www.ensembl.org/ for more information.
COMMENT     All feature locations are relative to the first (5') base of the
            sequence in this file.  The sequence presented is always the
            forward strand of the assembly. Features that lie outside of the
            sequence contained in this file have clonal location coordinates
in
            the format: .:..
COMMENT     The /gene indicates a unique id for a gene,
            /note="transcript_id=..." a unique id for a transcript,
/protein_id
            a unique id for a peptide and note="exon_id=..." a unique id for
an
            exon. These ids are maintained wherever possible between
versions.
COMMENT     All the exons and transcripts in Ensembl are confirmed by
            similarity to either protein or cDNA sequences.
FEATURES             Location/Qualifiers
     source          1..31136
                     /organism="Homo sapiens"
                     /db_xref="taxon:9606"
     gene            complement(4939..26136)
                     /gene=ENSG00000116745
     mRNA            join(complement(26072..26136),complement(24801..24883),
                     complement(22887..23037),complement(20953..21060),
                     complement(20708..20849),complement(17030..17177),
                     complement(15738..15819),complement(15368..15500),
                     complement(15119..15258),complement(14364..14493),
                     complement(7648..7762),complement(7459..7553),
                     complement(7242..7353),complement(4939..6104))
                     /gene="ENSG00000116745"
                     /note="transcript_id=ENST00000262340"
     CDS             join(complement(26072..26082),complement(24801..24883),
                     complement(22887..23037),complement(20953..21060),
                     complement(20708..20849),complement(17030..17177),
                     complement(15738..15819),complement(15368..15500),
                     complement(15119..15258),complement(14364..14493),
                     complement(7648..7762),complement(7459..7553),
                     complement(7242..7353),complement(5953..6104))

BASE COUNT  9220 a 5818 c 6020 g 10078 t
ORIGIN
     1 caattctaca tccttccctg ttttcatgac ctctgcagtg tgacactgta gtccttctca
    61 c^tgaagacta ccaaggtcaa agctattttc accccactca aagtctaagc tggccacatg
       (62)(a/g)(dbSNP:rs429404)
   ...
  4741 tgacgctggc cactcccacc tagctccttt ctttctaatc tgttctcatt ctccttggga
  4801 aggattgagg tctctggaaa acagccaaac aactgttatg ggaacagcaa gcccaaataa
  4861 agccaagcat caggggatc tgagagctga aagcaacttc tgttccccct ccctcagctg
                              exon1_fwd
  4921 aaggggtggg gaagggctcc caaagc^cata actcctttta agggatttag aaggcataaa
       (4947)(branch site - YRYRAY)
RPE65-Exon1 (5001-5065 -> 65bp)
  4981 aagg^cccctg gctgagaact TCCTTCTTCA TTCTGCAGTT GGTGCCAGAA CTCTGGATCC
```

(4985-5137)(STS:PMC19699P2)
5041 TGAACTGGAA GAAA^ATGTCT ATCCAgtaag tatctctggg agacttttt aaaacacctt
start MLPA exon1, IVS1+1G>T
5101 catggattca tgataaatgt gctatttcaa gggctcctga agagaaggca ttgattaatg
exon1_rev
5161 acc^tgtttag gaaagaccct tgaattttgg tggggaaatt ttttcctgct tatgtattaa
(5164)(a/t)(dbSNP:rs3125907)
5221 agcaactagc tagaattcag gtccaaatta tgggggttt gtgtgactta gcccggggtt
...
6121 ctgagactag accggcagga gtgaacaggc tttgagccag ccctagagtg ccttctctcc
6181 tgcagctctg cctctatctc tgcggacttt gag^catcaac at^gggcttct tccttattct
exon2_fwd,(6214)(branch site - YNYYRAY), (6223-6378)(STS:PMC19699P3)
RPE65-Exon2 (6254-6336 -> 83bp)
6241 tccaccattt cagGGTTGAG CATCCTGCTG GTGGTTACAA GAAACTGTTT GAAACTGTGG
6301 A[illegible]TGTC CTCGCCGCTC ACAGCTCATG TAACAGgttg gtctcgccca tcttgaagcc
Glu21Lys, Leu22Pro
6361 atcctctttt atgtcagtct ctcttctctg gcttcctatt ccttggcgtc tctcagtgca
exon2_rev
6421 gactcccccc gtgggtcaca gggagaggga tcaggtggtt tgggcttttt atagcaagaa
6481 cccattttct ttcattcgcc tttactttat ttctctaatg cagaaaaacg ctacctaatt
6541 ggaattccct aaggatcagg attacacctt tgtcatcttc aaactgtcag tgcctagctc
...
7981 tgccagctct atgaggaaga agctgcccaa tcaggctgct gatatactct gccttaccaa
RPE65-Exon3 (8100-8250 -> 151bp)
8041 ggacaagcct agcccaa^ggc agggataaga acaatgttc tgtctccccc tcatcacagG
(8058-8287)(STS:PMC19699P4), exon3_fwd, IVS2-18G>A
8101 CAGGATCCCC CTCTGGCTCA CCGGCAGTCT CCTTCGATGT GGGCCAGGAC TCTTTGAAGT
8161 TGGATCTGAG CCATTTTACC ACCTGTTTGA TGGGCAAGCC CTCCTGCACA AGTTTGACTT
MLPA exon3
8221 TAAAGAAGGA CATGTCACAT ACCACAGAAG gtaaagcagc actccatgcc actcctcctc
8281 ctcaaagtag ggcctagctt ggctcctcct cccatgtgag ttttcacctc tgactca g
exon3_rev, IVS3+80T>A
8341 aggcaaccta tatacccact ttcttctcac aatgtacctg ggccagtgtg tctgcatgga
8401 tacgagggaa cattatcatg gaataaagga gaagctaaag cctagggctt tggggtccaa
...
9901 gtgtctgccc tgcttggtca ccccaagaaa gtgagctaat aaaacccttt attcttcatg
9961 ttgtgcattt atgattgtga cttgatgagg acacatagaa tggccattct aagctccaca
RPE65-Exon4 (10077-10184 -> 108bp)
10021 tgggctgtac ^ggattgctcc tgtctata^ct cttccctatg ^tttcaatgtc cttcagGTTC
(10031)(c/t)(dbSNP:rs3790471),exon4/5_fwd,(10049-10210)(STS:PMC19699P5),
(10061)(branch site - YNYYRAY)
10081 ATCCGCACTG ATGCTTACGT ACGGGCAATG ACTGAGAAAA GGATCGTCAT AACAGAATTT
10141 GGCACCTGTG CTTTCCCAGA TCCCTGCAAG AATATATTTT CCAGgttact gaacccaaac
10201 tgaatgttac tcaagacatt ttatattagc ccttttttctc tcatg^gcttg aaaattactg
(10246-10465)(STS:PMC19699P6)
RPE65-Exon5 (10288-10429 -> 142bp)
10261 gactgaaaaa ttcatttgtt tctacagGTT TTTTTCTTAC TTTCGAGGAG TAGAGGTTAC
10321 TGACAATGCC CTTGTTAATG TCTACCCAGT GGGGGAAGAT TACTACGCTT GCACAGAGAC
Ala132Thr MLPA exon5
10381 CAACTTTATT ACAAAGATTA ATCCAGAGAC CTTGGAGACA ATTAAGCAGg tgggacacag
10441 tgctaggtga tgttcaggaa tttagaattt ggaacttaaa attaattcaa cataaattat
exon4/5_rev
10501 tcatgctgcg aatgtatgat tctaacttga tgaggacaca cagaatgacc attccaagct
10561 ccaaatggac tgcactgatt gcctctgtct atgatgcttt cctgcaattg gtgtaatttg
...
13801 gaactcaagg tgaaagaggg tagaatccag agagatggga agaaagagaa gtgcacttag
13861 gatgagagtt caaggggtag tgatgacctc agacctaggg acaaaggtat aatgtatctt
RPE65-Exon6 (13960-14107 -> 148bp) exon6_fwd
13921 cctt^c^tctca actggaggac attcacttta cttccgtagG TTGATCTTTG CAACTATGTC
(13925-14135)(STS:PMC19699P7),(13926)(branch site - YNYYRAY)
13981 TCTGTCAATG GGGCCACTGC TCACCCCCAC ATTGAAAATG ATGGAACCGT TTACAATATT
14041 GGTAATTGCT TTGGAAAAAA TTTTTCAATT GCCTACAACA TTGTAAAGAT CCCACCACTG
14101 CAAGCAGgtg agtttaccaa tctgtcct^ct tctgaaaata agtgtctata ttgtgagaaa
IVS6+22C>T(14129)(g/a)(dbSNP:rs2274321), exon6_rev
14161 gttactgtat tgtgagaaag ataagaatgt cagaatagca ttttgtgcat agttaaatta
14221 ctctcagact tcagaagtac tgccctcatc cctaccctat aaggggccta atgcccaagg
...
15181 atgatggaga aaatgaaaat aacccctcta aaacaatcaa aatgtgtttc tttgcctgta
15241 taagctgttc taaatgcttt gtattaaaaa aaaataaga g^gct^cttcca aagcctttta
exon7/8_fwd,(15282-15466)(STS:PMC19699P8), (15285)(g/c)(dbSNP:rs1925955)
RPE65-Exon7 (15318-15399 -> 82bp)

15301 aaaccacttt atttcagACA AGGAAGATCC AATAAGCAAG TCAGAGATCG TTGTACAATT
MLPA exon7
15361 CCCCTGCAGT GACCGATTCA AGCCATCTTA CGTTCATAGg taacttgaag gcctgctatg
15421 aatcttcagg aaaactcaag tttaaagatt tgctttgcct acctttgata ccaatcctga
15481 tcacaatatt ttttt[redacted]aga aaactaaatt taaacataat taatttcatg taggcactgt
IVS7+98C>T
15541 tgattcttgt aactgagcat accacatata tacaaatttg tctgtggctt gagaat^cagc
(15597-15810)(STS:PMC19699P9)
**RPE65-Exon8** (15637-15769 -> 133bp)
15601 cctttcattc acaagcccat ttgtgtttct gaacagTTTT GGTCTGACTC CCAACT[redacted]TAT
Tyr249Cys
15661 [redacted]GCTTTTTGTG GAGACACCAG TCAAAATTAA CCTGTTCAAG TTCCTTTCTT CATGGAGTCT
T>A250T
15721 TTGGGGAGCC AACTACATGG ATTGTTTTGA GTCCAATGAA ACCATGGGGg taagtcttag
15781 atatatttgt caagttt^**gtg attctgaaga agatgtgttt aa**taat**gtac actttttcc**
(15798-16063)(STS:PMC19699P10, **exon7/8_rev** **exon9_fwd**
**RPE65-Exon9** (15879-16018 -> 140bp)
15841 **tttttaaatg catc**aaaata ttttcctcat ttttcaagGT TTGGCTTCAT ATTGCTGACA
15901 AAAAAAGGAA AAAGTACCTC AATAATAAAT ACAGAACTTC TCCTTTCAAC CTCTTCCATC
MLPA exon9
15961 ACATCAACAC CTATGAAGAC AATGGGTTTC TGATTGTGGA TCTCTGCTGC TGGAAAGGgt
16021 aagaaaggac actggacaaa tgggacacct cccattgttc ctggaaatta cggggttttt
16081 acagagctgc tg**cactcaat ctgaatcaca tctaaaac**ag caagagtta^t gtgagagtca
**exon9_rev,** (16130)(a/g)(dbSNP:rs12138573)
16141 aaaatgtaat tggaaaagca gtaggaagtt taggtaatct gaacatcaaa actatgatta
16201 atgattgaaa actaatcggg tcca^tgaacc ccacctgaaa acaaaagatt ctgtgacctg
(16225)(a/g)(dbSNP:rs3118421)
16261 agaggtgaac ttaaagttga caaaatc^cct ggggaaactt gagccccatc ttctctgcga
(16288)(g/t)(dbSNP:rs3118420)
16321 agggagaaaa agacatgcgt ttttcagact agccaaatca ctaagaagac aacagc^acct
(16377)(t/c)(dbSNP:rs3125902)
16381 aaggacacaa acagaatggt gacaggaaga cagaacagaa agtatctcaa ggctgctgtt
16441 acttaaacaa atcc^attaag caggttgctt tattttcatc ctgtgtgggc gggaggaaat
(16455)(t/a)(dbSNP:rs12083082)
16501 ggctctgata cacctggctc aatagcagtt tctgggttgt ggaataaaga acaggcaggc
16561 acttgtgctt aaaaaggcaa gaatcatctc tctaaaatta t^ttgtcat**tg cctgtgctca**
(16602-16799)(STS:PMC19699P11), **exon10_fwd**
**RPE65-Exon10** (16644-16773 -> 130bp)
16621 **tgtttgac**tt tttatttttg cagATTTGAG TTTGTTTATA ATTACTTATA TTTAGCCAAT
16681 TTACGTGAGA ACTGGGAAGA ^GGTGAAAAAA AATGCCAGAA AGGCTCCCCA ACCTGAAGTT
Glu352Gln(16701)(c/t)(dbSNP:rs121459C4)
16741 AGGAG[redacted]TATG TACTTCCTTT GAATATTGAC AAGgtaacct gcttctctgt agatttcaga
Tyr368His
16801 tttaa**ccaga atgtttcatc tctctca**gga attgtcctcc tgcctcatgt ttatacataa
**exon10_rev**
16861 agtcttgaaa tttgagagct agaaggagct ttaaaaatag gctaattaaa cttccttcat
16921 tttacaaatg aaactaaagc tcagaagagt actgtgactt agcaaaagca actgcagcag
...
23221 tc^gcgtccca gggtccctca tctatagctt cctgcagttc ctccctgcat gttgacctaa
(23223)(c/t)(dbSNP:rs17130685)
23281 aaaagaactt aggagccaag acttaagaac tctgcatttc tggct**gtttg aattctttcc**
**RPE65-Exon11** (23375-23489 -> 115bp) **exon11/12/13_fwd**
23341 **tgctcac**tga ggtttctgtt atcttctctc ctagGCTGAC ACAGGCAAGA ATTTAGTCAC
23401 [redacted]GCTCCCCAAT ACAACTGCCA CTGCAATTCT GTGCAGTGAC GAGACTATCT GGCTGGAGCC
Rh2H1hr
23461 TGAAGTTCTC TTTTCAGGGC CTCGTCAAGg tgagatgatc tagagaaaac ttcacac[redacted]gg
IVS11+29G>T
23521 agtgaacaaa tgtttctttc aaagagatta a^gagttttcc taagcatgtg ctctatttcg
(23552-23727)(STS:PMC19699P12)
**RPE65-Exon12** (23584-23678 -> 95bp)
23581 tagCATTTGA GTTTCCTCAA ATCAATTACC AGAAGTATTG TGGGAAACCT TACACATATG
23641 CGTATGGACT TGGCTTGAAT CACTTTGTTC CAGATAGGgt aattaatcct tcttact[redacted]aat
IVS12+20A>G(23698)(t/g)(dbSNP:rs2564647)
23701 atttgaacag tgctttgagt atatgcta^gt caagtaaa^gc a^tat[redacted]gactg attgcttgat
(23729)(c/t)(dbSNP:rs12046996), (23739-23937)(STS:PMC19699P13),
(23742)(branch site - YNYYRAY), IVS12-39T>C(23745)(a/g)(dbSNP:rs1537546)
**RPE65-Exon13** (23784-23895 -> 112bp)
23761 tgatttttct ttctcacaaa cagCTCTGTA AGCTGAATGT CAAAACTAAA GAAACTTGGG
23821 TTTGGCAAGA GCCTGATTCA TACCCATCAG AACCCATCTT TGTTTCTCAC CCAGATGCCT
23881 TGGAAGAAGA TGATGgtaat gaaagcaatt gttgtgtctg aatactctt**c ttactgcagt**
**exon11/12/13_rev**

```
 23941 tctgtatgtt agttcgttag gaaaggagta aggttgatta ttccgtaata ctcaaaaa^cg
       (23999)(g/a)(dbSNP:rs932783)
 24001 atccttaata ctataaatag agcagtactg aagaattaaa actatttgaa aagaattttc
 24061 atcttttgat caggtcttgt tgattaccag tagaaaaaaa tgtcaaagca ccatttggtg
 24121 tcacgatgga gcatcaatgt ctgctctaga tgagggagag aaggaagaaa aaatggcaac
 24181 atgaattcca ggtttttgcc ttccctgacg aagacatatt tctaaaaaaa tagcaattct
 24241 aagaagggaa gctaagtatt atagcagata caagagaatg agaaggcagg tctgtgaata
 24301 gttttcattt gttctttcgg taagctaggg cttgaaaaaa gacccagagc cacaccagca
 24361 actcagatac agcaactcaa attacaaatc agcagtgtta gttaactgct tctcagctgg
 24421 gacagggaga tagattacac tctatgggat taaaatatgg aaaactaaga gaaatgagat
 24481 aaagaattgt ggagccattt cacactaaat gattttatat atccagtcga agctgtaaaa
 24541 gatttgttct cattttgttc gaaggaagtc agcttccaga tcgatcttag aaaccataca
 24601 tatgatatat cttactgctt tcaaagtctt ttaatacaca cttttcatgt gatcatcaca
 24661 gtttttacag tatgtacagc aggtgctata tttgtatgtt acagataaga acagatccat
 24721 ttattcagtg agtatttaat gagtacctac tacgctgtag tcattgtcat agaatattga
 24781 gatatagcag tgagcaagac agacaagact ctcccttctt agagcttaca gtgtaggtag
 24841 ggtacataaa gagtttaaaa gacctcttta ggtggtctca tgccaggtgg tacaagagt^c
                                exon14_fwd,(24900-25077)(STS:PMC19699P14)
 24901 agaaaaagaa gtcaggtcat atggttttct atatttgtca atgtaatacc tcctatatta
 24961 tttcaatgac attcaatcta tagcttgggc ttttaaaaac tcaatattgc ctaatttac^t
       (25020)(branch site - YNYYRAY)
RPE65-Exon14 (25033-26136 -> 1104bp)
25021 tctgataaac ag^GTGTAGTT CTGAGTGTGG TGGTGAGCCC AGGAGCAGGA CAAAAGCCTG
       (25033-25241)(STS:PMC19699P15), MLPA exon14
 25081 CTTATCTCCT GATTCTGAAT GCCAAGGACT TAAGTGAAGT TGCCCGGGCT GAAGTGGAGA
 25141 TTAACATCCC TGTCACCTTT CATGGACTGT TCAAAAAATC T^TGAGC^ATAC TCCAGCAAGA
       stop, (25187-25410)(STS:SHGC-31528)
 25201 TATGTTTTTG GTAG^CAAAAC TGAGAAAATC AGCTTCAGGT CTGCAATCAA ATTCTGTTCA
       (25215-25461)(STS:RH80292)
 25261 ATTTTAGCCT GCTATATGTC ATGGTTTTAA CTTGCAGATG CGCACAATTT TGCAATGTTT
 25321 TACAGAAAGC ACTGAGTTGA GCAAGCAATT CCTTTATTTA AAAAAAAAAG TACGTATTTA
             exon14_rev
 25381 GATAATCATA CTTCCTCTGT GAGACAGGCC ATAACTGAAA AACTCTTAAA TATTTAGCAA
 25441 TCAAATAGGA AATGAATGTG GACTTACTAA ATGGCTTTTA ATTCCTATTA TAAGAGCATA
 25501 TTTTAGGTAC CTATCTGCTC CAATTATATT TTTAACATTT AAAAACCAAA GTCCTCTACA
 25561 CTTGATTTAT ATTATATGTG GCTTTGCTGA GTCAAGGAAG TATCATGCAA TAAGGCTTAA
 25621 TTACTAAATG TCAAACCAAA CTTTTTCTCA AACCAGGGAC TATCATCTAA GATTAATTAC
 25681 AGTAATTATT TTGCGTATAC GTAACTGCTC AAAG^ATTATG AATCTTATGA ATGTTAACCT
       (25715)(t/c)(dbSNP:rs3118418)
 25741 TTCCGTTTAT TACAAGCAAG TACTATTATT TCTGATTTTA TAATAAGAAA ATCT^GTGTTT
       (25795)(c/t)(dbSNP:rs2182315)
 25801 AATCAACTGA GGCCTCTCAA CCAAATAACA TCTCAGAGAT TAAGTTATAT ATTAAAAGCT
 25861 TATGTAACAT AAAAGCAAGT ACATATAGTA GTGACTATAT TTAAAAAAA^C AGCATAAAAT
       (25910)(-/ct)(dbSNP:rs10626313)
 25921 GCTTAAAAAT GTAATATTTA CTAAAATCAG ATTATGGGAT AATGTTGCAG GATTATACTT
 25981 TATTGCATCT TTTTTGTTTA ATTGT^ATTTA AGCATTGTGC AATCACTTGG GAAAAATATT
       (26006)(t/c)(dbSNP:rs3118417)
 26041 AAATTATTAA CATTGAGGTA TTAATACATT TTAAGCCTTT TGTTTTTAAA TTTCTTTT^CT
       (26099)(g/c)(dbSNP:rs3118416)
 26101 TCCAGAGATT GTTTAAAAAT AAATATTGAC AAAAATaatg ttttatatct taattctagt
 26161 atctgtttta tgcttgaaag cattacagat catgatacct aagatgatca gcatggtgtt
 ...
 31021 tgttcttcag tatcctcaac attagagtaa ttgaatgatg cattagtcca ttttcacact
 31081 cttacaaaga taccacctga gaaggggat ttttgaagaa aagaggttta attgac
//
```

# Appendix 2: General Recipes and protocols

## Tris borate EDTA Buffer (TBE)

10X TBE buffer: 0.98M Tris[hydroxymethyl]aminomethane (Tris) (108g); 0.89M boric acid (55g); 0.02M Ethylenediaminetetra-acetic acid (EDTA) (7.4g) made up to a final volume of 1 litre with water

Dilute to 0.5X, 0.6X, 1X, or 1.5X as required

## Agarose loading dye

0.25% bromophenol blue (0.125g); 40% sucrose (20g) made up to a final volume of 50ml with water.

## Agarose gels (0.8%, 1%; 2%, 3.5%)

**0.8% gel:**

0.4g agarose in a final volume of 50ml 1XTBE buffer; with 2µl ethidium bromide (10mg/ml)

**1% gel:**

0.5g agarose in a final volume of 50ml 1XTBE buffer; with 2µl ethidium bromide (10mg/ml)

**2% gel:**

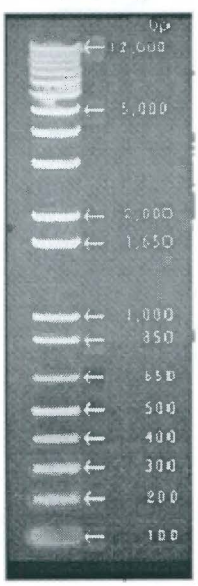
1g agarose in a final volume of 50ml 1XTBE buffer; with 2µl ethidium bromide (10mg/ml)

**3.5% gel:**

1.75g agarose in a final volume of 50ml 1XTBE buffer; with 2µl ethidium bromide (10mg/ml)

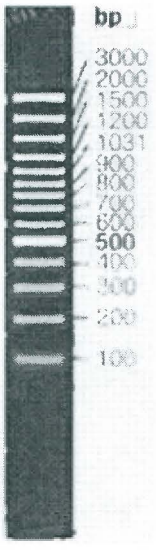
## DNA size standards

**1kb plus (Invitrogen™ Life Technologies, UK)**



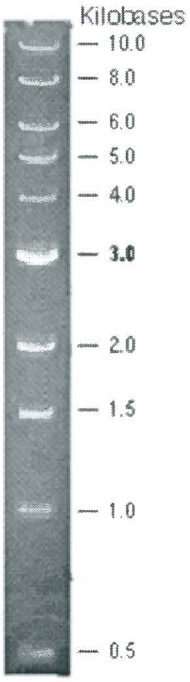

0.7 µg/lane; 0.9% agarose gel stained with ethidium bromide

**100bp Generuler™ plus (Fermentas International Inc, Canada)**




0.5 µg/lane; 1.7 % agarose gel stained with ethidium bromide

**1kb ladder (New England Biolabs, USA)**




0.5 µg/lane; 0.8% agarose gel stained with ethidium bromide

## SSCP loading dye

95% formamide (47.5ml); 10mM sodium hydroxide (0.02g); 20mM EDTA (0.372g); 0.02% bromophenol blue (0.01g); 0.02% xylene cyanol (0.01g) in a final volume of 50ml

## SSCP polyacrylamide gels

**40% Polyacrylamide stock**
99% acrylamide (39.6g) and 1% bis-acrylamide (0.4g) made up to a final volume of 100ml with water

**10% Polyacrylamide gel with 7.5% Urea**
15ml of 40% polyacrylamide stock; 4.5g of urea; 9ml of 10X TBE buffer; 35ml of water; 300µl of 10% Ammonium Persulphate (APS); 30µl of NNN'N'-Tetramethylethylenediamine (TEMED)

**10% Polyacrylamide gel with 5% Glycerol**
15ml of 40% polyacrylamide stock; 3ml of glycerol; 3ml of 10X TBE buffer; 39ml of water; 300µl of 10% APS; 30µl of TEMED

**0.5X MDE™ gel**
15ml MDE™ (BMA, USA); 3.6ml of 10X TBE buffer; 41.4ml of water; 300µl of 10% APS; 30µl of TEMED

**0.5X MDE™ gel with 10% glycerol**
15ml MDE™ (BMA, USA); 6ml of glycerol; 3.6ml of 10X TBE buffer; 35.4ml of water; 300µl of 10% APS; 30µl of TEMED

## Silver staining

Separate the glass plates and rinse the gel plate with water
Soak the gel plate in 0.1% Silver nitrate solution (2g silver nitrate in 2 litres of water) for 10-15 minutes
Remove the silver nitrate solution and rinse the gel plate with water
Soak the gel plate in fixing solution (30g sodium hydroxide pellets; 20ml of 15% formaldehyde; made up to 2 litres with water) for 10-15 minutes or until bands are suitably visible
Remove the fixing solution and rinse the gel plate with water
Place the gel on chromatography paper for viewing

## Multiphor™ loading dye

95% formamide (47.5ml); 100mM sodium hydroxide (0.16g); 0.25% bromophenol blue (0.125g); 0.25% xylene cyanol (0.125g) in a final volume of 50ml

## Multiphor™ II gels

**8% Polyacrylamide stock for size-based separation**
200ml of 40% Acrylogel (BDH Laboratory Supplies, England); 100ml of 10X TBE buffer; 422g of Urea made up to a final volume of 1 litre with water

**40% Acrylamide – PDA stock for SSCP**
99% acrylamide (396g) and 1% piperazine diacrylamide (PDA) (4g) made up to a final volume of 1 litre with water

**0.75M Tris formate buffer**
90.8g Tris in 600ml of water; pH to 9.0 with formic acid and made up to a final volume of 1 litre with water

**Tris Borate buffer**
125.9g Tris; 17.3g boric acid; dissolved in 950ml water; add 50µl of 4% bromophenol blue solution; made up to a final volume of 1 litre with water.

**8% polyacrylamide gel for Multiphor™ II size-based separation**
17-20ml of 8% polyacrylamide stock; 200µl of 10% APS; 20µl of TEMED

**12% non-denaturing gel for Multiphor™ II SSCP**
5.3ml of 40% acrylamide-PDA stock; 8.5ml of 0.75M Tris formate buffer; 3ml of 41% glycerol; 200µl of 10% APS; 20µl of TEMED

## ABI Prism™ 377 Sequencer

**Sequencing gel**
18g Urea; 5ml of 10X TBE; 5 ml of deionised 40% Acrylogel (BDH Laboratory Supplies, England); made up to a final volume of 50ml with water; degassed; 250µl of 10% APS; 25µl of TEMED

**Loading dye for sequencing**
5:1 ratio of deionised formamide: ABI Prism™ loading buffer containing 25mM EDTA and 50mg/ml Blue Dextran (Applied Biosystems, USA)

**Genotyping gel**
3.6g Urea; 1ml of 10X TBE; 1ml of 40% Acrylogel (BDH Laboratory Supplies, England); made up to a final volume of 10ml with water; 50µl of 10% APS; 7µl of TEMED

**Loading dye for genotyping**
5µl of deionised formamide; 1µl Rox™ 500 molecular weight marker (Applied Biosystems, USA); 1µl ABI Prism™ loading buffer (Applied Biosystems, USA)

# Appendix 3: Protocol for delivery of results and DNA consent form


## THE GENETICS OF RETINAL DEGENERATIVE DISORDERS IN SOUTHERN AFRICA

Division of Human Genetics, Faculty of Health Sciences

**University of Cape Town,**

**PRINCIPAL INVESTIGATORS**

Professor Raj Ramesar Ph.D.

Professor Jacquie Greenberg Ph.D.

rr@cormack.uct.ac.za

jg@cormack.uct.ac.za

Clinical Research Co-ordinator

**Lecia Bartmann SRN/SRM.**

lb@cormack.uct.ac.za


---

## Protocol for the delivery of genetic results arising from the research study of

***"The Genetics of Retinal Degenerative Disorders (RDD) in Southern Africa."***

Informed consent has to be obtained from all individuals from whom DNA has been isolated and on whom mutation screening or linkage analysis has been undertaken. Once a genetic mutation has been identified and confirmed in a subject/family with a history of RDD, the delivery of results will be as follows:

Sister Bartmann will contact the family (individual or principal family member) by telephone, followed by a letter, informing them that the disease-causing mutation has been found in their family. Included with this letter will be the option to receive their detailed results. Their signature on a consent form will be required along with details of the clinician/ophthalmologist of their choice who they wish to have deliver their results to them. The patient thereby gives us consent to release their molecular diagnostic results to the clinician/ophthalmologist/genetic counsellor as stated on the form.

Professor Greenberg will contact the professional of the individual's choice and send the results to her/him. Attached to this will be a letter of recommendation regarding the delivery of the genetic test results, counsellor information for post-result follow-up, together with references, where applicable. Should there be any need to study additional family members, this will be discussed with the clinician and a course of action will be decided upon as to who makes the contact and what the family is told.

The individual or principal family member will be contacted by their managing clinician and personally given the genetic result. Thereafter, the family members who would like to receive their results need to contact this division or their doctor. Sister Bartmann will follow-up within a month of the sending the report to ascertain what has transpired. A plan of action as to how the results are delivered and whether predictive counselling is required will depend on the nature of the results and also the decision of who else in the family will and should be told. This will be discussed with the individual and decided upon, in consultation with the clinician/counsellor. **The individual retains the right at all times to either receive or refuse the results.**

All costs involved in this process of informing subjects of their molecular results ought to be borne by the participants.

All genetic information is regarded as strictly confidential. Only information relating to the potential genetic cause of the inherited RDD will be released to the individual concerned, personally. No other genetic information or family information produced by this study will be released to any one else, including other family members. At this stage, it needs to be stressed and understood that no additional treatments will be available as a result of these test results.

Participants need to be informed that there is no financial compensation, commercial benefits or other rewards for participation in this programme. There may be no direct benefit as a participant other than the possible medical advances and greater understanding of the genetic condition in the family that may result if causative genes are found.

Individuals will be informed and assured that only other qualified research investigators may study their DNA samples, if necessary, but only for the purpose of further investigations of genetic factors causing inherited retinal eye disease.
1/11/2004

# REQUEST FOR MOLECULAR STUDIES (DNA)

**Molecular Laboratory**
**Division of Human Genetics**
**1st Floor, Anatomy Building,**
**Faculty of Health Sciences,**
**UCT , Observatory 7925**

**Tel: (021) 406-6425 Fax: (021) 448-0906**

10ml blood should be drawn in 5ml EDTA Tubes

(Purple top)

Each tube should be inverted to mix and should be clearly labelled with the patient's name and DOB

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

**Please DO NOT send specimens on ice or frozen.**

***Please fill in all the information requested:***

SURNAME:_____ FIRST NAMES:_____

NEW FAMILY: YES ☐ NO ☐ (IF NO, PLEASE FILL IN FAMILY NAME) FAMILY NAME: _____

MEDICAL AID: _____MEDICAL AID NO: _____

SEX: MALE ☐ FEMALE ☐ DATE OF BIRTH: YEAR:_____ MONTH: _____ DAY: _____

NUMBER OF CHILDREN: _____

ETHNIC ORIGIN : (AS IN ANCESTRAL, AS DETAILED AS POSSIBLE)_____

CONTACT ADDRESS: _____ FAX: TOWN: _____ TEL: _____

REFERRING DOCTOR/SISTER: _____ FAX: TOWN: _____ TEL: _____

HOSPITAL OR ADDRESS: _____ FAX: TOWN: _____ TEL: _____

**REASON FOR REFERRAL (CLINICAL DIAGNOSIS):**

AFFECTED ☐ AT RISK ☐ CARRIER ☐ SPOUSE ☐ QUERY ☐ UNAFFECTED ☐

| | | | | | |
|---|---|---|---|---|---|
| RETINITIS PIGMENTOSA | ☐ | USHER SYNDROME | ☐ | DOMINANT INHERITANCE | ☐ |
| STARGARDT DISEASE | ☐ | ONSET AGE | ☐ | RECESSIVE INHERITANCE | ☐ |
| MACULAR DYSTROPHY | ☐ | DIAGNOSTIC AGE | ☐ | X-LINKED INHERITANCE | ☐ |

ISOLATED CASE ☐

ADDITIONAL FAMILY HISTORY _____

ADDITIONAL DISORDERS (APPARENT OR PREVIOUSLY TREATED): _____

**RELEVANT CLINICAL DETAILS:**

PHYSICAL DISABILITY ☐ MENTAL RETARDATION ☐ DEAFNESS ☐ IMPAIRED VISION ☐

NIGHT BLINDNESS ☐ OTHER: _____

HAVE SAMPLES FROM THIS PATIENT BEEN SENT TO A DNA LAB BEFORE? YES / NO / DON'T KNOW
(DELETE WHERE NOT APPLICABLE)

IF YES, WHERE: _____

***For Laboratory use only:***

*DNA number:* _____ *Vol.Blood:* _____ *(ml)* *Other:* _____

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

# CONSENT FOR DNA ANALYSIS AND STORAGE

1. I, _____ , request that an attempt be made using genetic material to assess the probability that:
I / my child / my unborn child (DELETE WHERE NOT APPLICABLE)
might have inherited a disease-causing mutation in the gene for:

_____

2. I understand that the genetic material for analysis is to be obtained from:
blood cells/skin sample/other (specify) (DELETE WHERE NOT APPLICABLE)

3. I request that **no** portion of the sample be stored for later use. ☐ (MARK IF APPLICABLE )
Or
I request that a portion of the sample be stored indefinitely for (DELETE WHERE NOT APPLICABLE)
( a ) possible re-analysis
( b ) analysis for the benefit of members of my immediate family
( c ) research purposes, subject to the approval of the University of Cape Town Research Ethics Committee, provided that any information from such research will remain confidential.

4. The results of the analysis carried out on this sample of stored biological material will be made known to me, via my doctor, in accordance with the relevant protocol, if and when available.
In addition, I authorise that they may be made known to: (DELETE WHERE NOT APPLICABLE)

(a) other doctors involved in my care _____

(b) the following family members: _____

other:_____

5. I authorise / do not authorise my doctor(s) (DELETE WHERE NOT APPLICABLE)
to provide relevant clinical details to the Division of Human Genetics, UCT.

6. I have been informed that:
(a) There are risks and benefits associated with genetic analysis and storage of biological material and these have been explained to me.
(b) The analysis procedure is specific to the genetic condition mentioned above and cannot determine the complete genetic makeup of an individual.
(c) The genetics laboratory is under an obligation to respect medical confidentiality. .
(d) Genetic analysis may not be informative for some families or family members.
(e) Even under the best conditions, current technology of this type is not perfect and could lead to incorrect results.
(f) Where biological material is used for research purposes, there may be no direct benefit to me.

7. I understand that I may withdraw my consent for any aspect of the above at any time without this affecting my future medical care.

8. **ALL OF THE ABOVE HAS BEEN EXPLAINED TO ME IN A LANGUAGE THAT I UNDERSTAND AND MY QUESTIONS ANSWERED BY:**

**Signature**_____ **Date:** _____

**Patient signature** _____ **Witnessed consent**_____

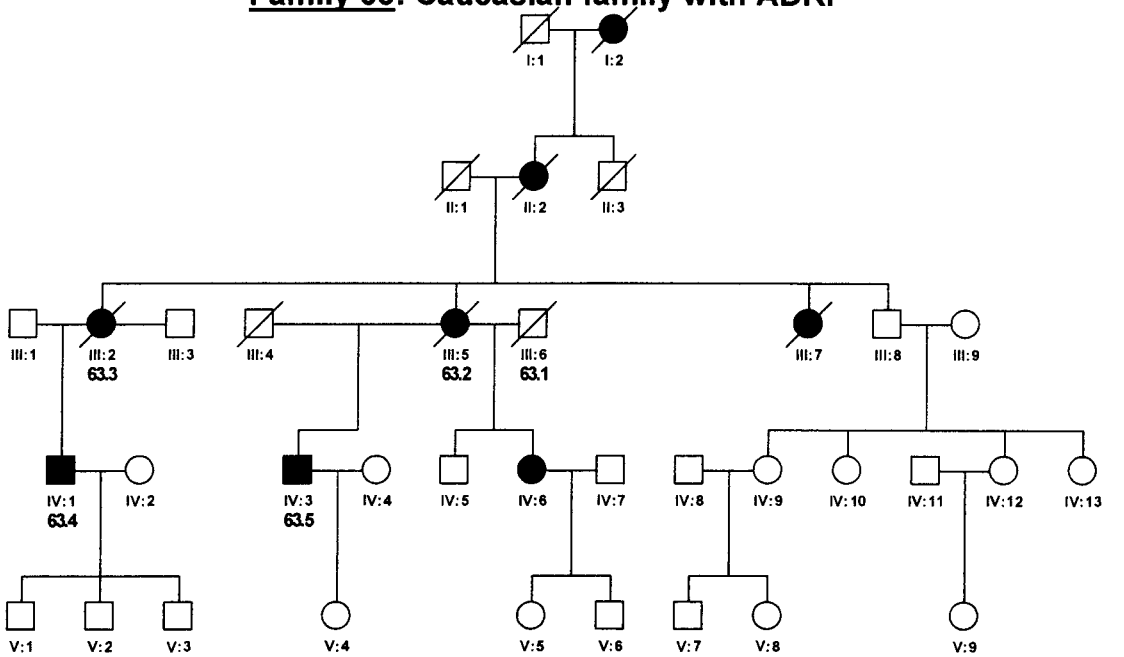
*NOTE - PLEASE INSERT A FAMILY PEDIGREE DRAWING ON THE REVERSE OF THIS FORM*

# Appendix 4: Pedigrees and clinical information

In the pedigrees shown, standard symbols are used - squares represent males, circles represent females, and a diamond annotated "SB" indicates a stillbirth. Shaded symbols indicate affected individuals, and a symbol with a line through it indicates that the individual is deceased. Siblings connected by diagonal lines represent twins. Marriage lines with 2 lines crossing through them indicate a divorce, and double marriage lines indicate a consanguineous marriage.

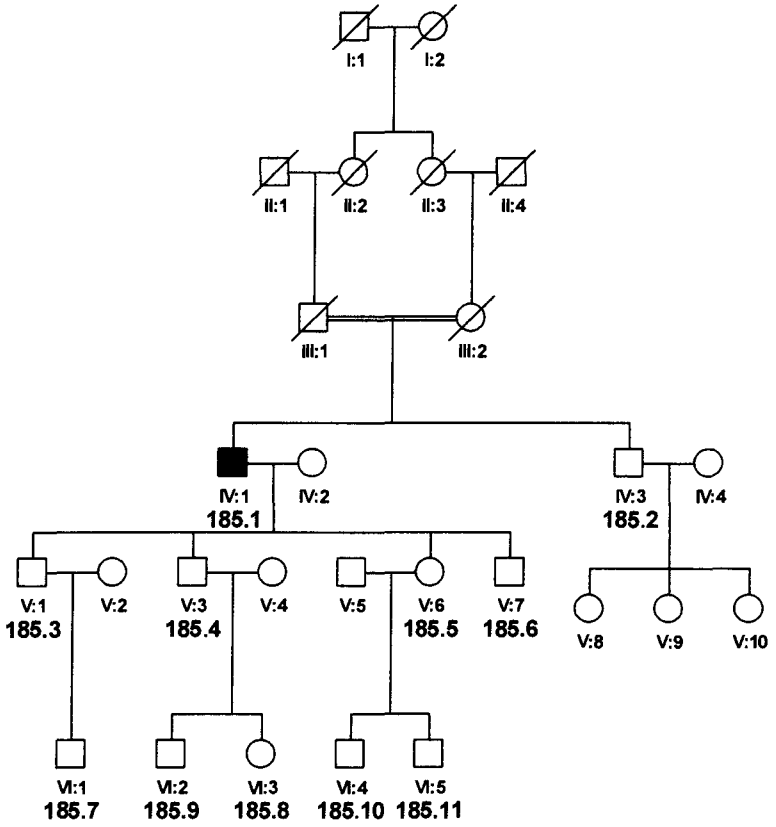
Visual acuities are described in the metric system. A visual acuity of 6/6 indicates that the individual can see detail from the same distance as a person with normal sight. The lowest measured visual acuity is usually 6/60, indicating that the individual can see detail from 6 metres which a person with normal sight could see at 60 metres. If visual acuity is lower than 6/60, the descriptions are given as (in order of severity): counting fingers, hand motions, light perception and no light perception (total blindness).

**Family 63: Caucasian family with ADRP**



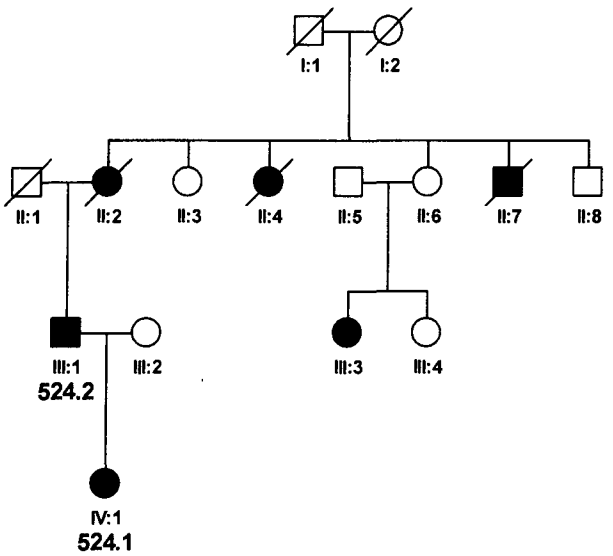

| Individual | Mutation | Current Age | Age of Onset | Visual Acuity | Other clinical information |
|---|---|---|---|---|---|
| 63.1 | None | Deceased | | | |
| 63.2 | Heterozygous *RHO* Thr17Met | Deceased | | | |
| 63.3 | Not tested | Deceased | 16 | | Nightblindness noticed when young, subsequent central vision loss, very poor vision at age 77. |
| 63.4 | Heterozygous *RHO* Thr17Met | 66 | 20 | | Diffuse RP, nightblindness, restricted visual field and myopia, loss of central vision in left eye at age 64. Vitamin A therapy from 1999-2006, vision stable during this time. |
| 63.5 | Not tested | 64 | | | |

## Family 185: Caucasian family with ARRP



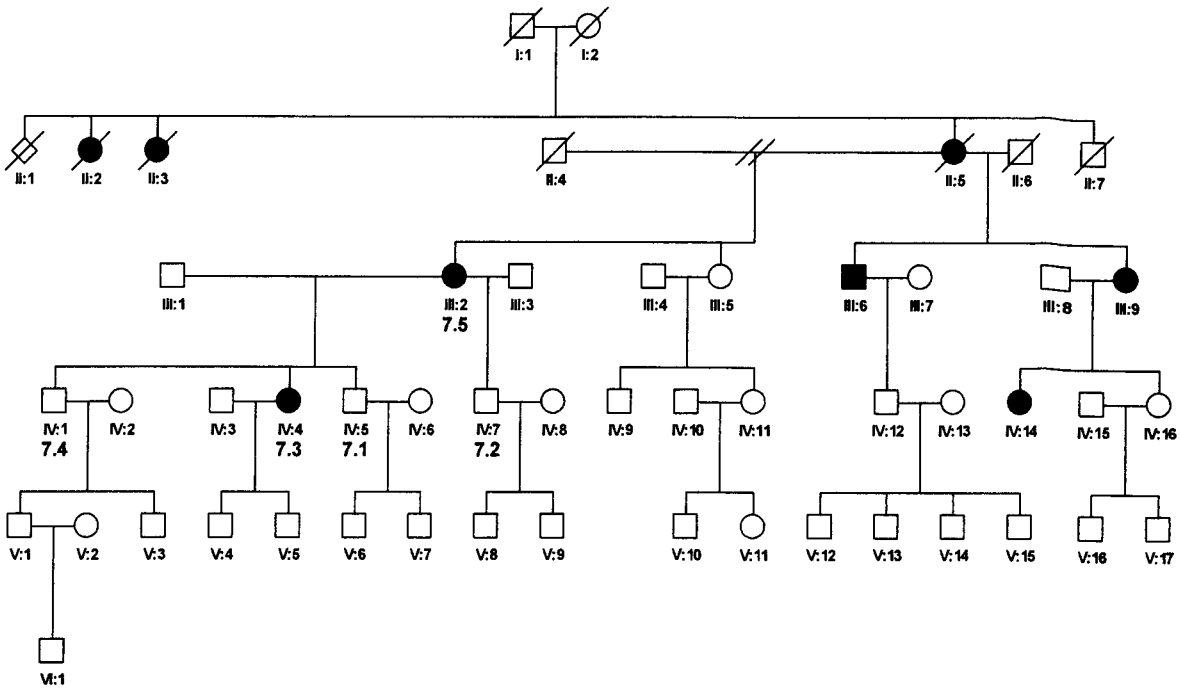

| Individual | Mutation | Current Age | Age of Onset | Visual Acuity | Other clinical information |
|---|---|---|---|---|---|
| 185.1 | Homozygous *RHO* IVS4+1G>T | 74 | 10 | | |
| 185.2 | Heterozygous *RHO* IVS4+1G>T | 66 | No apparent manifestation | | |
| 185.3 | Heterozygous *RHO* IVS4+1G>T | 49 | No apparent manifestation | | |
| 185.4 | Heterozygous *RHO* IVS4+1G>T | 47 | No apparent manifestation | | |
| 185.5 | Heterozygous *RHO* IVS4+1G>T | 45 | No apparent manifestation | | |
| 185.6 | Heterozygous *RHO* IVS4+1G>T | 39 | No apparent manifestation | | |
| 185.7 | None | 15 | | | |
| 185.8 | None | 19 | | | |
| 185.9 | Heterozygous *RHO* IVS4+1G>T | 18 | No apparent manifestation | | |
| 185.10 | Heterozygous *RHO* IVS4+1G>T | 18 | No apparent manifestation | | |
| 185.11 | Heterozygous *RHO* IVS4+1G>T | 15 | No apparent manifestation | | |

## Family 524: Caucasian Family with ADRP



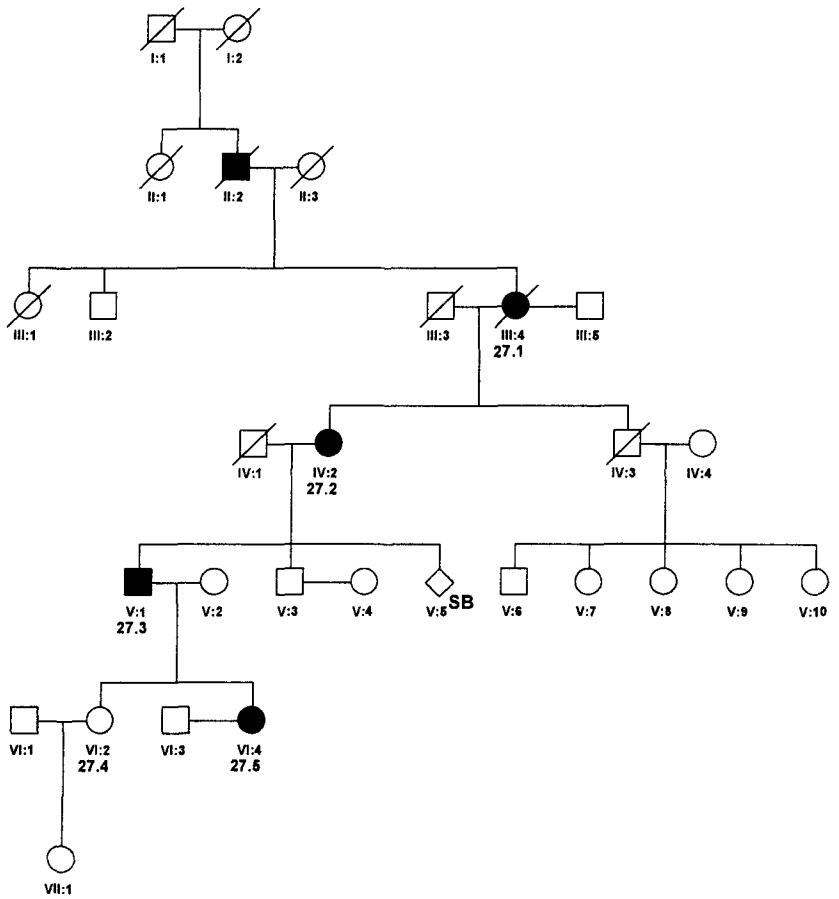

| Individual | Mutation | Current Age | Age of Onset | Visual Acuity | Other clinical information |
|---|---|---|---|---|---|
| 524.1 | Heterozygous *RP1* Arg677X | 32 | Early twenties | Age 22: 6/6<br>Age 27: 6/7.5 | Diffuse RP. |
| 524.2 | Heterozygous *RP1* Arg677X | 59 | 34 | Age 48: 6/24<br>Age 53: hand movements (both eyes) | Diffuse RP with macular oedema, rapid progression with the onset of macular degeneration, cataracts, waxy pallor of discs, attenuated vessels, extensive distribution of bone spicule in retinal periphery, vitreal floaters. |

## Family 7: Caucasian family with ADRP



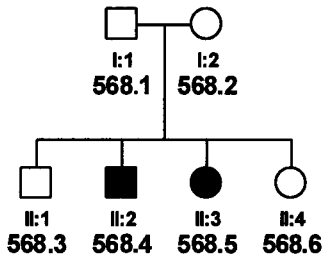

| Individual | Mutation | Current Age | Age of Onset | Visual Acuity | Other clinical information |
|---|---|---|---|---|---|
| 7.1 | Heterozygous *RP1* Ser911X | 41 | No apparent manifestation | | |
| 7.2 | Heterozygous *RP1* Ser911X | 35 | No apparent manifestation | | |
| 7.3 | Heterozygous *RP1* Ser911X | 44 | 19 | | Nightblindness began at age 19, followed by loss of visual fields in early 20s. |
| 7.4 | None | 48 | Not applicable | | |
| 7.5 | Heterozygous *RP1* Ser911X | 69 | About 40 | Age 64: 6/18 (left eye) 6/9 (right eye) Age 68: 6/60 (Both eyes); improved after 6 months on Diamox to 6/21 (left eye) 6/15 (right eye) | Early onset of nightblindness, diffuse RP, cystoid macular oedema, mild nuclear sclerosis, pale and waxy discs, bone spicule scattered peripherally in the retina, floaters and asteroid hyalosis of the vitreous. |

## Family 27: Caucasian family with ADRP



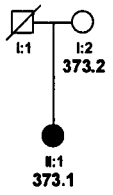

| Individual | Mutation | Current Age | Age of Onset | Visual Acuity | Other clinical information |
|---|---|---|---|---|---|
| 27.1 | Heterozygous *RP1* 2590-2599del ATAACTTTAA | Deceased | Thirties | | Nightblindness began in thirties, peripheral vision poor at age 48, unable to read at age 80. |
| 27.2 | Heterozygous *RP1* 2590-2599del ATAACTTTAA | 72 | 17 | | Nightblindness began at age 17, by the age of 40 peripheral vision was very poor, hyperopia at age 60. |
| 27.3 | Heterozygous *RP1* 2590-2599del ATAACTTTAA | 52 | 10 | Age 50: 6/9 (left eye) 6/15 (right eye) | Diffuse RP with slow progression, cataracts, waxy pallor of the discs, attenuated vessels, granularity of the macula, bleakish fovea, bone spicule and white dots on retina, no cellularity or vitreous floaters. |
| 27.4 | Heterozygous *RP1* 2590-2599del ATAACTTTAA | 29 | No apparent manifestation | | Retinoblastoma in one eye at 6 months of age. |
| 27.5 | Heterozygous *RP1* 2590-2599del ATAACTTTAA | 27 | 24 | | Nightblindness, diffuse RP with slow progression, waxy pallor of discs, attenuated vessels, normal macula and fovea, occasional and diffuse pigment in the periphery of the retina, bone spicule, normal vitreous. |

## Family 568: Indian family with early onset ARRP



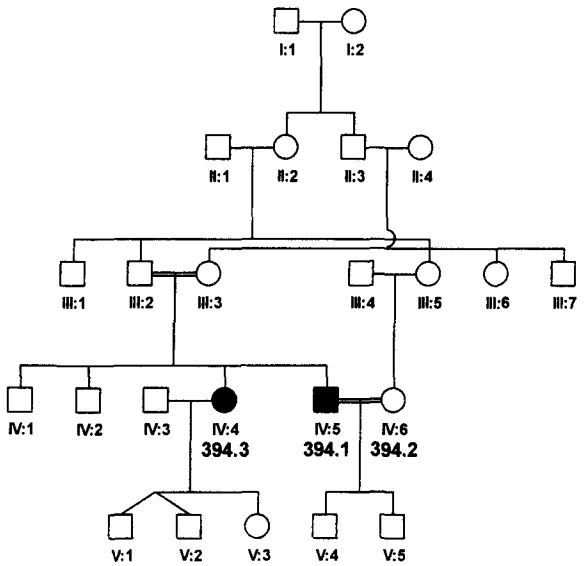

| Individual | Mutation | Current Age | Age of Onset | Visual Acuity | Other clinical information |
|---|---|---|---|---|---|
| 568.1 | Heterozygous *RPE65* IVS1+1G>T | 55 | No apparent manifestation | | |
| 568.2 | Heterozygous *RPE65* Tyr249Cys + Ala132Thr | 55 | No apparent manifestation | | |
| 568.3 | Heterozygous *RPE65* IVS1+1G>T | 32 | No apparent manifestation | | |
| 568.4 | Compound Heterozygote *RPE65* IVS1+1G>T; Tyr249Cys + Ala132Thr | 29 | 2 months | Age 27: Light perception | |
| 568.5 | Compound Heterozygote *RPE65* IVS1+1G>T; Tyr249Cys + Ala132Thr | 26 | 2 | Age 25: 6/60 (left eye) 6/120 (right eye) | Photophobia, Diffuse RP, waxy pallor of discs, attenuated arteries, bony spicules, markedly thinned retinas, atrophic macula, rapid deterioration of vision from the age of 7, Keratoconus, ERG at age 22 shows advanced rod and cone dysfunction. Vision appeared stable at age 26. |
| 568.6 | None | 15 | | | |

## Family 373: Caucasian family with early onset ARRP



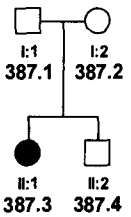

| Individual | Mutation | Current Age | Age of Onset | Visual Acuity | Other clinical information |
|---|---|---|---|---|---|
| 373.1 | Compound Heterozygote *RPE65* Glu21Lys; Leu22Pro | 44 | 3 | Age: 36: 6/15 (left eye) 6/24 right eye Age 38: 6/30 (left eye) 6/40 (right eye) | High myopia and nightblindness at age 3, Flat rod and cones ERG response by age 8. Progressive central vision loss at age 37 with typical waxy white discs, attenuated vessels, choroidal atrophy of the right macula, singular bony spicules and significant white punctuate lesions. Cataracts extracted at age 38. |
| 373.2 | Heterozygous *RPE65* Glu21Lys | No apparent manifestation | | | |

## Family 394: Indian family with early onset ARRP



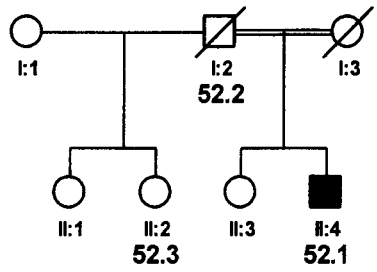

| Individual | Mutation | Current Age | Age of Onset | Visual Acuity | Other clinical information |
|---|---|---|---|---|---|
| 394.1 | Heterozygous *RPE65* Ala132Thr | 31 | 5 | Age 29: 6/120 (left eye) 6/72 (right eye) | Glaucoma at age 29. |
| 394.2 | None | 29 | No apparent manifestation | | |
| 394.3 | Heterozygous *RPE65* Ala132Thr | 35 | 8 | Age 32: 6/18 (left eye) 6/70 (right eye) | Diffuse RP, Less than 10° visual fields left at age 32, slow progression in the last 10 years. Cataracts. |

## Family 387: Caucasian family with early onset ARRP




| Individual | Mutation | Current Age | Age of Onset | Visual Acuity | Other clinical information |
|---|---|---|---|---|---|
| 387.1 | Heterozygous *RPE65* Ala132Thr | 49 | No apparent manifestation | | |
| 387.2 | None | 44 | | | |
| 387.3 | Heterozygous *RPE65* Ala132Thr | 18 | 2 | Age 4: 6/108 (left eye) 6/36 (right eye) Age 16: 6/60 (both eyes) | Markedly abnormal (flat) ERG at age 2, less than 5° visual field at age 16. |
| 387.4 | Heterozygous *RPE65* Ala132Thr | 14 | No apparent manifestation | | |

## Family 52: Mixed Ancestry family with early onset ARRP




| Individual | Mutation | Current Age | Age of Onset | Visual Acuity | Other clinical information |
|---|---|---|---|---|---|
| 52.1 | Homozygous *RPE65* Tyr368His | 49 | 4 | Age 36: 6/60 (left eye), counting fingers (right eye) | Early loss of fields, loss of colour vision, RP tigroid fundus, drusen in macula, white dots, waxy pallor, attenuated vessels, choroidal atrophy. |
| 52.2 | Heterozygous *RPE65* Tyr368His | Deceased | No apparent manifestation | | |
| 52.3 | None | 69 | | | |

# Appendix 5: Publications generated by this work

All the bench work described in all four publications is the sole work of the candidate.
Publications one and four were written by the candidate, and significant input to the writing of publications two and three was made by the candidate.

Clin Genet 2000: 58: 77–78
Printed in Ireland. All rights reserved


CLINICAL GENETICS
ISSN 0009-9163

## Letter to the Editor

# Low frequency of rhodopsin mutations in South African patients with autosomal dominant retinitis pigmentosa

*To the Editor:*

Retinitis pigmentosa (RP) is a group of hereditary retinal degenerative disorders characterised by night-blindness and gradual constriction of the visual fields, resulting in partial or total blindness. The reported frequency of RP is about 1 in 3000 (1); however, in South Africa (SA) no accurate incidence of the condition is known (2).

Localisation of the first autosomal dominant retinitis pigmentosa (ADRP) locus was reported in 1989 (3). Mutations in the rhodopsin gene were subsequently identified in many patients with RP (4). Thus far, the reported frequencies of rhodopsin mutations in ADRP patients throughout the world range from 20 to 31%, while in the UK, the figure appears to be as high as 50% (5). Of particular note was the observation that one single base-change (Pro23His) correlated with the disease in about 12% of US ADRP patients. This mutation has not been reported in such a high frequency in other populations and is believed to represent a founder effect for RP in the US, originating from the UK (6).

More than 80 different mutations in the rhodopsin gene have been identified in RP patients (1); however, only two mutations have been reported in multiple populations: the Pro-347-Leu amino acid substitution has been reported in the USA, UK, Germany and Japan; and the Thr-58-Arg substitution in the USA and UK. It has thus been suggested that these might possibly be mutation hotspots in the rhodopsin gene (5). The codon 347 mutation (CCG-CTG) destroys an *Msp*I site, while the codon 58 mutation (ACG-AGG) creates a *Dde*I restriction site (7). It was thus proposed that screening AD families (especially sectorial RP cases), as well as simplex cases of RP, using a simple restriction enzyme digest assay, would be a worthwhile approach. Such a screen of 120 patients in the UK revealed about 6% of ADRP patients with the aforementioned mutations (5).

In the study reported here, a total of 120 SA patients with retinal degeneration were screened for the loss of an *Msp*I restriction site, or the gain of a *Dde*I restriction site. Sixty of these patients had a family history indicating dominant RP, while 60 were sporadic cases. One ADRP patient was found to carry the codon 347 mutation, and another, the codon 58 mutation. The respective extended family members available for study were then examined with the same rapid screen approach and the disease-causing mutation was confirmed in each family. This detection of only two rhodopsin mutations in 60 ADRP individuals yields a frequency of 3.3%. The individual found to carry the codon 347 mutation is of black African origin and this was the first report of a disease-causing rhodopsin mutation in an indigenous black African family with RP (7). Interestingly, the patient of Caucasian origin found to carry the codon 58 mutation exhibits diffuse retinal degeneration.

Mutation detection screening has also been undertaken using all five exons of the rhodopsin gene and single stranded conformational polymorphism analysis (SSCP). To date, 227 individuals have been studied, of whom 60 have a family history indicating dominant RP. Only four rhodopsin disease-causing mutations have been identified in this patient cohort (8). These mutations include a novel disease-causing mutation in exon one of the rhodopsin gene in one SA ADRP family (9), as well as a known codon 190 mutation (unpublished data). Of the 60 ADRP families from whom DNA was available for this study, only ten are of indigenous black African origin, whereas 45 are of Caucasian origin and the rest, of mixed ancestry. Total ascertainment of the prevalence of RP in SA is incomplete and it must be emphasised that the figures quoted here are not necessarily a reflection of an absolute population prevalence of the condition in this country.

In summary, this study supports the findings of Tarttelin et al., that ADRP families can be rapidly and efficiently assessed for two common rhodopsin disease-causing mutations (5). In addition, we also report a lower incidence (3.3%) of these two muta-

tions in this cohort of SA ADRP patients, as well as a relatively low frequency (6.6%) of rhodopsin mutations, in general, identified in SA patients with ADRP.

## Acknowledgements

This research was supported by grants from the RP Foundation of South Africa and the Foundation Fighting Blindness (USA). We are most grateful to Sister Lecia Bartmann for her efforts in tracing family members and the Genetic Sisters of the Department of National Health for visiting family members and collecting blood. We are indebted to the SA RP family members for their participation in the study. We are grateful to Professor T. Dryja for assistance and advice with the exon 5 rhodopsin primers.

*L Roberts*
*R Ramesar*
*J Greenberg*

## References

1. Sullivan LS, Daiger SP. Inherited retinal degeneration: exceptional genetic and clinical heterogeneity. Mod Med Today 1996: 2 (Sept): 380–386.
2. Greenberg J, Bartmann L, Ramesar R, Beighton P. Retinitis pigmentosa in Southern Africa. Clin Genet 1993: 44: 232–235.
3. McWilliam P, Farrar J, Kenna P et al. Autosomal dominant retinitis pigmentosa (ADRP): localisation of an ADRP gene to the long arm of chromosome 3. Genomics 1989: 5: 619–622.
4. Dryja TP, McGee TL, Reichel E et al. A point mutation of the rhodopsin gene in one form of retinitis pigmentosa. Nature 1990: 343: 364–366.
5. Tarttelin E, Al-Maghtheh M, Keen J, Bhattacharya S, Ingelhearn C. Simple tests for rhodopsin involvement in retinitis pigmentosa. J Med Genet 1996: 33: 262–263.
6. Dryja TP, McGee TL, Reichel E et al. Mutations within the rhodopsin gene in patients with autosomal dominant retinitis pigmentosa. New Engl J Med 1990: 323 (19): 1302–1307.
7. Greenberg J, Franz T, Goliath R, Ramesar R. A photoreceptor gene mutation in an indigenous black African family with retinitis pigmentosa using a rapid screening approach for common rhodopsin mutations. South Afr Med J 1999: 89 (8): 877–878.
8. Greenberg J, Rebello G, Ramesar R. Ophthalmic Genetics: A review of the Molecular Genetics of Familial Retinal Dystrophies in Southern Africa. Specialist Medicine 1999: 21:2: 108–112.
9. Goliath R, Bardien S, September A, Martin R, Ramesar R, Greenberg J. Rhodopsin mutation Gly109Arg in a family with autosomal dominant retinitis pigmentosa. Hum Mutat 1998: Supplement 1: 40–41.

*Correspondence:*
Jacquie Greenberg
Human Genetics Laboratory
UCT Medical School
First Floor, Anatomy Building
Observatory
South Africa 7925
Tel.: + 21-406-6299
Fax: + 21-448-0906
E-mail: jg@anat.uct.ac.za

New insights into retinal degenerative diseases. Proceedings of the 9th International Symposium on Retinal Degeneration. 2000. Durango, CO: Kluwer Academic/Plenum Publishers. pp 329-333

# UNUSUAL FREQUENCIES OF RHODOPSIN MUTATIONS AND POLYMORPHISMS IN SOUTHERN AFRICAN PATIENTS WITH RETINITIS PIGMENTOSA

Jacquie Greenberg, Lisa Roberts and Rajkumar Ramesar[1]

## 1. INTRODUCTION

The average adult eye contains approximately 92 000 000 rods and 5 000 000 cones distributed throughout the retina. Cone cells function in colour perception, while rod cells are required for vision in low light. Degeneration of primarily the rod photoreceptor cells is the cause of Retinitis Pigmentosa (RP). This is a group of hereditary disorders characterised by night-blindness and gradual constriction of the visual fields, resulting in partial or total blindness. RP can be inherited in dominant, recessive, X-linked, mitochondrial or digenic modes. It may also present as part of a syndrome.[1]

The first autosomal dominant retinitis pigmentosa (ADRP) locus was mapped to the long arm of chromosome 3 in 1989.[2] Shortly thereafter, a disease-causing mutation in the rhodopsin (RHO) gene, which also maps to the long arm of chromosome 3, was identified in a patient with ADRP.[3] RHO encodes a G-protein coupled receptor (GPCR) which acts in the first stage of the signal transduction cascade in rods, mediating vision in dim light. Mutations in RHO have been reported as the most common cause of RP and account for 20-30% of ADRP cases,[4] although this figure could be as high as 50%, as has been reported in the UK.[5] About 100 different mutations in RHO have been identified in RP patients around the world.[6] The majority of these are in ADRP families, but also include two mutations known to cause recessive RP.[7]

Two mutation hotspots have been described in the RHO gene. Pro-347-Leu has been seen in patients from the US, UK, German and Japan and Thr-58-Arg has been reported in ADRP families from the US and UK. It has been proposed that screening these hotspots using a simple restriction enzyme-based assay (the rapid screen) in cases of sporadic RP and ADRP would be a worthwhile approach.[5]

[1] Department of Human Genetics, UCT Medical School, Observatory, South Africa.

## 2. METHOD

For a mutation screening programme, six pairs of primers were designed to cover the 5 exons of RHO, including the intron/exon boundaries[8] (table 1).

**Table 1.** Primer sequences used for mutation screening of the RHO gene.

| Primer | Primer Sequence (5' → 3') |
|---|---|
| Exon 1a (fwd) | ttc gca gca ttc ttg ggt gg |
| Exon1a (rev) | agc agg atg tag ttg aga gg |
| Exon1b (fwd) | caa ctt cct cac gct cta cg |
| Exon 1b (rev) | cat tga cag gac agg aga ag |
| Exon 2(fwd) | ccg cct gct gac tgc ctt gca g |
| Exon 2(rev) | gct tct tcc ctt ctg ctc agt g |
| Exon 3 (fwd) | ttg gct gtt ccc aag tcc ct |
| Exon 3 (rev) | tcc aga cca tgg ctc ctc ca |
| Exon 4 (fwd) | tca cgg ctc tga ggg tcc ag |
| Exon 4 (rev) | gag tag ctt gtc ctt ggc ag |
| Exon 5 (fwd) | act caa gcc tct tgc ctt cc |
| Exon 5 (rev) | gcc aca gag tcc tag gca gg |

### 2.1. The Rapid Screen

Primers for exons 1a and 5 were used for the 2 hotspot mutations:

The Pro-347-Leu mutation (CCG-CTG) in exon 5 destroys a *Msp1* restriction site, and the Thr-58-Arg mutation (ACG-AGG) in exon 1a creates a *Dde1* restriction site. (Boehringer Mannheim restriction enzymes were used.) The amplification of exons 1a and 5 was followed by restriction enzyme digestion, and electrophoresis on 4% agarose gels visualised with ethidium bromide staining.

The rapid screen was used to test 152 patients for the 2 hotspot mutations. Eighty of these patients had a family history indicating ADRP, while 72 were sporadic cases.

### 2.2. SSCP Analysis

Mutation detection screening was undertaken using all 5 exons of the Rhodopsin gene and single stranded conformational polymorphism analysis (SSCP). Two SSCP gel conditions were used for each exon to ensure reproducibility and high confidence levels. Polymorphisms or disease-causing mutations detected in this manner were sequenced either manually or on an ABI 377 Automated Sequencer (using the PE BIOSYSTEMS BigDye™ Terminator Cycle Sequencing kit), and sequence changes were confirmed with restriction enzyme digestion.

The SSCP method was used to test 60 South African individuals with a family history indicating ADRP.

## 3. RESULTS

A total of four disease-causing mutations have been identified in the cohort of SA ADRP patients screened to date (Table 2).

**Table 2.** Disease-causing mutations in the RHO gene in South African ADRP patients.

| Exon | Mutation | Base change | RFLP |
|---|---|---|---|
| 1a | Thr58Arg | C to G | +*Dde1* |
| 1b | Gly109Arg | G to A | -*Fok1* |
| 3 | Asp190Asn | G to A | -*Rsa1* |
| 5 | Pro347Leu | C to T | -*Msp1* |

Two of these mutations were detected by the rapid screen; one codon 347 mutation, and one codon 58 mutation.

In addition, a donor splice site mutation in intron 4 was detected in an individual with a family history indicating recessive RP. This G to T transversion at position 4335 was present in the homozygous state as confirmed by the loss of a *Ban1* restriction site.

Furthermore, an unusually high frequency of a polymorphism in the 5' non-coding region of RHO was detected. This single nucleotide substitution of a G for an A at position 269 of RHO can be confirmed by the creation of a *Ksp1* site.

A total of 71 patients with RP, as well as 54 known ADRP patients, were investigated for the presence of the A269G polymorphism (table 3). In addition, 53 unaffected unrelated Caucasian controls were studied, as well as 54 indigenous black African controls (Figure1).

**Table 3**. Frequency of A269G in affected and unaffected population groups

| Phenotype | No. individuals | No. Black individuals | Heterozygotes (%) | Homozygotes (%) |
|---|---|---|---|---|
| RP | 71 | 3 | 37 | 10 |
| ADRP | 54 | 10 | 26 | 20 |
| Caucasian Controls | 53 | - | 30 | 4 |
| Black Controls | 54 | - | 15 | 79 |

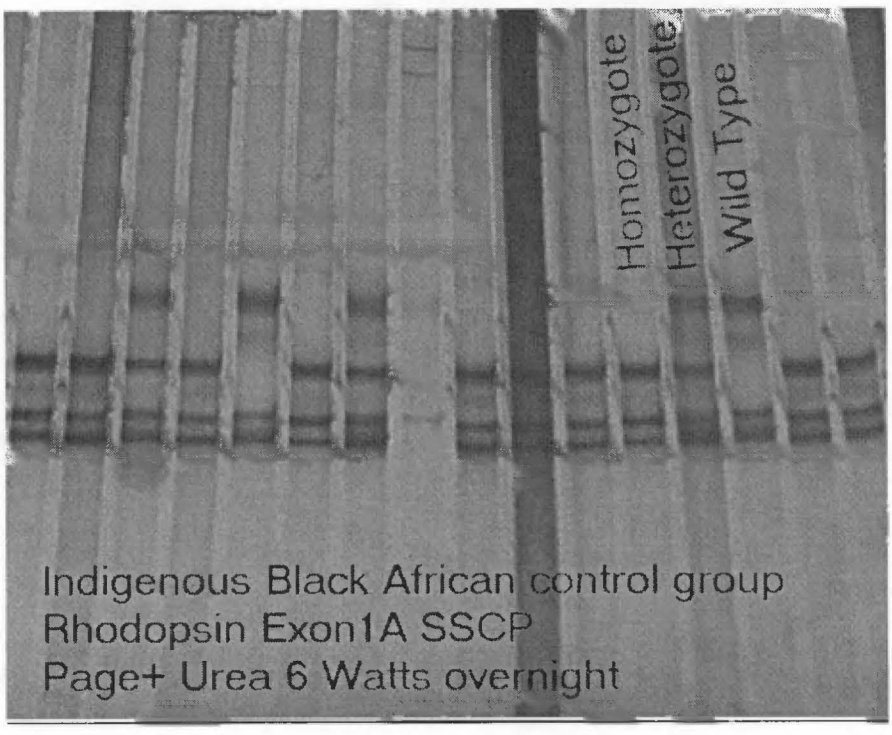



**Figure 1.** Digital image of banding pattern of A269G polymorphism detected on SSCP.

## 4. DISCUSSION

The rapid screen resulted in the detection of only 2 rhodopsin mutations in 80 ADRP individuals (2.5%). A similar screen of 120 patients in the UK revealed that 6% of ADRP patients carried the codon 347 or codon 58 mutations.[5] The one SA ADRP patient found to carry the codon 347 mutation is of black African origin. This was the first report of a disease-causing RHO mutation in an indigenous black African family with RP.[9,10]

Only 4 mutations (including the novel Gly109Arg mutation[11]) have been identified in a cohort of 60 ADRP patients in whom the full RHO gene was screened, using SSCP analysis and the rapid screen. This reflects a low frequency of RHO mutations in SA ADRP patients (6.6%) when compared to 50% in the UK[5] and 20-30% globally.[4]

In addition, a homozygous donor splice site mutation was found in intron 4 of a patient with a family history indicating recessive RP. There has been some discussion as to whether this mutation results in a dominant or recessive allele,[12] but to our knowledge, it has never been reported in the homozygous state. Further investigation of extended family members is being undertaken.

Finally, an unusually high frequency of the A269G polymorphism in the 5' non-coding region of the RHO gene was identified. This polymorphism was detected in 46% of SA ADRP patients, as opposed to 14% of a similar cohort reported in the US.[13] Interestingly, not only is the frequency high in SA Caucasian controls (34%), but it is present in 94% of indigenous black African unaffected, unrelated individuals. The significance of this high frequency should be investigated, as polymorphisms have been found to be extremely useful in studies of protein structure and function. RHO is thought to constitute up to 85% of the total protein present in the rod outer segment,[6] implying a structural role which may be functionally affected by this polymorphism.

These unusual findings together with the two novel ADRP genes that were localised in SA (RP13 and RP17 in 1994 and 1995 respectively)[14,15] clearly emphasise the genetic heterogeneity of Retinal Degenerative Diseases (RDD). In addition, the 'molecular demography' for RDDs in SA may be different from other international study sites, and future genetic analyses could yield further interesting findings.

## 5. ACKNOWLEDGEMENT

This research was supported by grants from the Retinal Preservation Foundation of South Africa and the Foundation Fighting Blindness (USA). We are most grateful to Sister Lecia Bartmann for her efforts in tracing family members and the Genetic Sisters of the Department of National Health for visiting family members throughout the country and collecting blood. We are indebted to the SA RP family members for their participation in the study. We are grateful to Professor T. Dryja for assistance and advice with the exon 5 rhodopsin primers.

## 6. REFERENCES


1. G. Clarke, E. Heon, and R. R. McInnes, Recent Advances in the molecular basis of inherited photoreceptor degeneration, *Clin. Genet.* **57**: 313-329 (2000).
2. P. McWilliam, J. Farrar, P. Kenna, D. G. Bradley, M. M. Humphries, E. M. Sharp et al., Autosomal Dominant Retinitis Pigmentosa (ADRP): localisation of an ADRP gene to the long arm of chromosome 3, *Genomics* **5**: 619-622 (1989).
3. T. P. Dryja, T. L. McGee, E. Reichel, L. B. Hahn, G. S. Cowley, D. W. Yandell et al., A point mutation of the rhodopsin gene in one form of retinitis pigmentosa, *Nature* **343**:364-366 (1990).
4. J. Phenan, and D. Bok, A brief review of retinitis pigmentosa and the identified retinitis pigmentosa genes, Mol. Vis. **6**: 116-124 (2000). http://www.molvis.org/molvis/v6/216/.
5. E. Tarttelin, M. Al-Maghtheh, J. Keen, S. Bhattacharya, and C. Ingelhearn, Simple tests for rhodopsin involvement in retinitis pigmentosa, *J. Med. Genet.* **33**: 262-263 (1996).
6. S. Van Soenst, A. Westerveld, P. T. V. M. de Jong , E. M. Bleeker-Wagemakers, and A. A. B. Bergen, Retinitis Pigmentosa: Defined from a molecular point of view, *Surv. Ophth.* **43**(4): 321-359 (Jan-Feb 1999).
7. C. Inglehearn, Molecular Genetics of human retinal dystrophies, *Eye* **12**: 571-579 (1998).
8. C. Inglehearn, T. Keen, R. Bashir, M. Jay, F. Fitzke, A. C. Bird et al., A completed screen for mutations of the rhodopsin gene in a panel of patients with autosomal dominant retinitis pigmentosa, *Hum. Mol. Genet.* **1**(1): 41-45 (1992).
9. J. Greenberg, T. Franz, R. Goliath, and R. Ramesar, A photoreceptor gene mutation in an indigenous black African family with retinitis pigmentosa using a rapid screening approach for common rhodopsin mutations, *South Afr. Med. J.* **89**(8): 877-878 (1999).
10. L. Roberts, R. Ramesar, and J. Greenberg, Low frequency of rhodopsin mutations in South African patients with autosomal dominant retinitis pigmentosa, *Clin. Genet.* **58**: 77-78 (2000).
11. R. Goliath, S. Bardien, A. September, R. Martin, R. Ramesar, and J. Greenberg, Rhodopsin mutation Gly109Arg in a family with autosomal dominant retinitis pigmentosa, *Hum. Mut.* Supplement 1: 40-41 (1998).
12. P. Rosenfeld, L. Hahn, M. Sandberg, T. Dryja, and E. Berson, Low incidence of retinitis pigmentosa among heterozygous carriers of a specific rhodopsin splice site mutation, *I. O. V. S* **36** (11): 2186-2192 (October 1995).
13. C. Sung, C. Davenport, J. Hennessey, I. Maumenee, S. Jacobson, J. Heckenlively et al., Rhodopsin mutations in ADRP, *Proc. Natl. Acad. Sci. USA* **88**: 6481-6485 (August 1991).
14. J. Greenberg, R. Goliath, P. Beighton, and R. Ramesar, A new locus for autosomal dominant retinitis pigmentosa on the short arm of chromosome 17, *Hum. Mol. Genet.* **3**: 913-918 (1994).
15. S. Bardien, N. Ebenezar, J. Greenberg, C. F. Inglehearn, L. Bartmann, R. Goliath et al., An eighth locus for autosomal dominant retinitis pigmentosa is genetically linked to chromosome 17q, *Hum. Mol. Genet.* **4**: 1459-1462 (1995).

Ophthalmic Genetics 1381-6810/03/
US$ 16.00

*Ophthalmic Genetics – 2003, Vol. 24*
*No. 4*, pp. 225–232


Accepted 5 March 2003

# A rare homozygous rhodopsin splice-site mutation: the issue of when and whether to offer presymptomatic testing

**J. Greenberg**
**L. Roberts**
**R. Ramesar**

Division of Human Genetics, Department of Clinical Laboratory Medicine, University of Cape Town Medical School, Cape Town, South Africa

**Abstract** Having identified a disease-associated rhodopsin mutation in a patient with retinitis pigmentosa (RP), the issue is to address the question of whether to offer genetic testing to at-risk family members. Two members of a South African (SA) family, one of whom suffers from RP, as well as 54 unrelated SA RP patients from the same population group were investigated using single-stranded conformational polymorphism analysis followed by DNA sequencing. A rare homozygous mutation at the intron-exon boundary of exon 4 in the rhodopsin gene was identified in the proband. One of his siblings was found to be heterozygous for the same mutation. The mutation was not detected in the 54 unrelated SA RP patients examined, 11 of whom were sporadic cases. A low incidence of RP amongst heterozygous carriers of this mutation has been reported; however, in the past it has been unclear whether the mutation has an effect in single copy or dual copy. To the best of our knowledge, this is the first time that this mutation has been reported as homozygous in an affected individual, thereby resolving the issue and confirming that it is a recessive disease-associated mutation. This is also the first autosomal recessive RP disease-causing rhodopsin mutation that has been identified in Southern Africa. Analysis of the extended pedigree indicated obligate heterozygous carriers of the mutation, without obvious signs of visual impairment in early adulthood. The extent to which potential heterozygous carriers should be pursued and clinically examined is discussed and the question is addressed as to whether to inform the family of these molecular findings.

**Key words** Rhodopsin; genetic counselling; ethics; presymptomatic testing

**Introduction** Retinitis pigmentosa (RP) comprises a group of inherited degenerative diseases primarily involving loss of photore-

*Correspondence and reprint requests to:*
Jacquie Greenberg, Ph.D.
Division of Human Genetics
Molecular Laboratory
1st Floor, Anatomy Building
UCT Medical School
Penzance Road
Observatory, 7925
Cape Town
South Africa
Tel: 021 406 6299
Fax: 021 448 0906
E-mail: jg@cormack.uct.ac.za

*Acknowledgements:*
This research was supported by grants from Retina South Africa, the South African Medical Research Council, and the Foundation Fighting Blindness (USA). We are most grateful to Sister Lecia Bartmann and the Genetic Nursing Sisters of the Department of National Health for visiting family members and collecting blood samples. We thank Professor Ann Peters for referring the index patient to us. We are indebted to all RD family members in SA for their participation in this research programme.

ceptors, specifically the rods. RP is reported to affect 1:5000 live births in developed countries, causing night blindness and progressive loss of peripheral vision.[1] Mutations in the rhodopsin (RHO) gene have been found in families with dominant and recessive forms of RP.[2,3] The RHO gene encodes a G-protein-coupled receptor which acts in rods in the first stage of the visual transduction cascade. RHO is thus structurally important in rod photoreceptors, and functionally essential for mediating vision in dim light.[3]

A homozygous IVS4+1 G-T mutation in the RHO gene was detected in a South African (SA) patient with RP. A low prevalence of RP amongst heterozygous carriers of this mutation has been previously reported and it has been unclear whether the mutation results in a dominant or recessive allele.[2–5]

This specific G-T mutation was first reported in the intronic donor splice site of RHO intron 4 in 1992.[2] The heterozygous mutation was identified in a 28-year-old female with no other family history of RP, who had normal visual field, normal fundus, and ERG within normal limits. This heterozygous carrier was identified amongst a group of unrelated volunteers with no personal or family history of RP, who had provided DNA samples to be used as normal controls.[2] The subject did, however, on closer examination exhibit a subtle abnormality of rod function and was thus thought to be a possible carrier of autosomal recessive retinitis pigmentosa (ARRP).[5] Furthermore, it has been proposed that the mutation interferes with the splicing of intron 4 and may cause truncation of the protein such that it is functionally inactive. Similar splice mutations have resulted in low-level protein expression or none at all.[2–5]

The mutation was subsequently reported to co-segregate with the disease in a family with autosomal dominant RP (ADRP).[3] Affected heterozygotes in that report were 76, 46, and 21 years old.[4] The 76-year-old female had severely abnormal visual acuity, a greatly reduced kinetic field, and undetectable ERG. Her 46-year-old son had a normal fundus and visual acuity with slightly subnormal kinetic field and ERG. His 21-year-old daughter had normal fundus, visual acuity, kinetic field, and ERG. All three individuals showed rod sensitivity losses. The three different phenotypes resulted in the proposal that the younger patients represented the earlier stages of RP, and that the effect of the mutation was either variable or delayed.

In 1995, a third family, with 23 heterozygotes carrying this splice-site mutation, was identified.[5] Only one heterozygote, a 45-year-old male, was clinically affected with night blindness, loss of visual acuity, and loss of peripheral vision during adolescence. Although older and younger heterozygous family members were clinically unaffected and unaware of the changes in their retina, some of these individuals were found on closer examination to have mild rod functional abnormalities.

These observations were given several possible explanations:

- the mutation results in a protein product with a dominant disease-associated effect, with reduced expression;
- the mutation results in a protein product with a recessive disease-associated effect, with the mutation in the other rhodopsin allele undetected;

- the mutation, together with a recessive mutation in another gene, leads to recessive or digenic RP; and
- the mutation is not, in fact, disease-causing and the patient's clinical phenotype is due to a mutation in another gene/s.[5]

More recently, it has been noted that the varying rates of disease progression of the retina within a family with a rhodopsin mutation may be due to factors other than the mutation itself. Examples of these factors include modifier genes, diet, and light exposure, and thus the clinical course of the disease is difficult to predict, even when the disease-causing rhodopsin mutation is known.[6]

To the best of our knowledge, this mutation has not yet been reported in the homozygous state. The finding reported here was made during an ongoing screening programme of SA patients with inherited retinal degeneration, and no RHO mutation of this nature has been previously reported in SA.[7,8] The question now is whether to inform the family of these molecular findings and the extent to which potential heterozygous carriers should be investigated. Although ethical considerations regarding genetic testing and rare recessive mutations have previously been discussed at length, this is the first publication to address the issue in the context of retinal dystrophies (RD). There are no references on any presymptomatic testing ever having been offered to at-risk family members with RHO gene mutations, apart from a recent report of the first prenatal diagnosis of a RHO mutation.[9] In fact, there are few papers on predictive testing in RP in general and very few on predictive testing in children.

## Materials and methods

FAMILY HISTORY AND CLINICAL PHENOTYPE OF THE SA RP PATIENT Individuals were invited to participate in a study investigating the genetics of RD in SA in an attempt to define the genetic profile of all SA subjects who suffer from inherited retinal degeneration. An information sheet outlining the project was supplied to all subjects and a consent form was completed and accompanied all blood samples that were sent to the laboratory for DNA isolation. The DNA consent form and information sheet were submitted to and approved by the Institutional Research Ethics Committee, both of which comply with the tenets of the most recent Declaration of Helsinki. The proband presented with a typical advanced stage of RP at the age of 68 years. He was initially diagnosed with RP at 12 years of age, and suffers from night blindness, sensitivity to bright light, visual field loss, and loss of visual acuity. Consanguinity in the family suggests that the mode of inheritance is recessive (Figure 1). The four children of the affected patient are therefore obligate heterozygotes (assuming positive paternal status), and the five grandchildren each have a 50:50 chance of inheriting the single mutant allele. Details on the clinical phenotype of these potential 'carriers' in this family are not available; this is part of the ethical dilemma of the study.

PCR AMPLIFICATION Peripheral blood samples were obtained with informed consent and genomic DNA was isolated from white blood

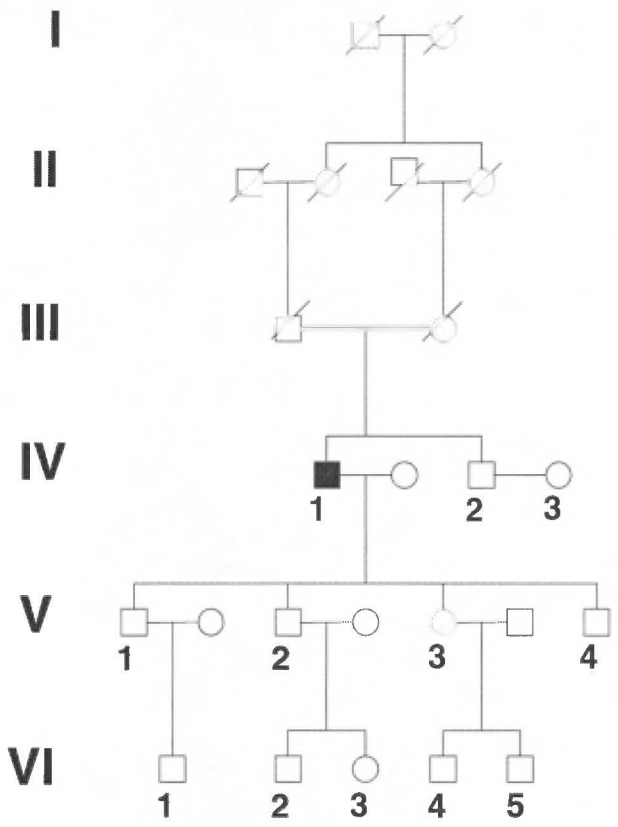



*Fig. 1.* Pedigree of the South African family indicating autosomal recessive retinitis pigmentosa. Squares represent males and circles females. Symbols for affected persons are solid and a diagonal line through the symbol indicates that the individual is deceased. The proband is individual IV-1. The sibling who was also tested is IV-2.

cells by standard methods. PCR was performed in a 25 µl reaction volume and both PCR and single-stranded conformational polymorphism analysis (SSCP) conditions were optimized for RHO exon 4. PCR amplification was performed with primers which amplify across the intron/exon boundaries of exon 4 (Table 1).

The mutation screening was undertaken using the following PCR conditions: PCRs containing 200 ng DNA, 10 pmoles fwd and rev primer, 200 µM dNTPs, 1× Invitrogen Standard PCR buffer (Life Technologies), 1.5 mM $MgCl_2$, 0.5 U Invitrogen *Taq* (Life Technologies), in a 25 µl total reaction volume, were cycled at 94°C for 3 min; (94°C for 30 sec, 60°C for 30 sec, 72°C for 40 sec) × 30; and 72°C for 7 min. Due to the size of the exon 4 fragment (402 bp, which is slightly large for effective SSCP analysis), 17 µl PCR product was subjected to restriction enzyme digestion at 60°C for 1.5 h with 12 Units of *Bst*EII (Promega).

MUTATION SCREENING AND SEQUENCE ANALYSIS *Bst*EII-digested PCR products were diluted in an equal volume of loading dye (95% formamide, 10 mM NaOH, 20 mM EDTA, 0.02% bromophenol blue, 0.02% xylene cyanol). Then, 10 µl were loaded onto the acrylamide gel. Two conditions were used to ensure SSCP sensitivity: 10% polyacrylamide with 7.5% urea was run with 1.5× TBE for 16 h at 3 W, and MDE (FMC Bioproducts) with 10% glycerol was run with 0.6× TBE buffer for 16 h at 4 W. All gels were run at room temperature.

| *Exon* | *Forward primer sequence (5′→3′)* | *Reverse primer sequence (5′→3′)* |
|---|---|---|
| 4 | TCA CGG CTC TGA GGG TCC AG | GAG TAG CTT GTC CTT GGC AG |

TABLE 1. Primers used to amplify across the intron/exon boundaries of exon 4.

The mobility shift observed in the DNA sample from the proband was further investigated with direct sequencing on an ABI Prism 377 automated sequencer according to standard procedures and with the same primers as in the SSCP analysis.

Following sequencing, a restriction enzyme digest was performed to confirm the loss of a *BanI* site caused by the identified mutation. To do this, 17μl of the PCR products were restricted with 12 Units *BanI* (Promega) in a 20μl reaction volume at 50°C for 4h. The digested samples were then size-fractionated on a 3% agarose gel and visualized with ethidium bromide.

GENOTYPING Genotyping of the proband was performed on an ABI Prism 377 automated sequencer with the ABI Prism Linkage Mapping Set Version 2 Panels 5, 6, and 7 (PE Applied Biosystems). These panels contain markers spanning chromosomes 3 and 4. PCR amplification was performed according to manufacturers instructions, and standard procedures were used for electrophoresis.

**Results** An altered migration pattern, suggesting a possible sequence variation in the DNA from the proband, was observed on SSCP analysis of the RHO exon 4 PCR amplification product. Direct sequencing of the PCR product revealed a homozygous G-to-T transversion (IVS4+1 G-T) in the consensus splice donor site of intron 4 in this 68-year-old South African male (Figure 2). The G-to-T transversion destroys a *BanI* restriction endonuclease cleavage site. *BanI* was used to demonstrate the presence of the heterozygous G-to-T mutation in another family member who was available for investigation. This mutation was not seen in the SSCP analysis of 54 other SA RP patients from the same population group. Of the 14 genotypes that were generated around the RHO locus on 3q21 from the ABI Prism Linkage Mapping Set panels, all but four were heterozygous for the markers examined in the proband (data not shown).

**Discussion** To our knowledge, this is the first report of a homozygote with this splice-site mutation and the first autosomal recessive SA RP family to be identified with a RHO disease-causing mutation.

A phenotype-genotype correlation is not yet clear for this RHO splice-site mutation. It appears that the mutation has a recessive effect, although heterozygous carriers can be clinically affected.[2–5] Since the RHO mutation in the affected individual reported here is homozygous, unless this is a rare case of the cumulative effect of two dominant mutations with reduced penetrance, it will be presumed that the identified allele is recessive. Haplotype analysis to differentiate homozygosity by descent from other possible explanations for the putative homozy-

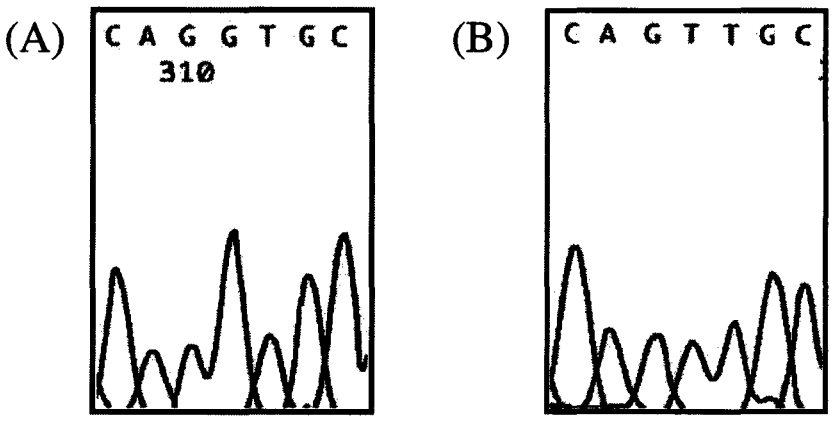



*Fig. 2.* The sequence of the rhodopsin (RHO) donor splice-site mutation, obtained by automated sequencing of RHO, exon 4. (A) Electropherogram showing the wild-type sequence of an unaffected spouse. (B) Electropherogram showing the homozygous mutation in the affected male.

gosity, such as a deletion on the other allele or uniparental disomy (UPD), was not possible as both the proband's parents are deceased. Given the consanguinity in the family, we believe that the lack of homozygosity on 3q, around the RHO locus, is sufficient evidence to argue against UPD. Therefore, members of this family who are heterozygous for this specific G-T mutation in the RHO gene probably have a very low risk of developing RP (if at all). Of course, if they inherit a second recessive RHO allele or if they have mutant alleles causing RP unrelated to this RHO mutation, then they might still develop RP. These risks, however, are the same as for the general population. Taking into account the genetic heterogeneity of RP, it must be remembered that this recessive rhodopsin mutation does not protect against the effect of mutations in other RP genes and so once again, the risks for the family members remain the same as for the general population.

From a research point of view, it would be most valuable to make a careful comparison of the clinical phenotype in the heterozygous individuals in this family. However, this is part of the ethical dilemma of the present report: should the family be told of these molecular results, bearing in mind that this is an extremely rare finding? One of our main concerns is that we could offer to confirm the heterozygous carrier status (assuming paternity) for the four children of the proband, but that would then mean that the five grandchildren might also request testing and they are all minors. The types of questions likely to be asked if genetic testing is offered are: at what age will vision loss become apparent in the heterozygous carriers (if, in fact, at all); how rapidly will the disease progress; and how severe will the symptoms become? Unfortunately, these are questions that will remain unanswered until more is understood about RP disease-causing genes and about this IVS4+1 mutation.[2–5]

Given that there is no treatment as yet and no beneficial knowledge to be imparted to these family members regarding the disease and mutation they have, should they actually even be offered testing at this stage? Should the grandchildren, all still in early puberty, be tested to

allow for informed reproductive decisions when the severity of the phenotype caused by the mutation is unknown? Although these ethical issues are not new, they have not been addressed in the context of RD and, in particular, recessive RP.

As has been stated, the RP symptoms in this family had an early onset (12 years of age) in the proband and a mutation has been identified in both alleles of the RHO gene. It is our belief that this information should now be imparted to the affected individual. His children should also have the right to pursue genetic testing, should they so desire. This is, however, a very personal decision, and it is obvious that a parent cannot request DNA testing for an adult child. The issue of a parent requesting predictive testing for a minor is often debated and in this case, if the information gained from such DNA testing can influence medical treatment, then genetic testing for minor children, when requested by a parent, should be considered.[10]

We regard genetic counselling in ophthalmic genetics as extremely important. It has been shown repeatedly that people's misconceptions and misinformation can cause some individuals to be so fearful of modern medical technology that they decline any opportunity for genetic testing. It follows, therefore, that a detailed description of the consequences of the different phenotypes, modes of inheritance, and molecular genetic information must be carefully explained by the professional doing the counselling. Knowledge itself without the ability to act on it may be comforting to some, but may simply aggravate the situation for others. Consequently, skilled counsellors should be able to determine each individual's tolerance level and respond accordingly on a case-by-case basis. However, a defined structured approach is nevertheless essential to manage the delivery of genetic testing results. Towards this end, a protocol has been prepared by our laboratory for both the delivery of genetic results and the subsequent management of individuals who are at risk of genetic disease. This document has recently been approved by our Institutional Review Board. The detailed protocol will be available shortly for scrutiny on our www site and will be adapted for different genetic conditions that are being investigated by our laboratory.[11]

It is the conclusion of this case presentation that the proband needs to be informed of his genetic results and understand these complex concepts as well as the implications for his family. He needs to be adequately equipped to be able to use this information to make an informed decision regarding the dissemination of the information to his immediate family. Subsequently, family members need to be assisted to understand the risks, benefits, and limitations of genetic testing, wherever possible. Genetic testing to identify heterozygous carriers could be offered to all of the children of the affected parent as well as their extended families, should they request it.

Finally, regarding this rare homozygous rhodopsin splice-site mutation and the question of whether or not to test, there is no definitive answer. This particular case, however, highlights some of the problems associated with genetic testing in general and how we have attempted to address at least some of these difficult but universal issues.

## References


1 Jay M, Evans K. Retinal dystrophies and genetic counselling. *Acta Ophthalmol Scand.* 1996;74(Suppl 219):5–7.

2 Rosenfeld P, Cowley G, McGee T, Sandberg M, Berson E, Dryja T. A null mutation in the rhodopsin gene causes rod photoreceptor dysfunction and autosomal recessive retinitis pigmentosa. *Nat Genet.* 1992;1:209–213.

3 Mack J, Davenport C, Jacobson S, Hennessey J, Gonzalez-Fernandez F, Conway B, et al. Identification of novel rhodopsin mutations responsible for retinitis pigmentosa: implications for the structure and function of rhodopsin. *Am J Hum Genet.* 1993;53(1): 80–89.

4 Jacobson S, Kemp C, Cideciyan A, Macke J, Sung C, Nathans J. Phenotypes of stop codon and splice site rhodopsin mutations causing retinitis pigmentosa. *Invest Ophthalmol Vis Sci.* 1994;35(5): 2521–2534.

5 Rosenfeld P, Hahn L, Sandberg M, Dryja T, Berson E. Low incidence of retinitis pigmentosa among heterozygous carriers of a specific rhodopsin splice site mutation. *Invest Ophthalmol Vis Sci.* 1995;36(11):2186–2192.

6 Berson EL, Rosner B, Weigel-DiFranco C, Dryja TP, Sandberg MA. Disease progression in patients with dominant retinitis pigmentosa and rhodopsin mutations. *Invest Ophthalmol Vis Sci.* 2002;43(9):3027–3036.

7 Greenberg J, Rebello G, Ramesar R. Ophthalmic genetics: a review of the molecular genetics of familial retinal dystrophies in Southern Africa. *Spec Med.* 1999;21:108–113.

8 Roberts L, Ramesar R, Greenberg L. Low frequency of rhodopsin mutations in South African patients with autosomal dominant retinitis pigmentosa. Letter to the Editor. *Clin Genet.* 2000;58:77–78.

9 Tessitore A, Toniato E, Gulino A, Frati L, Ricevuto E, Vadala M, Vingolo E, Martinotti S. Prenatal diagnosis of a rhodopsin mutation using chemical cleavage of the mismatch. *Prenat Diagn.* 2002;22:380–384.

10 Wertz DC, Fanos JH, Reilly PR. Genetic testing for children and adolescents. Who decides? *J Am Med Assoc.* 1994;272(11):875–881.

11 Greenberg J, Beatty S, Soltau H, Bryer A. A predictive testing service for Huntington disease (HD) and late onset spinocerebellar ataxia (SCA1) in Cape Town. *CME Rev.* 1996;14:1364–1367.

*Molecular Vision* 2006; 12:177-83 <http://www.molvis.org/molvis/v12/a19/>
Received 14 July 2005 | Accepted 7 March 2006 | Published 15 March 2006



# Novel variants in the hotspot region of RP1 in South African patients with retinitis pigmentosa

**Lisa Roberts, Lecia Bartmann, Rajkumar Ramesar, Jacquie Greenberg**

*Division of Human Genetics, Department of Clinical Laboratory Sciences, Faculty of Health Sciences, University of Cape Town, Cape Town, South Africa*

**Purpose:** Mutations in the hotspot of *RP1* are reportedly responsible for 4-7% of autosomal dominant retinitis pigmentosa (ADRP) in the United States, Canada, and Europe. South Africa (SA) has unique subpopulations and a comparatively low observed frequency of rhodopsin mutations, which lead to this investigation of the contribution of *RP1* mutations to the ADRP disease burden in SA.

**Methods:** Fifty-seven affected, unrelated South African individuals with ADRP were selected for mutation screening of the *RP1* hotspot, using denaturing high performance liquid chromatography (HPLC). Variants were identified by direct sequencing, after which cosegregation analysis and population frequency studies were performed using restriction fragment length polymorphism analysis, nondenaturing HPLC, or denaturing HPLC.

**Results:** Three mutations were identified, including two novel sequence variations and the common Arg677X mutation. A wide spectrum of disease severity was observed in the families with these *RP1* gene mutations. Two nondisease-associated polymorphisms were also detected, with the frequency of one of these variants being significantly low in Black African individuals.

**Conclusions:** Mutations were only found in Caucasian families with origins in the British Isles. The observed *RP1* mutation frequency of 5.3% in SA ADRP patients is comparable to the frequency reported in other populations.

Defects of the eye account for approximately one-third of all human inherited diseases [1]. One in 3,500 people live with retinitis pigmentosa (RP), the most common inherited retinal dystrophy [1], characterized by nightblindness and progressive constriction of the visual fields. Total blindness occurs in approximately 30% of cases.

Approximately 15% of RP is inherited in an autosomal dominant manner [2]. The major genes contributing to autosomal dominant retinitis pigmentosa (ADRP) described to date are Rhodopsin (*RHO*), *RDS*, and *RP1*. In the United States, United Kingdom, and Europe, mutations in *RHO* account for 20-25% of all ADRP cases [3]. *RDS* mutations were identified in 8% of ADRP cases in the United States [4]. Mutations in a hotspot region of *RP1* are reported to be responsible for 4-7% of ADRP in the United States, Canada, and Europe [4,5].

In 1977, RP1 on chromosome 1 was the first locus reported to be associated with RP [6]. This finding was later retracted, and the locus reassigned to chromosome 8 [7]. Twenty years later, the gene "Oxygen-regulated protein 1" (*ORP-1* or *RP1*) was identified, and mutations therein were found to correlate with the ADRP phenotype [8,9]. The RP1 protein is expressed in the photoreceptor connecting cilia, and appears to be required for the correct orientation and stacking of discs during photoreceptor outer segment morphogenesis [10]. In addition, it is reported to be modulated by retinal oxygen levels [8].

Although the *RP1* gene has four exons (GenBank accession number NM_006269), reports have indicated that pathogenic mutations cluster in a small region of exon 4 [10,11]. These mutations include deletions, insertions, and substitutions and cause a wide spectrum of disease severity [12]. The Arg677X mutation in *RP1* accounts for 2% of ADRP, making it the third major contributor to this disease after the two *RHO* mutations, Pro23His and Pro347Leu [8,13]. South Africa (SA) has subpopulations including Black (people of indigenous Black African origin); mixed ancestry (representing an admixture of San, Khoi-Khoi, West African, Madagascan, Javanese, and Western European populations), and Caucasian (in SA, immigrants mainly of Western European origin, including Dutch, French, German, and British). This provides a novel gene pool with which one can study inherited disorders [14]. Given these population demographics, detection of mutations previously identified in Europe and the United Kingdom is expected in some of the subpopulations. The surprisingly low frequency of the two common *RHO* mutations in the SA population with ADRP [15], despite a strong immigrant history, lead to this investigation of the contribution of *RP1* mutations to the ADRP disease burden in SA.

## METHODS

*Subjects:* Patients with inherited retinal degenerative disorders and their families are referred to the Division of Human Genetics at the University of Cape Town from throughout

Correspondence to: Associate Professor Jacquie Greenberg, UCT/MRC Human Genetics Unit, Division of Human Genetics, Level 3, Wernher and Beit North, Institute of Infectious Disease and Molecular Medicine, Faculty of Health Sciences, University of Cape Town, Observatory, 7925, South Africa; Phone: 27 21 406-6299; FAX: 27 21 406-6826; email: jg@cormack.uct.ac.za

South Africa by ophthalmologists, health care professionals, nurses from the Department of Health, and the lay organization, Retina South Africa (from whom most of the referrals are received). Affected individuals and their families are recruited into a research program investigating the genetics of retinal degenerative disorders in this country, in an attempt to define the genetic profile of all subjects who suffer from inherited forms of retinal degeneration. Informed consent is obtained using the tenets of the most recent Declaration of Helsinki (2000). Fifty-seven affected, unrelated individuals from known families with ADRP were selected for mutation screening of the *RP1* hotspot.

The ethnic breakdown of these families was as follows: thirty-six Caucasian, fifteen Black, three mixed ancestry, two Indian, and one of unknown origin. A general population control cohort of 103 individuals was selected for population frequency studies of novel *RP1* polymorphisms. These individuals were not assessed specifically for the presence of disease, and were classified ethnically as belonging to Caucasian or Black population groups based on the same system used to classify the sample cohort.

Peripheral blood samples were obtained and genomic DNA was isolated from white blood cells using standard methods.

*Polymerase chain reaction (PCR) amplification:* The *RP1* region of interest was amplified using three overlapping sets of primers [5]. All polymerase chain reaction (PCR) amplification was undertaken using the following conditions, unless otherwise stated in Table 1: 0.2 mM dNTPs, 1X Invitrogen™ Buffer (Invitrogen™ Life Technologies, Paisley, UK), 1.5 mM magnesium chloride, 1 U Invitrogen™ Taq polymerase, 200 ng genomic DNA. Final reaction volumes were made up to 50 µl with distilled water, and thermal cycling conditions were as follows: 95 °C for 5 min, followed by 30 or 35 cycles of 94 °C for 5 min, 50 °C for 30 s, 72 °C for 40 s, and a final single incubation at 72 °C for 7 min.

An aliquot of 5 µl of PCR product was subjected to agarose gel electrophoresis on 2% agarose gel to confirm successful amplification, prior to denaturing high performance liquid chromatography (dHPLC).

*denaturing High Pressure Liquid Chromatography:* Mutation screening was performed according to the manufacturer's instructions, using the WAVE® Nucleic Acid Fragment Analysis System (Transgenomic Inc., Omaha, NE). An aliquot of 10-20 µl of each PCR product was heat-denatured at 95 °C for 5 min, and heteroduplex formation was promoted by allowing the tube temperature to reach room temperature over 45 min. PCR products were then separated into hetero- and homoduplexes on the WAVE®, using an acetoni-

**Table 1. Primers and amplification conditions**

| Gene | Primer (5'-3') | Amount | Additive | Cycles |
|---|---|---|---|---|
| 4F | F: tgctcagtgtggtttaacaaaac<br>R: ctatggaaattcttggaaatcg | 25 pmol | 1% glycerol | 30 |
| 4G | F: ggaagacctccagaaaagtgatac<br>R: cattcctctcaaatacccagatg | 20 pmol | 1% glycerol | 35 |
| 4H | F: ccaaagatttttatgcaccg<br>R: caatttaccacactcgtttcatttc | 20 pmol | - | 30 |

The table indicates polymerase chain reaction conditions and 5'-3' primer sequences for three overlapping fragments spanning the RP1 hotspot region.

**Table 2. Mutation screening conditions**

| 4F | 4G | 4H |
|---|---|---|
| 54 °C at 0.9 ml/min | 54 °C at 0.9 ml/min | 55 °C at 1.5 ml/min |
| 56.1 °C at 1.5 ml/min | 54.5 °C at 0.9 ml/min | 56 °C at 1.5 ml/min |
| | 57.5 °C at 0.9 ml/min | 57 °C at 1.5 ml/min |

The table indicates the temperatures and flow rates used to optimally separate hetero- and homoduplexes by denaturing High Performance Liquid Chromatography for each fragment spanning the RP1 hotspot.

**Table 3. Frequencies of the five variations detected in RP1**

| Autosomal dominant retinitis pigmentosa cohort | Mutation | | | | |
|---|---|---|---|---|---|
| | Arg677X | Ser911X | 2590-2599del ATAACTTTAA | Thr752Met | Arg872His |
| **Patients** | | | | | |
| 72 Caucasian | 1 | 1 | 1 | 2 | 14 |
| 30 Black | 0 | 0 | 0 | 1 | 4 |
| 6 Mixed ancestry | 0 | 0 | 0 | 1 | 1 |
| 4 Indian | 0 | 0 | 0 | 0 | 1 |
| 2 Unknown | 0 | 0 | 0 | 0 | 0 |
| **Controls** | | | | | |
| 102 Caucasian | - | 0 | - | 1 | 24 |
| 104 Black | - | 0 | - | 3 | 12 |

The table shows the number of chromosomes screened in each ethnic group of patient and control cohorts, indicating the number of variant chromosomes detected. The hyphen indicates that the variation was not screened in the control group.

trile gradient. In the partially denaturing mode, in which heteroduplexes emerge prior to homoduplexes, separation chemistry is based on sequence, size of the fragment, and analysis temperature. Gradient parameters, temperatures, and flow rates were developed empirically based on the size and melting profile of the fragments (Table 2).

Nondenaturing conditions on the WAVE® (in which the acetonitrile gradient is determined by the number of base pairs of the fragment) allowed size-based separation of PCR products.

When numerous samples appeared to have the same heteroduplex profiles, these samples were tested using a second dHPLC analysis to confirm whether they were due to the same sequence variation. This was done by mixing equal volumes of the PCR product being queried with that of a sample of a known DNA sequence variant. This mixture was subjected to heteroduplex formation and dHPLC analysis with the appropriate gradient condition. Lack of additional peaks indicated that sequences of the PCR product being queried and the known sample were identical. This highly effective secondary dHPLC was found to be cost efficient when compared to direct sequencing of multiple samples with the same variant. It is a convenient and rapid way of testing for common sequence variants that do not alter restriction enzyme sites.

*Direct sequencing:* DNA samples that exhibited variant elution profiles on dHPLC were characterized by direct sequencing on an ABI Prism™ 3100 Automated Sequencer (Applied Biosystems, Foster City, CA) using the above described standard procedures and the same primers.

*Restriction fragment length polymorphism (RFLP) analysis:* Where possible, sequence variations were confirmed and tested for cosegregation with disease by restriction fragment length polymorphism (RFLP) analysis. Restriction enzyme digests were performed for approximately 15 h, in a final volume of 20-25 µl. Five units of the appropriate enzyme were used to digest 10 µl of PCR product. Incubation temperatures of 65 °C, 37 °C, and 50 °C were used for *TaqI*, *Hsp92II*, and *AcsI* enzymes, respectively.

*Proof of disease association:* Disease association of novel variants was judged by the following criteria: (1) whether the protein would be significantly altered and thus likely to modify the phenotype; (2) whether there was co-segregation of the variant and the phenotype; and (3) whether the variant occurred in less than one percent of the population tested [16].

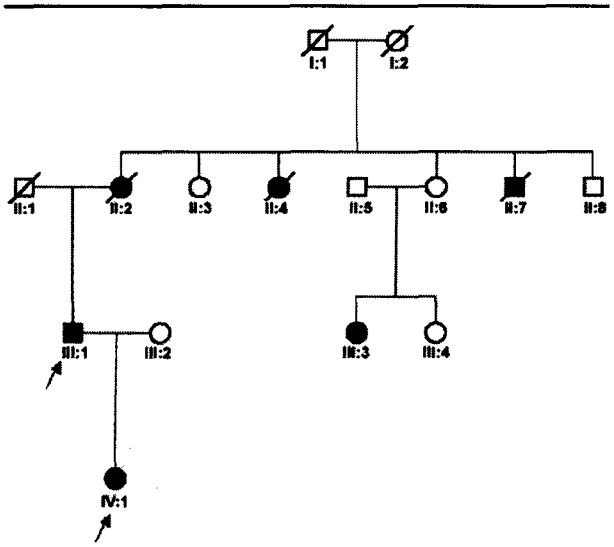



Figure 1. Pedigree of the South African family with Arg677X in *RP1*. Squares and circles represent males and females, respectively. Symbols for affected persons are solid. A diagonal line through the symbol indicates that the individual is deceased. The two individuals tested for the mutation are indicated by arrows. Both individuals are mutation carriers.

## RESULTS

Of the 57 affected, unrelated individuals screened for variants in the hotspot of *RP1*, three were determined to carry disease associated mutations (5.26%) and two of these mutations are novel. These results and the frequencies of variant sequences are summarized in Table 3.

## DISCUSSION

*RP1* mutations are reported to cause 4-7% of ADRP in the United States, Canada, and Europe [4,5] and thus could be considered one of the major identifiable causes of this form of retinal degeneration. There are no reports of the mutation spectrum nor disease burden of the *RP1* gene in Africa. This led to the investigation of the role of *RP1* in this cohort.

Several factors resulted in mutation screening being taken as a direct approach, rather than linkage analysis. These factors include: first, the lack of comprehensive family information, and second, the fact that all disease associated mutations reported to date, have been found in a hotspot region encompassing 553 codons of exon 4 of *RP1* [10].

The hotspot region of *RP1* was investigated in 57 SA individuals with pedigrees exhibiting an autosomal dominant mode of inheritance of retinitis pigmentosa. Variations were detected by dHPLC, and these were confirmed by sequencing or restriction enzyme analysis. Further investigation revealed that three of the changes contributed toward disease, while two of them were nondisease associated. The findings indicate that *RP1* mutations are responsible for approximately 5.26% of ADRP cases in the SA population screened to date. It is interesting to note that all three families identified with *RP1* mutations have origins in either the United Kingdom or Ireland, and two of these families carry novel mutations.

*Disease associated variants:* The Arg677X mutation has previously been reported as the most common *RP1* disease associated variant [8,13]. Arg677X was detected in a Caucasian family of British origin (Figure 1). The presence of the mutation was confirmed in two affected individuals by RFLP analysis using *TaqI* as the mutation (c.2029C>T) destroys a cutting site of this enzyme.

Ser911X was detected in a Caucasian family of British origin (Figure 2). This novel mutation is predicted to truncate the protein by 1,246 amino acids, and was not detected in 102 chromosomes investigated in the Caucasian controls or 104

chromosomes investigated in the Black indigenous controls. Ser911X (c.2732 C>A) creates a cutting site for *AcsI*, and its presence was thus confirmed by a restriction enzyme digest. The novel Ser911X mutation co-segregates with the disease in the family: It was detected in two affected individuals (IV:4 and III:2 of Figure 2, aged 43 and 69, respectively), and two at-risk individuals (IV:7 and IV:5, aged 34 and 40, respectively), who showed no signs of the disorder at the time of recruitment.

A novel 10 bp deletion (c.2590-2599delATAACTTTAA) was detected in a Caucasian family originating from Dublin (Figure 3). The presence of the mutation was confirmed by

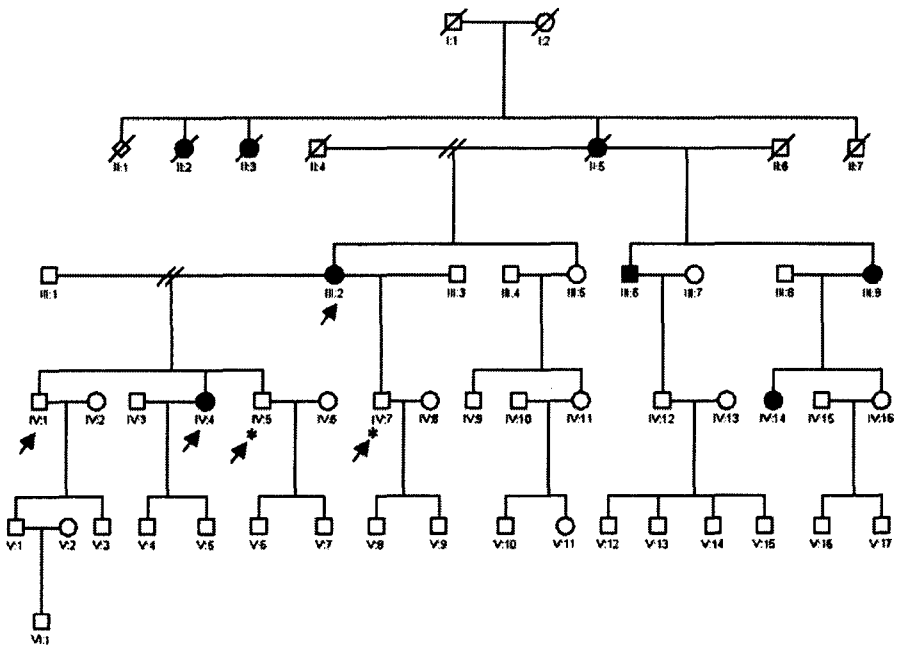


Figure 2. Pedigree of the South African family with Ser911X in *RP1*. Squares and circles represent males and females, respectively. The diamond indicates several unaffected offspring who were not involved in this research project. Symbols for affected persons are solid. A diagonal line through the symbol indicates that the individual is deceased. Divorces are indicated by the double slashes. The individuals tested for the mutation are indicated by arrows, and the clinically asymptomatic mutation-carriers are indicated by asterisks.

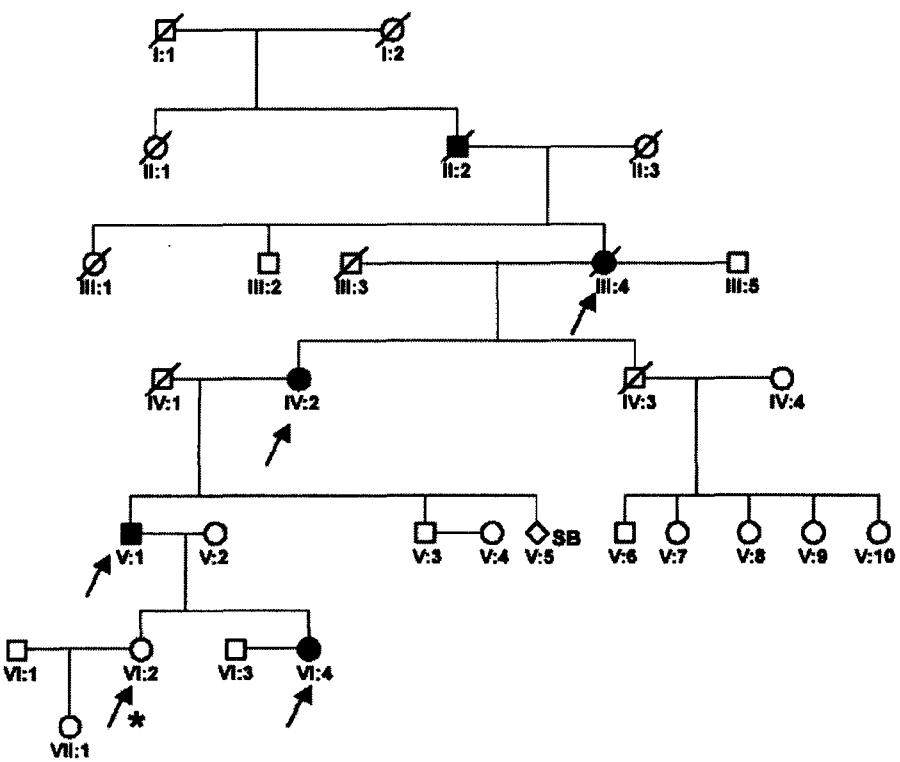



Figure 3. Pedigree of the South African family with 2590-2599delATAACTTTAA in *RP1*. Squares and circles represent males and females, respectively. "SB" indicates a stillbirth. Symbols for affected persons are solid, and a diagonal line through the symbol indicates that the individual is deceased. The individuals tested for the mutation are indicated by arrows and the clinically asymtomatic mutation-carrier is indicated by an asterisk.

using nondenaturing, size-based separation on the WAVE. The mutation is predicted to cause a frameshift at codon 864, truncating the protein by 1,280 amino acids. Due to these great effects on RP1, it was not necessary to perform population frequency analysis. The mutation cosegregates with the disease in this family: it is present in four affected members in four successive generations of the family; these are individuals III:4 (deceased), IV:2 (age 71), VI (age 51), and VI:4 (age 26). This mutation was also detected in one at-risk individual VI:2 (age 28) clinically unaffected at the time of recruitment.

Clinical categorization of families with *RP1* mutations is confounded by the variability of disease severity in the *RP1* form of ADRP, ranging from nyctalopia at age 11 to asymptomatic carriers at age 66, and variability within families has been reported [11,12]. This phenomenon of variable expressivity was also evident within the three families identified in this study, as described in Table 4. It should be noted that we often only have access to individuals' clinical test results via their ophthalmologists and only when they have had a routine check-up. We do not ask asymptomatic individuals

Table 4. Clinical details of RP1 mutation carriers

| Person | Age | Age of onset | Visual acuity | Other clinical information |
|---|---|---|---|---|
| Mutation Arg677X (Figure 1) | | | | |
| III:1 | 58 | 34 | Age 48: 6/24<br>Age 53: Hand motions | Diffuse RP with macular edema, rapid progression with the onset of macular degeneration, cataracts, waxy pallor of discs, attenuated vessels, extensive distribution of bone spicule in retinal periphery, vitreal floaters. |
| IV:1 | 31 | Early 20s | Age 22: 6/6<br>Age 27: 6/7.5 | Diffuse RP |
| Mutation Ser911X (Figure 2) | | | | |
| III:2 | 69 | About 40 | Age 68: 6/60 OU, which improved with Diamox to 6/21 OS after 6 months | Early onset of nyctalopia, diffuse RP, cystoid macular edema, mild nuclear sclerosis, pale and waxy discs, bone spicule scattered peripherally in the retina, floaters and asteroid hyalosis of the vitreous. |
| IV:4 | 43 | 19 | | Nyctalopia began at age 19, followed by loss of visual fields in early 20s. |
| IV:5 | 40 | | | No apparent manifestation |
| IV:7 | 34 | | | No apparent manifestation |
| Mutation 2590-2599del ATAACTTTAA (Figure 3) | | | | |
| III:4 | | 30s | | Nyctalopia began at age 48. Deceased |
| IV:2 | 71 | 17 | | Nyctalopia began at age 17, by the age of 40 peripheral vision was very poor, hyperopia at age 60. |
| V:1 | 51 | Early teens | Age 50: 6/15 OD, 6/9 OS | Diffuse RP with slow progression, cataracts, waxy pallor of the discs, attenuated vessels, granularity of the macula, bleakish fovea, bone spicule and white dots on retina, no cellularity or vitreous floaters. |
| VI:2 | 28 | | | No apparent manifestation. Retinoblastoma in one eye at 6 months of age. |
| VI:4 | 26 | 24 | | Nyctalopia began in 20s, diffuse RP with slow progression, waxy pallor of discs, attenuated vessels, normal macula and fovea, occasional and diffuse pigment in the periphery of the retina, bone spicule, normal vitreous. |

Clinical details of all mutation carriers, showing inter- and intra-familial variations in phenotype. RP represents retinitis pigmentosa. Visual acuities are provided in metric units.

to have any additional evaluations done unless they request molecular testing as part of our genetic service and are then informed about their mutation-carrier status, accompanied by appropriate genetic counseling. The policies and guidelines used for the genetic testing of retinal degenerative disorders are available on the Services page of our division's website, under the link "Genetic testing protocols and policy guidelines," Genetics.

This wide spectrum of severity of disease in these families will be an important issue for genetic counseling when providing diagnostic advice to the individuals concerned.

*Polymorphic variants:* Two nondisease-associated polymorphisms were detected in the cohort screened, and these were further investigated to determine the frequencies in the general population. Over 100 chromosomes were screened for these two polymorphisms in each ethnic group (Caucasian and Black) of unaffected, unrelated individuals.

A heterozygous polymorphism, Thr752Met was detected in 4 of the 57 ADRP individuals screened. Two subjects were Caucasian, one was Black, and one was of mixed ancestry (4/114 chromosomes=3.51%). The presence of this polymorphism was confirmed by performing an *Hsp92II* restriction enzyme digest, as the mutation (c.2255C>T) creates a cutting site for this enzyme. This sequence variation was found to be nonpathogenic as it did not cosegregate with the disease. The variant was detected in 1 of 102 chromosomes investigated in the Caucasian controls (0.98%) and 3 of 104 chromosomes investigated in the Black controls (2.83%).

Arg872His was previously reported as a nonpathogenic variant occurring in 25% of the population [9]. This variant (c.2615 G>A) was investigated in several cohorts, as described in Table 3, and was found to occur in 20/114 chromosomes (17.54%) of the total ADRP cohort; 19.44% of the Caucasian individuals with ADRP, and 23.53% of the Caucasian control chromosomes. These frequencies correlate with those reported previously; however, this variant was only detected in 11.5% of Black control chromosomes. There is thus a significant (p=0.037) association between ethnicity and the prevalence of this polymorphism. The difference in gene variant frequencies between these South African (SA) ethnic groups has been noted previously [17] and should be investigated further.

In conclusion, this is the first report describing the role of the *RP1* gene in SA patients with ADRP. We have found that the rapid, large-scale screening of the hotspot region of *RP1* using the WAVE is a viable initial approach in the routine screening of ADRP families for defects in candidate genes, as this technique is reported to have a specificity and sensitivity greater than 96% [18]. The results of this study could also indicate merit in screening sporadic individuals for the hotspot of *RP1* as suggested previously [13]. The observed *RP1* mutation frequency of 5.3% in SA ADRP patients is comparable to the 4-7% frequency reported elsewhere [4,5], however, mutations were only found in Caucasian families with origins in the British Isles. The frequency did not compensate for, nor did it explain, the reported low frequency of *RHO* mutations in SA ADRP families.

## ACKNOWLEDGEMENTS

We are grateful to Annapurna Hazra for her assistance with the statistical calculations. This research was supported by grants from the University of Cape Town, Retina South Africa, the South African Medical Research Council, and Technology and Human Resources for Industry Programme (THRIP, South Africa). We are indebted to all RD family members in SA for their participation in this research program.

## REFERENCES

1. Hims MM, Diager SP, Inglehearn CF. Retinitis pigmentosa: genes, proteins and prospects. Dev Ophthalmol 2003; 37:109-25.
2. Phelan JK, Bok D. A brief review of retinitis pigmentosa and the identified retinitis pigmentosa genes. Mol Vis 2000; 6:116-24.
3. van Soest S, Westerveld A, de Jong PT, Bleeker-Wagemakers EM, Bergen AA. Retinitis pigmentosa: defined from a molecular point of view. Surv Ophthalmol 1999 Jan-Feb; 43:321-34.
4. Sohocki MM, Daiger SP, Bowne SJ, Rodriquez JA, Northrup H, Heckenlively JR, Birch DG, Mintz-Hittner H, Ruiz RS, Lewis RA, Saperstein DA, Sullivan LS. Prevalence of mutations causing retinitis pigmentosa and other inherited retinopathies. Hum Mutat 2001; 17:42-51.
5. Bowne SJ, Daiger SP, Hims MM, Sohocki MM, Malone KA, McKie AB, Heckenlively JR, Birch DG, Inglehearn CF, Bhattacharya SS, Bird A, Sullivan LS. Mutations in the RP1 gene causing autosomal dominant retinitis pigmentosa. Hum Mol Genet 1999; 8:2121-8.
6. Spence MA, Sparkes RS, Heckenlively JR, Pearlman JT, Zedalis D, Sparkes M, Crist M, Tideman S. Probable genetic linkage between autosomal dominant retinitis pigmentosa (RP) and amylase (AMY2): evidence of an RP locus on chromosome 1. Am J Hum Genet 1977; 29:397-404. Erratum in: Am J Hum Genet 1977; 29:592.
7. Blanton SH, Heckenlively JR, Cottingham AW, Friedman J, Sadler LA, Wagner M, Friedman LH, Daiger SP. Linkage mapping of autosomal dominant retinitis pigmentosa (RP1) to the pericentric region of human chromosome 8. Genomics 1991; 11:857-69.
8. Pierce EA, Quinn T, Meehan T, McGee TL, Berson EL, Dryja TP. Mutations in a gene encoding a new oxygen-regulated photoreceptor protein cause dominant retinitis pigmentosa. Nat Genet 1999; 22:248-54.
9. Sullivan LS, Heckenlively JR, Bowne SJ, Zuo J, Hide WA, Gal A, Denton M, Inglehearn CF, Blanton SH, Daiger SP. Mutations in a novel retina-specific gene cause autosomal dominant retinitis pigmentosa. Nat Genet 1999; 22:255-9.
10. Liu Q, Lyubarsky A, Skalet JH, Pugh EN Jr, Pierce EA. RP1 is required for the correct stacking of outer segment discs. Invest Ophthalmol Vis Sci 2003; 44:4171-83.
11. Jacobson SG, Cideciyan AV, Iannaccone A, Weleber RG, Fishman GA, Maguire AM, Affatigato LM, Bennett J, Pierce EA, Danciger M, Farber DB, Stone EM. Disease expression of RP1 mutations causing autosomal dominant retinitis pigmentosa. Invest Ophthalmol Vis Sci 2000; 41:1898-908.
12. Berson EL, Grimsby JL, Adams SM, McGee TL, Sweklo E, Pierce EA, Sandberg MA, Dryja TP. Clinical features and mutations in patients with dominant retinitis pigmentosa-1 (RP1). Invest Ophthalmol Vis Sci 2001; 42:2217-24.
13. Schwartz SB, Aleman TS, Cideciyan AV, Swaroop A, Jacobson SG, Stone EM. De novo mutation in the RP1 gene (Arg677ter) associated with retinitis pigmentosa. Invest Ophthalmol Vis Sci 2003; 44:3593-7.

14. Ramesar R, Roberts L, Rebello G, Goliath R, Vorster A, September A, Ehrenreich L, Gama D, Greenberg J. Retinal degenerative disorders in Southern Africa: a molecular genetic approach. In: LaVail MM, Hollyfield JG, Anderson RE, editors. Retinal degenerations: mechanisms and experimental therapy. Proceedings of the 10th International Symposium on Retinal Degenerations; 2002 Sep 30-Oct 5; Burgenstock, Switzerland. New York: Kluwer Academic/Plenum Publishers; 2003. p.35-40.

15. Roberts L, Ramesar R, Greenberg J. Low frequency of rhodopsin mutations in South African patients with autosomal dominant retinitis pigmentosa. Clin Genet 2000; 58:77-8.

16. Cotton RG, Scriver CR. Proof of "disease causing" mutation. Hum Mutat 1998; 12:1-3.

17. Greenberg J, Roberts L, Ramesar R. Unusual frequencies of Rhodopsin mutations and polymorphisms in Southern African patients with retinitis pigmentosa. In: Anderson RE, LaVail MM, Hollyfield JG, editors. New insights into retinal degenerative diseases. Proceedings of the 9th International Symposium on Retinal Degeneration; 2000 Oct 9-14; Durango, CO. New York: Kluwer Academic/Plenum Publishers; 2001. p.329-33.

18. Xiao W, Oefner PJ. Denaturing high-performance liquid chromatography: A review. Hum Mutat 2001; 17:439-74.

The print version of this article was created on 15 Mar 2006. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.