Rhodopsin mutations in a Scottish retinitis pigmentosa population, including a novel splice site mutation in intron four

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Abstract
Retinitis pigmentosa (RP) is the name given to a group of disorders, both clinically and genetically heterogeneous, that primarily affect the photoreceptor function of the eye. Mutations in the genes encoding for rhodopsin, RDS-peripherin, or the β subunit of the cGMP phosphodiesterase enzyme can be responsible for the phenotype. In this study the rhodopsin gene has been screened for mutations in a panel of RP individuals and five different sequence changes have been detected to date in three dominantly inherited and two unclassified families. One of these, a base substitution in the 3'UTR, has not yet been confirmed as disease specific, while three missense substitutions have previously been reported and are likely to be responsible for the phenotype. The fifth change, a base substitution at the intron 4 acceptor splice site, represents a novel mutation and is assumed to be the causative mutation.

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The term retinitis pigmentosa (RP) describes a group of retinal degenerations that affect the photoreceptor function of the eye and are heterogeneous in their genetic and clinical presentation. The disorder may be inherited in an autosomal dominant, autosomal recessive, or X linked recessive manner, or occur as an isolated case with no previous family history. Within these subgroups the clinical picture may differ; from mild impairment to severe visual handicap in combination with an early or late age of onset. The symptoms include night blindness, commonly occurring early in the disease, followed by a progressive loss of peripheral vision and characteristic changes in the fundus. The disorder has been calculated to affect approximately one in 3500 of the population and thus constitutes a major cause of visual impairment.

Recent advances in molecular genetics have led to the identification of several genes that are responsible for the phenotype. The first of these followed the demonstration of linkage of the RP gene in a large Irish family with the marker CRI-C17 on 3q.1 Dryja and colleagues soon reported a mutation in the rhodopsin gene, also on 3q, in 17 autosomal dominant RP patients.1 Further studies have revealed over 40 different mutations in this gene which can be responsible for the disorder in dominant RP families. A mutation in the rhodopsin gene has also been shown to account for the phenotype in an individual with autosomal recessive RP.2 Non-allelic genetic heterogeneity has been shown in these two subtypes by the demonstration that a mutation in the peripherin-RDS gene may also result in dominant RP,4,7 and that mutations in the gene which codes for the β subunit of the rod phosphodiesterase enzyme may result in autosomal recessive RP.4

Other linkage studies in several dominant families indicate that the respective RP genes are located on chromosome 7p, 7q, 8q, and 19q.9-12 There is also evidence supporting the involvement of the ROM-1 gene on chromosome 11q in a dominant RP family. Considering that at least two genes are responsible for the X linked form of the disease,13 it is clear that a mutation in one of numerous genes is capable of producing the classic RP phenotype. In addition to causing RP, mutations in these genes may result in other retinal degenerations, including macular degeneration, congenital stationary nightblindness, and retinitis punctata albescens.14-17

We have collected DNA from members of 30 RP families and from 23 isolated cases of retinal degenerations, and have screened these patients using single stranded conformation polymorphism (SSCP) analysis and direct sequencing to identify the causative gene. Here we present the results from the study of the rhodopsin gene.

Patients and methods
Samples were collected from patients with RP, who presented to the RP clinic in Tennent Institute, Western Infirmary as and when referred by their general practitioners and ophthalmologists. Alternatively, patients were recruited when referred to the genetic clinic in Aberdeen Royal Hospitals by their ophthalmologists. The samples comprised a panel of RP patients representing 30 families and 23 isolated cases from a geographical area covering the west and north east of Scotland. Of the families, 12 have been confirmed as being dominant due to the observation of male to male inheritance, three are considered to be recessive families, and 15 are designated unclassified. These unclassified families have affected members in several generations but no observed male to male inheritance. Genomic DNA was extracted from 20 ml whole blood using a method based on that described by Kunkel.18

Five different sequence changes were detected, three of which were in designated dominant families. The fourth and fifth changes occur in unclassified families (Fig 1).

The first sequence change has been reported19 and occurs in a dominant family from the Tayside/Grampian region (referred to as adRP3 in Lester et al20) with a diffuse form of the
patients with gene. Individuals tested for the presence of the particular mutation are indicated (*). Arrows refer to individuals indicated in the text. Hatched symbols represent individuals in whom the RP diagnosis has not been confirmed.

Figure 1  Pedigrees of patients with mutations identified in the rhodopsin gene. Individuals tested for the presence of the particular mutation are indicated (*). Arrows refer to individuals indicated in the text. Hatched symbols represent individuals in whom the RP diagnosis has not been confirmed.

disease. Onset of symptoms (nightblindness) usually appeared in late childhood with preservation of central vision into the third or fourth decade. Clinical features include typical field losses, atrophic retinal pigment epithelial changes with characteristic bone spicule pigmentation. Electoretinographic (ERG) testing reveals grossly reduced rod and cone function, with flat responses having been recorded in one family member at age 12 years (arrowed in Fig 1). This individual had vision of 6/18 in both left and right eyes and marked constriction of visual fields (approximately 10 degrees) in each eye.

The second and third sequence changes were identified in two dominant families (M-6191 and K-6056) who come from the Strathclyde region and have typical RP symptoms including constricted fields, pale discs, and attenuated arteries. Both families have nightblindness as the onset symptom. Limited detailed clinical information is available for family K-6056.

The individual (48 years old) indicated by an arrow in family M-6191 presented to the RP clinic 7 years ago with a 20 year history of nightblindness, which had increased in severity in the past 5 years. At that time he had good central vision in each eye (6/6 NS) and early retinal and visual field changes. ERG testing revealed a normal photopic signal, but a markedly abnormal scotopic signal. Recent examinations confirm the retention of central vision, with a progression of fundus changes and very marked constriction of visual fields (approximately 15 degrees in each eye). His photopic ERG is now reduced to approximately 60% of normal and scotopic signals to below 10% of normal.

The fourth change was identified in two affected members of an unclassified family (B-6183) that appears to suffer a mild form of sectoral RP with a later age of onset. The individual indicated by the arrow in family B-6183, presented 6 years ago (age 58) at a general ophthalmic clinic after referral from her general practitioner, complaining of hazy vision in both eyes. She was subsequently referred to the RP clinic, where examination revealed vision of 6/9 and 6/12 in the right and left eyes, respectively. Fundus findings showed a typical area of sector RP affecting the inferior fundus, particularly along the inferotemporal vessels and visual field changes corresponded with this in that there was a marked loss of vision in the upper field in each eye. Her photopic ERG was recorded at 43% and 37% of normal, while her scotopic ERG was 41% and 29% of normal in right and left eyes respectively. Her affected daughter (asterisked) shows early but similar features affecting the lower fundus.

Figure 2  SSCP shift (A) and sequence (B) of the mutation (agg>atg in codon 17) detected in family B-6183. The mobility shift is indicated by the arrow (lane number indicated beside arrow) in lane 1 in A, and the corresponding sequence in B(i). Control sequence is shown in B(ii).
The fifth sequence change was observed in one individual representing a small unclassified family (F-6246). This man was first examined 3 years ago (age 25) when he attended a general clinic with a divergent squint. On noting signs of the disease, he was then referred to the RP clinic. Only on close questioning did he admit to having some visual field defect and difficulty in dark conditions. His visual acuities were at that time, 6/9 N5 right and 6/18 N14 left. He has the classic fundus appearance of RP with markedly constricted fields to less than 10 degrees in each eye. ERG testing revealed flat photopic responses and a flat left scotopic, with a small response (10% of normal) in right scotopic. He is also markedly hypermetropic with a moderate degree of astigmatism.

Both unclassified families have origins in the Strathclyde region.

Sequence changes were detected using SSCP analysis followed by direct sequencing. SSCP primers were designed to amplify the whole coding region of the rhodopsin gene and 10-20 bp of the intron/exon junctions and amplification products were electrophoresed in 5% non-denaturing acrylamide gels (with 5% glycerol at both 5°C and 20°C). Samples which displayed a mobility shift were amplified again using primers upstream of each sense and antisense primer, enabling the whole region under SSCP analysis to be sequenced.

Results
SSCP shifts were detected in exons 1, 2, 3, and 5 of the rhodopsin gene in five distinct families. No mobility shifts were identified in the isolated cases. Sequencing revealed the shift in exon 1 (Fig 2A) to originate from the heterozygous base substitution ACG to ATG (threonine to methionine) in codon 17 of the rhodopsin gene. The sequence change is shown in Figure 2B next to the normal sequence. This mutation was observed in two affected individuals in two generations of this family (B-6183), but was absent from an unaffected member.

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**Figure 3** SSCP shift (A) and sequence (B) of the mutation (g> t) in codon 135 detected in family F-6246. The mobility shift is indicated by the arrows (lane number indicated beside arrows) in lane 1 in A, and the corresponding sequence in B(i). Control sequence is shown in B(ii).

**Figure 4** SSCP shifts (A) and sequences (B-C) of the mutations detected in the families K-6056 (g>a transition at position 5299) and M-6191 (g>a at intron 4 acceptor splice site). The mobility shifts are indicated by the arrows (with lane numbers indicated beside arrows) in lanes 1 (K-6056) and 2 (M-6191) in A, and the corresponding sequences in B(i) and C(i) respectively. Control sequence at these points are also shown (B(ii) and C(ii)).
the mutation has been observed in other families with adRP\textsuperscript{23,24} and is therefore believed to be responsible for the phenotype.

The shift in exon 2 was observed in one individual, belonging to the family F-6246. The SSCP shift is shown in Figure 3A and sequencing revealed a heterozygous base substitution in codon 135: CGG (arginine) to TGG (tryptophan). The change is shown in Figure 3B. Although this sequence change has only been identified in one individual in an unclassified family, it is likely to be the responsible mutation, as it has previously been reported in dominant RP.\textsuperscript{2*}

The shift in exon 3 was observed in an affected member of the family designated adRP3 and found to be due to a base substitution in codon 178 (TAC (tyrosine) to TGC (cysteine) results not shown). This mutation abolishes a Rsa restriction site and restriction analysis shows the segregation of the mutation with the phenotype as reported previously.\textsuperscript{19}

Two different mobility shifts were observed near exon 5 of the rhodopsin gene. The two shifts are shown in Figure 4A. The shift shown in lane 1 was due to a heterozygous G to A substitution at position 5299 in the 3' untranslated region (3'UTR) of the gene. This mutation was detected in an affected individual of family K-6056 and is shown in Figure 4B. The shift in lane 2 was due to a heterozygous G to A (AG to AA) substitution at the 3' acceptor site of intron 4 and was observed in three affected family members of family M-6191. The sequence is shown in Figure 4C. Normal sequence at these points are shown alongside as a comparison.

Discussion

RHOODOPSIN MUTATIONS IN RP

The mutations identified in exons 1, 2, and 3 have all previously been identified in autosomal dominant cases of retinitis pigmentosa\textsuperscript{22-25} and therefore, although the exon 2 mutation in family F-6246 has only been identified in one individual, it is likely to be the causative mutation in that family. Similarly, the mutations identified in exons 1 and 3 are considered responsible for the phenotype in the respective families (B-6183 and adRP3).

The two sequence changes in exon 5 have not been reported previously. The first of these, a G to A substitution at position 5299 (family K-6056) has not been confirmed in any other affected family members owing to the limited availability of additional samples, and it is therefore conceivable that the change is merely a neutral polymorphism. We have screened a panel of 88 control samples (178 alleles) for the presence of this sequence change and have found the change to be absent in this sample population. However, until it can be shown to segregate with RP in a family it is best considered a (rare) polymorphism. Attempts are under way to locate additional family members to determine the nature of the change.

The novel splice site mutation was observed in three affected siblings and absent in a fourth unaffected sibling of a dominant family (M-6191). Although this mutation has not been confirmed through two generations it is likely that it is the disease specific mutation in the family. The sequence change destroys the conserved 3' splice acceptor site (AG) which is essential for accurate splicing of mRNA. Mutations within these splice sites are known to result in abnormal splicing and commonly result in disease. This has been observed in choroideremia and gyrate atrophy.\textsuperscript{26,27}

EFFECT OF MUTATION ON RHODOPSIN MOLECULE

Rhodopsin is the major protein of the rod outer segments and functions as the light absorbing protein, initiating a cascade of reactions that mediate dim vision. However, the mechanisms by which these and previously identified mutations in the rhodopsin gene result in disease are unclear.

Sung and colleagues have studied several mutant rhodopsin protein species using transfected cells and have designated two mutant classes: the first of these, known as class I, groups together those proteins which resemble wild type rhodopsin in yield, regenerability with the chromophore and subcellular localisation (the plasma membrane (PM)), while class II mutants refers to the proteins which accumulate to significantly lower levels than the wild type, regenerate the chromophore inefficiently and are retained predominantly in the endoplasmic reticulum (ER) (class IIa). Class IIb proteins are similar to class IIa except in their cellular localisation, which is distributed roughly between the ER and PM.\textsuperscript{28} Abnormal proteins that fail to fold correctly or assemble into the required complexes have been shown to be retained in the ER, in a manner similar to those described as class II mutants, and are degraded therein.\textsuperscript{29} That three of the mutations discussed here (codons 17, 135, and 178) have been assigned to the class II type\textsuperscript{29} and thus retained in the ER is not surprising if we consider the likely effects of each sequence change.

The first mutation, Thr 17 Met, destroys the three residue recognition sequence (Asn-'Ala-'Thr)\textsuperscript{30} that is required for glycosylation at Asn\textsuperscript{15} and the subsequent prevention of glycosylation presumably results in an abnormally folded protein. The close proximity of the second mutation (Tyr 178 Cys) to the conserved Cys\textsuperscript{16}, which is involved in forming the disulphide bridge with Cys\textsuperscript{16}, and crucial in the tertiary structure of rhodopsin, may be also be considered deleterious to correct folding. The mutation Arg 135 Trp occurs in one of the transmembrane segments, which contain several invariant glycine and proline residues and are thought to be important in forming the three dimensional structure of the protein, especially in forming a pocket for the invariant Lys\textsuperscript{27}. The substitution of this amino acid may again affect the proper folding of the protein.

The effect of the remaining sequence change is as yet unclear. Mutations in splice sites commonly lead to the use of cryptic sites, or exon skipping, which result in the production of an altered protein, either with several residues or large portions of the gene deleted. Alternatively, the intron may be retained in the processed transcript and if the transcript is stable then the intron sequence will be translated. We are currently
studying this mutation further using mRNA isolated from peripheral blood lymphocytes of an affected individual to determine the effect of the sequence change. Early studies indicate that the intron is retained in the transcript (unpublished results) which subsequently results in the formation of a premature stop codon in intron four. The protein produced as a result would be missing amino acids 313-348, the carboxyl terminal of the protein. This region contains several invariant serine and threonine residues, which are phosphorylated during the recovery phase to block the action of rhodopsin.

RHODOPSIN MUTATION AND ITS RELATION TO THE PHENOTYPE

The manner in which these mutations result in the disease phenotype is unclear. Although the mutations can be divided into class I/II mutants, this does not correlate with a particular phenotype. Within class I and class II mutants there exist both severe and mild phenotypes and this is further demonstrated in these results: the rhodopsin molecules with mutations in codons 17 and 135/178 belong to class II but are generally considered to have mild and severe phenotypes respectively. The further subdivision of the class II mutants does not correlate directly with phenotype either, as Thr17 Met (clinically mild) and Tyr178 Cys (clinically severe) both belong to class IIa. Additional factors may play a role in the progression of the disease in the more severe forms with mutations in this class. The Arg 135 Trp mutation belongs to class IIb, where equal amounts of the protein are located in the ER and the PM. The phenotypic severity resulting from this mutation may be due to the presence of the mutant protein in the outer segments, where it has been shown to be defective in activating nucleotide exchange by transducin, thus preventing further amplification of the response. Perhaps the combination of this defect, together with competitive binding with the wild type protein are sufficient to effect a severe phenotype.

Clearly, further studies are required to understand the pathogenic effect of these and similar rhodopsin mutations in rod photoreceptors and how these changes also affect, in some instances, the cone cells.

Although the pathogenesis of the condition is not fully understood, knowledge of the actual mutation may provide ophthalmologists with a prognostic indicator for specific families. The codon 17 mutation has previously been reported in families with a regional predilection for pigmentary changes and appears to correlate with a form of the disease having a moderately good prognosis, and the severity of the disease in the families with the codon 135/178 mutations also concur with previous reports.

It is apparent from the above discussion that some unknown factors influence the effect of the resulting mutant protein in the rod cells and the progression of the disease – the observation of decreased levels of certain polyunsaturated acids in the plasma of affected individuals in family aDRP suggests that a gene concerned with the transport or synthesis of fatty acids may be linked to the progression of the disease. In addition, light toxicity has been suggested as a secondary factor in RP.

Undoubtedly, as the number of causative genes increase, and their biochemical nature and/or structural significance are realised, the complex events of the photoreceptor cell will become clearer. Combined with an increase in knowledge of interacting factors, definition of the pathogenic mechanisms involved in the disease process may result and lead to some form of therapeutic intervention.

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