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Identification of Novel Rhodopsin Mutations Associated with Retinitis Pigmentosa by GC-clamped Denaturing Gradient Gel Electrophoresis

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Summary

Retinitis pigmentosa (RP) is a group of disorders characterized by progressive degeneration of the outer retina, resulting in night blindness, visual field loss, an abnormal electroretinogram, and characteristic retinal pigmentary changes. An important step in the understanding of RP has been the recognition that some cases of autosomal dominant RP (ADRP) are caused by mutations in the rhodopsin gene. Multiple different point mutations within the coding sequence of the rhodopsin gene have been associated with ADRP. We have developed a GC-clamped denaturing-gradient-gel electrophoresis (DGGE) assay for the coding region of the rhodopsin gene and have used this assay to screen ADRP patients for mutations. The assay consists of amplifying with PCR the five exons of the rhodopsin gene and then analyzing each PCR product by DGGE. We have used this assay to detect three previously unreported rhodopsin base substitutions associated with ADRP. The use of this assay to identify ADRP patients who have various rhodopsin mutations has allowed us to begin studies seeking to correlate molecular genotype with clinical phenotype. Furthermore, GC-clamped DGGE has allowed us to identify families with ADRP not caused by a rhodopsin mutation. Such families will be important in the search for other genes involved in ADRP.

Introduction

"Retinitis pigmentosa" (RP) is a general term used to refer to a group of hereditary diseases characterized by progressive night blindness, visual field loss, an abnormal electroretinogram, degeneration of the outer retina, and a characteristic ophthalmoscopic appearance of "bone-spicule-like" pigmentation. RP has three different modes of inheritance including autosomal dominant (ADRP), autosomal recessive, and X linked (Boughman et al. 1980; Bunker et al. 1984). The variation in mode of inheritance makes it difficult to counsel families about recurrence risk, particularly in sporadic cases and in cases for which family history is not available. In addition, counseling of affected individuals as to their visual prognosis is difficult because of the wide range of both age at onset and rate of visual deterioration known to occur in RP. The ability to make a molecular diagnosis of RP will greatly aid in counseling patients and family members as to recurrence risk. Moreover, as the phenotypes associated with specific mutations are characterized, molecular diagnosis may allow an accurate prognosis to be made. An understanding of the molecular defects that cause RP may also contribute to the understanding of the pathophysiology of this group of disorders.

An important step in the understanding of RP at the molecular level has been the recognition that some cases of ADRP are the result of a mutant rhodopsin gene (Dryja et al. 1990*a*, 1990*b*). Several point mutations within the coding sequence of the rhodopsin gene have been associated with ADRP (Dryja et al. 1990*a*, 1990*b*). In the present study, we report the develop-

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ment of a GC-clamped denaturing-gradient-gel electrophoresis (DGGE) assay which makes it possible to efficiently screen the rhodopsin coding sequence for point mutations. We have utilized this assay to identify three previously unreported rhodopsin gene nucleotide substitutions. These nucleotide substitutions are found in RP patients and not in normal controls, suggesting that these changes are mutations associated with ADRP. In addition, we identified a rhodopsin gene polymorphism found in both affected and control individuals. Furthermore, we have demonstrated that this DGGE assay is capable of detecting the previously reported mutations which cause ADRP.

Material and Methods

RP patients were recruited from the ophthalmology clinics at the University of Iowa and the University of Illinois. The diagnosis of RP was based on ophthalmoscopy, electroretinography, visual field testing, and dark adaptometry. Only patients with a family history of RP consistent with autosomal dominant inheritance were included in the study. To our knowledge, probands and kindreds utilized in the present study have not been investigated previously in other laboratories. A peripheral blood sample was drawn from probands as well as from all available family members. In addition, blood was obtained from 50 unrelated control individuals living in Iowa or Illinois.

DNA Preparation

DNA was prepared from blood samples by using the protocol of Grimberg et al. (1989). In brief, whole blood was mixed with a four-fold volume of cold CLB buffer (0.32 M sucrose, 10 mM Tris-HC1 pH 7.6, 5 mM $MgCl_2$, 1% Triton X-100). The resulting lysate was centrifuged to obtain a nuclear pellet. The pellet was dissolved in proteinase K buffer containing 10 μg proteinase K/ml and was digested for 2 h at 65°C. DNA concentration was measured using a Hoefer spectrofluorimeter in the presence of Hoechst dye.

DNA Amplification

Primers for DNA amplification with PCR were selected from the human rhodopsin gene sequence (Nathans and Hogness 1984) and were prepared using phosphoramidite chemistry with an Applied Biosystem model 391 oligonucleotide synthesizer. Two primer pairs were used to amplify overlapping fragments of exon 1. One primer pair was used to amplify each of the remaining four rhodopsin gene exons. PCR amplification with all primer pairs was performed under identical conditions by using minor modifications of a previously reported protocol (Sheffield et al. 1989). Specifically, approximately 500 ng of human genomic DNA was mixed with 50 pmol of each appropriate nucleotide primer and with 37.5 nmol of each deoxyribonucleoside triphosphate in 100 μl of PCR buffer (67 mM Tris-HC1 pH 8.8, 6.7 mM $MgCl_2$, 16 mM ammonium sulfate, 10 mM 2-mercaptoethanol, 10% dimethylsulfoxide). *Thermus aquaticus* DNA polymerase (1.5 units) was then added to each sample. The samples were overlaid with approximately 50 μl of mineral oil and incubated at 94°C, 58°C, and 72°C for 30 s at each temperature in a Cetus model TC1 thermocycler for a total of 40 cycles. Ten microliters of the resulting amplification product were electrophoresed on a 1.5% agarose gel to check for success of amplification.

DGGE

The optimum gradient of DNA denaturant for each PCR product was determined experimentally by analyzing each DNA fragment with perpendicular DGGE described elsewhere (Myers et al. 1987, 1988, 1989). Samples (10–20 μl) of each amplified DNA product to be tested for mutations were electrophoresed on a parallel denaturing gradient gel prepared as reported elsewhere (Myers et al. 1987, 1988). These gels consisted of 8% polyacrylamide (37:1 acrylamide: bis acrylamide) containing a linear gradient of DNA denaturants (formamide and urea). On the basis of results of the perpendicular DGGE, the range of DNA denaturant differed slightly for each specific rhodopsin gene fragment being analyzed (exons 1 and 5, 50%–75%; exon 2, 55%–80%; exons 3 and 4, 40%–70%. One-hundred-percent denaturant is defined as 7 M urea and 40% [v/v] formamide). Electrophoresis was performed at 150 V for 8 h at constant 60°C temperature. Gels were stained with ethidium bromide and were photographed by using Polaroid positive/negative film. An exon containing a mutation was recognized on the basis of the appearance of one or more new bands. Samples that, on the basis of DGGE analysis, were shown to contain a mutation were sequenced.

DNA Sequencing

Direct sequencing of PCR products was performed using a protocol described elsewhere (Dubose and Hartl 1990). In brief, the remaining amplified DNA was electrophoresed on a 1.5% preparative agarose

gel to separate the amplified product from the remaining primer. The fragment was recovered from the agarose by cutting the desired band from the agarose gel, freezing it either at −70°C for 15 min or at −20°C overnight, and centrifuging it in a Costar 0.22-μm cellulose acetate filter unit. The DNA was ethanol precipitated and resuspended in 15 μl of dH_2O. Sequencing was then performed with 7 μl of the sample by using a USB sequencing kit according to manufacturer's instructions, except that 10 pmol of primer was used. The sequencing reactions were electrophoresed on an 8% polyacrylamide sequencing gel. The gel was dried and autoradiographed overnight by using Kodak X-OMAT AR film.

Results

In order to screen ADRP patients for rhodopsin gene mutations, six pairs of primers for PCR were selected. Two primer pairs were used to amplify overlapping fragments of the largest exon, exon 1. One primer pair was used to amplify each of the remaining four exons. The primer sequences, their nucleotide positions within the rhodopsin gene (Nathans and Hogness 1984), and the size of each PCR product are listed in table 1. One primer of each primer pair contained a 5′ 40-bp GC-rich sequence (GC-clamp). This clamp sequence is incorporated into the PCR amplification product during the amplification reaction.

Each of the six rhodopsin gene fragments was amplified separately by PCR, from the genomic DNA of 34 unrelated ADRP patients, in preparations for analysis by DGGE. In order to optimize the DGGE conditions for each DNA fragment, we analyzed, by perpendicular DGGE (Myers et al. 1987, 1988, 1989), each rhodopsin-exon PCR product from a normal individual, to determine melting-domain structure. This allowed us to determine the ideal range of denaturant to be used for each DNA fragment, so that single base mutations could be detected by DGGE at the greatest level of sensitivity. Once the optimum conditions for DGGE were determined, the amplified DNA samples from each ADRP patient were analyzed on the appropriate denaturing gradient gel. Samples containing a nucleotide change were recognized by the presence of two or more DNA bands on the denaturing gradient gel, compared with a single band in normal samples. Multiple bands are the result of the normal

Table 1

Rhodopsin Oligonucleotides Used for Amplification of Rhodopsin Exons

Exon and Oligonucleotide[a]		Size of PCR Product (bp)	Denaturant Concentration[b] (%)
Exon 1a:			
Sense.....	GC-clamp-5′-CTGAGCTCAGGCCTTCGCAGCAT-3′ (228–250)	310	50–75
Antisense.....	5′-GCTTCTTGTGCTGGACGGTGAC-3′ (498–477)		
Exon 1b:			
Sense.....	5′-TCTGCTGATCGTGCTGGGCTT-3′ (428–449)	400	50–75
Antisense.....	GC-clamp-5′-GAGGGCTTTGGATAACATTG-3′ (788–769)		
Exon 2:			
Sense.....	GC-clamp-5′-GGCAGTGGGGTCTGTGCTGAC-3′ (2396–2417)	295	55–80
Antisense.....	5′-AAGAGCCCCCGGAGCTTCTTC-3′ (2651–2630)		
Exon 3:			
Sense.....	5′-CAGCGCTCGGCAGCCACCTT-3′ (3741–3760)	321	40–70
Antisense.....	GC-clamp-5′-CTGGACCCTCAGAGCCGTGA-3′ (4022–4002)		
Exon 4:			
Sense.....	GC-clamp-5′-CTGGAGGAGCCATGGTCTGGA-3′ (4053–4073)	417	40–70
Antisense.....	5′-TGGCAGGCAGCGCCTGTGGCCT-3′ (4390–4369)		
Exon 5:			
Sense.....	GC-clamp-5′-CGTGCCAGTTCCAAGCACTGTGG-3′ (5106–5130)	320	50–75
Antisense.....	5′-ACTTCGTTCATTCTGCACAGGCG-3′ (5386–5364)		

[a] Numbers in parentheses indicate nucleotide position of oligonucleotide sequence within rhodopsin gene. GC-clamp = sequence 5′-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCG-3′.

[b] 100% denaturant = 7 M urea and 40% (v/v) formamide.

allele being separated from the mutant or polymorphic allele and the heteroduplexes being separated from homoduplexes. Heteroduplexes are the result of a normal DNA strand being annealed with a mutant or polymorphic complementary DNA strand during the last cycle of the PCR reaction in samples containing both a normal and mutant allele (i.e., heterozygous samples) (Sheffield et al. 1989).

DGGE analysis of the five rhodopsin exons from 34 unrelated ADRP patients identified eight patients with nucleotide changes. Six of these patients had a change in exon 1. Sequence analysis revealed that four of these six patients were heterozygous for a codon 23 mutation (Pro23His) reported elsewhere by Dryja et al. (1990*b*). One patient was heterozygous for a codon 58 mutation (Thr58Arg) also reported elsewhere (Dryja et al. 1990*a*). The remaining patient had a C-to-T transition at codon 17, which resulted in a threonine-to-methionine change (Thr17Met). Each of these exon 1 changes was detected by DGGE analysis of the 5′ half of exon 1 (exon 1a primer pair), and each has a distinctive band pattern on a denaturing gradient gel (fig. 1). Since codon 58 lies within the region of overlap of the 5′ and 3′ PCR amplification products spanning exon 1, a change in this codon was detected by DGGE analysis of both of these fragments in the single patient with a base change in this codon (data not shown).

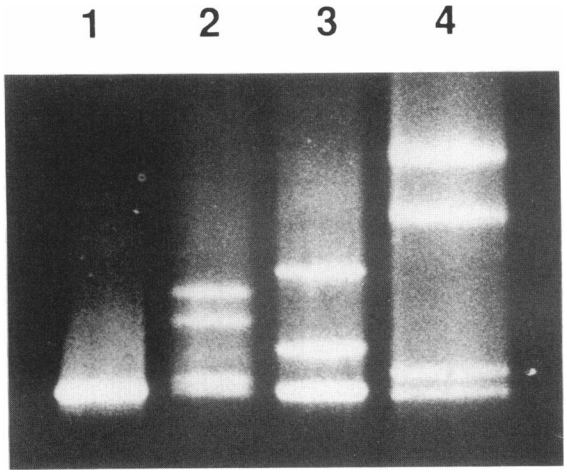

Figure 1 PCR-amplified exon 1 fragment (primer pair 1a) from three ADRP patients and normal individual. The amplified products were electrophoresed on a 50%–75% denaturing gradient gel for 7 h at 150 V at 60°C. The photograph is the positive image of an ethidium bromide–stained gel. The results show a normal control (lane 1), Thr17Met heterozygote (lane 2), Pro23His heterozygote (lane 3), and Thr58Arg heterozygote (lane 4).

In addition to the exon 1 changes, DGGE analysis of the 34 patients also revealed one patient heterozygous for an exon 3 base change and one patient heterozygous for an exon 4 base change. Sequence analysis identified these changes to be a G-to-A transition at codon 182, which causes a glycine-to-serine substitution (Gly182Ser), and a C-to-T transition at codon 267 in exon 4, which causes a proline-to-leucine change (Pro267Leu). Figure 2 shows the direct sequence analysis of the Thr17Met, Gly182Ser, and Pro267Leu changes identified in the present study. It should be noted that, although all five exons were screened for mutations in these patients, no other changes were detected in the rest of the coding sequence of the rhodopsin gene.

In order to determine whether the nucleotide changes identified in the ADRP patients were likely to be disease-causing mutations or benign polymorphisms, we analyzed 50 control individuals by DGGE. No base changes were found within the coding region in any of these control individuals. Thus, in summary, a total of eight base changes resulting in amino acid substitutions were identified in 34 ADRP patients, but no such changes were identified in 50 controls (P = .0012 by Fisher's exact probability as calculated on the 2 × 2 table).

To investigate further whether the Thr17Met and Gly182Ser changes are mutations, we looked for segregation of the changes with the RP phenotype within the affected families. DNA was prepared from other available members of the families, and the rhodopsin gene was analyzed by using the PCR-DGGE assay. In all cases, affected individuals were shown to be heterozygous for the change as analyzed by DGGE (fig. 3). This analysis was not performed for the Pro267Leu substitution because additional family members were not available for study.

DGGE analysis of the PCR amplification product containing exon 5 sequences from control and ADRP patients revealed an altered band pattern in several individuals. This alteration was found in both control and affected individuals and is thus a polymorphism. This polymorphism was the only change detected in 50 unrelated controls. We used this polymorphism for linkage analysis in a portion of a large ADRP pedigree whose members did not have a DGGE detectable mutation in any exon. The results show a recombination event between the rhodopsin gene and the RP pheno-

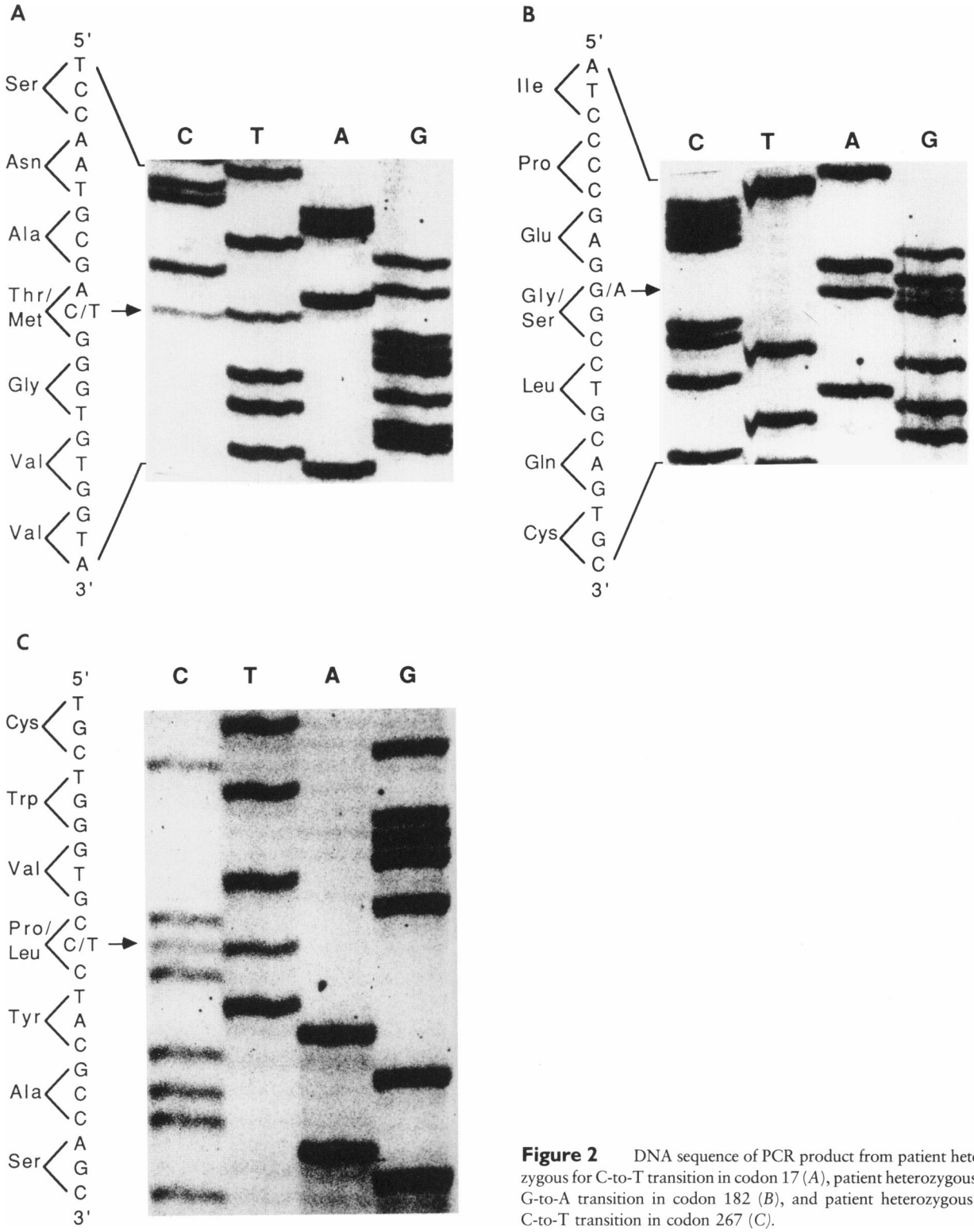

Figure 2 DNA sequence of PCR product from patient heterozygous for C-to-T transition in codon 17 (*A*), patient heterozygous for G-to-A transition in codon 182 (*B*), and patient heterozygous for C-to-T transition in codon 267 (*C*).

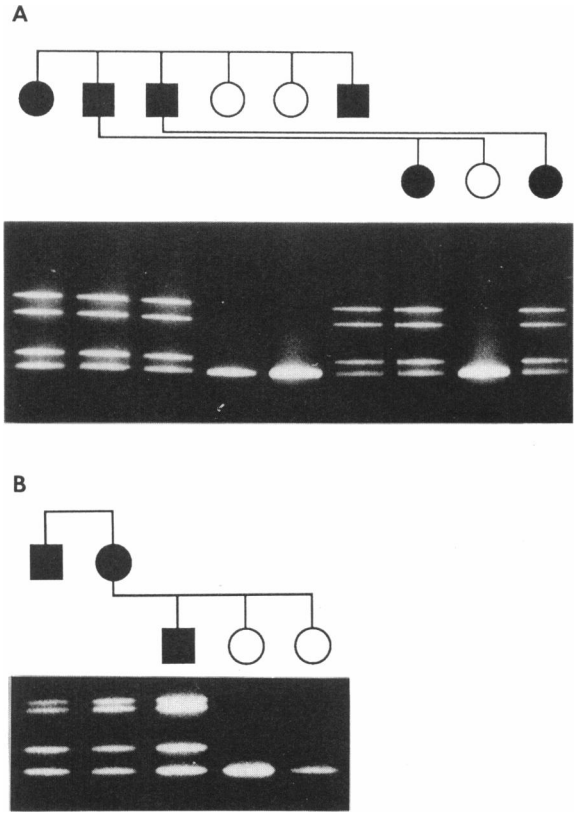

Figure 3 DGGE analysis of families identified in present study as having Thr17Met (*A*) and Gly182Ser (*B*) mutations. In each case, clinically affected members of the family (blackened symbols) carry the base change while clinically normal individuals (unblackened symbols) do not.

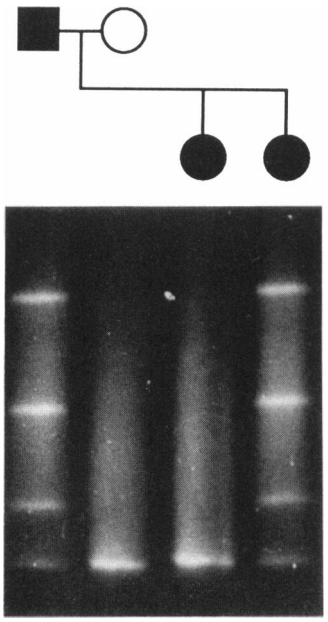

Figure 4 ADRP family with polymorphism in PCR product that contains exon 5 of rhodopsin gene. In this instance, the base change does not segregate with the disease, as demonstrated by the fact that the two affected offspring received different rhodopsin alleles from their affected father. This nuclear family is part of a larger extended pedigree (not shown).

type. This demonstrates that ADRP is not likely to be rhodopsin associated in this family (fig. 4).

Discussion

RP is a potentially blinding disease that affects 1/3,500 patients in the United States (Bunker et al. 1984). Both for patients and for their physicians one of the most frustrating aspects of the disease is that the visual prognosis varies widely, making it difficult for patients to plan their lives and for physicians to counsel them accurately. The recognition of specific mutations in the rhodopsin gene that cause some forms of RP is encouraging from three perspectives. First, it allows some forms of ADRP to be diagnosed rapidly even in the absence of a family history. Second, it raises the possibility that specific phenotypes will be associated with several of the mutations, so that much more accurate and timely counseling can be provided to affected patients. Third, it is possible that, by correlation of specific mutations with the clinical phenotype, some clues to the pathogenic mechanism of the disease will be found that will eventually allow the course of the disease to be altered by physical or pharmacologic means.

In the present study, we have developed a GC-clamped DGGE assay which can be used to screen efficiently for rhodopsin mutations in RP patients. The assay consists of using PCR to amplify separately the five exons of the rhodopsin gene by primers which flank the exon sequence, followed by analysis of each exon PCR product with DGGE. One primer of each primer pair used for PCR amplification contains a 40-bp GC-rich sequence (GC-clamp) which becomes incorporated into the PCR product. The presence of the GC-clamp has been shown to increase the sensitivity of DGGE, allowing for detection of nearly 100% of all possible base changes (Myers et al. 1985; Sheffield et al. 1989). This assay can be utilized effectively to screen patients for rhodopsin mutations lying

anywhere within the coding region. By using this approach, we identified patients with the known exon 1 point mutations (Pro23His and Thr58Arg). In addition, we identified previously unreported base changes in exons 1, 3, and 4. The three base changes reported in the present study result in amino acid changes—from threonine to methionine, from glycine to serine, and from proline to leucine, in codons 17, 182, and 267, respectively. We did not identify any patients who had the two previously reported exon 5 mutations (both in codon 347), nor did we identify a patient with the reported 3-bp deletion (Inglehearn et al. 1991). However, computer analysis (Lerman and Silverstein 1987) indicates that all of these mutations can be detected by our DGGE assay.

In total, in 34 patients tested, we identified eight (23.5%) who had base changes which result in amino acid substitutions. We did not identify any such changes in 50 control individuals. Statistical analysis reveals this difference to be highly significant (P = .0012). These data support the previous Dryja et al.'s (1990*b*) finding that mutations in the rhodopsin gene cause some cases of ADRP. However, these data do not prove that any individual base change identified in our study is a disease-causing mutation. Such proof awaits the development of a functional assay for rhodopsin.

The three novel substitutions (Thr17Met, Gly182Ser, and Pro267Leu) identified in the present study are likely to be rhodopsin mutations resulting in RP, for three reasons. First, they change an amino acid which is evolutionarily conserved in both the chicken and bovine rhodopsin protein sequence (Nathans and Hogness 1983; Takao et al. 1988). Second, we have not found these alterations in control individuals. Third, the Thr17Met and Gly182Ser mutations have been shown to segregate with the disease within an affected family (affected individuals have the base change, and unaffected individuals do not) (see fig. 3). Segregation with the disease phenotype could not be demonstrated for the Pro267Leu change because other members of the family were not available to study. However, this proline lies within a highly conserved region of the protein, a region which is conserved even in *Drosophila* (Zuker et al. 1985).

The fact that these rhodopsin gene changes are likely to be mutations is further supported by the finding that no other changes were detected within the coding region of the rhodopsin gene in patients with one of these nucleotide substitutions. Previous studies have demonstrated that GC-clamped DGGE can be used to detect nearly 100% of single base changes (Myers et al. 1985; Sheffield et al. 1989). Thus it is unlikely that other changes exist within the coding region of this gene in the patients examined. It should be noted, however, that mutations in regulatory sequences lying upstream from the initiation site would not be detected by the assay used in the present study. Such mutations would result in a change in the amount of rhodopsin synthesized rather than in the synthesis of an abnormal rhodopsin protein. A change in the amount of rhodopsin synthesized would be unlikely to result in a dominant phenotype. All mutations identified in the rhodopsin gene to date have been within the coding sequence (Dryja et al. 1990*a*, 1990*b*; Inglehearn et al. 1991).

The fact that multiple different nucleotide changes within the rhodopsin gene are associated with ADRP serves to underscore the need for an effective method of detecting all point mutations, if ADRP is to be diagnosed efficiently at the molecular level. The DGGE assay described in the present paper has the advantage of being able to detect both known and unknown base substitutions. In addition, this approach makes it possible to distinguish one change from another. For example, the codons 23, 58, and 17 nucleotide substitutions, all found in exon 1, give distinctly different patterns when analyzed by DGGE (see fig 1).

The ability to detect nearly all point mutations makes it feasible to identify patients whose RP is the result of a rhodopsin mutation. This is very useful when patients are counseled with respect to recurrence risk. In addition, results from our and other laboratories indicate that patients with the Pro23His rhodopsin change have relatively mild disease (Berson et al. 1991; Heckenlively et al. 1991; E. M. Stone and V. C. Sheffield, unpublished data). Such information is useful when prognosis is discussed with a patient. The DGGE assay described in the present study will also be of value both in identifying additional rhodopsin mutations and in studies seeking to correlate genotype with phenotype.

In addition to the mutations, we also identified one DNA polymorphism in the present study. The PCR amplification product containing exon 5 also contains approximately 100 bp of the 3′ untranslated region of the gene. We identified a DGGE polymorphism in this portion of the gene in several control and RP individuals. Excluding the unlikely possibility of an intragenic recombination event, we have eliminated the rhodopsin gene as the disease-causing gene in one ADRP family by using this polymorphism for linkage analysis.

These data illustrate the power of GC-clamped DGGE for identifying informative polymorphisms within specific genes, for use in linkage analysis in specific families. The DGGE assay described in the present study can be used in a two-step process to identify ADRP families in which rhodopsin is not the disease-causing gene. First, the presence of a mutation within the rhodopsin coding sequence can be ruled out by using the GC-clamped DGGE assay described, and, second, DGGE of amplified DNA from noncoding regions of the rhodopsin gene can identify polymorphisms for linkage studies in a specific family. Polymorphisms identified by DGGE can then be used to exclude linkage to the rhodopsin gene in these families.

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