Rhodopsin mutations in autosomal dominant retinitis pigmentosa

CHING-HWA SUNG*, CAROL M. DAVENPORT*, JILL C. HENNESSY†, IRENE H. MAUMENE†, SAMUEL G. JACOBSON§, JOHN R. HECKENLIVELY¶, RODNEY NOWAKOWSKI, GERALD FISCHMAN**, PETER GOURAS‡†, AND JEREMY NATHANS‡‡

*Howard Hughes Medical Institute, Department of Molecular Biology and Genetics, and Department of Neuroscience, Johns Hopkins University School of Medicine, 725 North Wolfe Street, Baltimore, MD 21205; †National Retinitis Pigmentosa Foundation, 1401 Mount Royal Avenue, Baltimore, MD 21217; ‡Wilmer Ophthalmologic Institute, Johns Hopkins University School of Medicine, 600 E. Monument Street, Baltimore, MD 21205; ¶Bascom Palmer Eye Institute, University of Miami School of Medicine, Miami, FL 33101; §Jules Stein Eye Institute, University of California at Los Angeles School of Medicine, Los Angeles, CA 90024; †School of Optometry, University of Alabama at Birmingham, Birmingham, AL 35294; **Department of Ophthalmology, University of Illinois College of Medicine, Chicago, IL 60612; and ‡‡Department of Ophthalmology, Columbia University College of Physicians and Surgeons, New York, NY 10032

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ABSTRACT DNA samples from 161 unrelated patients with autosomal dominant retinitis pigmentosa were screened for point mutations in the rhodopsin gene by using the polymerase chain reaction and denaturing gradient gel electrophoresis. Thirty-nine patients were found to carry 1 of 13 different point mutations at 12 amino acid positions. The presence or absence of the mutations correlated with the presence or absence of retinitis pigmentosa in 174 out of 179 individuals tested in 17 families. The mutations were absent from 118 control subjects with normal vision.

Human vision is mediated by four visual pigments. Rhodopsin, the pigment in rods, mediates vision in dim light; the red-, green-, and blue-sensitive pigments reside in the cones and mediate color vision. The visual pigments form a family of homologous proteins encoded by the corresponding members of a family of genes (1, 2).

The goal of the present study is to identify mutations in the genes encoding human rhodopsin. In planning this study, we first considered the phenotypes that result from alterations in the genes encoding the human cone pigments. One class of rearrangements in the red and green pigment gene cluster preserves the structural integrity of the encoded proteins and causes the benign anomalies of color vision commonly referred to as color blindness (3). A second class of mutations is responsible for blue cone monochromacy, a dysfunction of both red and green cones that is associated in some individuals with a progressive degeneration of the cone-rich central retina (4, 5); and a third type of mutation causes red cone dysfunction and a progressive central degeneration (6). Recent experiments show that point mutations in the blue pigment gene lead to defective blue cone function (C. Weitz and J.N., unpublished results).

By analogy with the known cone pigment gene defects, we reasoned that rhodopsin gene mutations would produce a deficit in rod photoreceptor function (i.e., night blindness) and might lead to a progressive degeneration and consequent loss of function in the rod-rich peripheral retina. Night blindness and progressive loss of peripheral vision are the hallmarks of retinitis pigmentosa, a group of inherited disorders that appear to affect the photoreceptors and underlying pigment epithelium (7). Retinitis pigmentosa is known to be genetically heterogeneous, comprising X chromosome-linked, autosomal recessive, and autosomal dominant types (8). Within each genetic type there is marked individual variation in natural history. Any particular mutation in a given candidate gene is, therefore, unlikely to be present in more than a fraction of the retinitis pigmentosa population.

To search efficiently for rhodopsin mutations, our strategy has been to collect DNA samples from a large number of unrelated patients with retinitis pigmentosa, amplify the rhodopsin gene exons by using PCR (9), and then assay the amplified products for sequence variation by using denaturing gradient gel electrophoresis (DGGE) (10). We focused initially on 161 unrelated patients with autosomal dominant retinitis pigmentosa (ADRP). This choice was prompted by the report of McWilliam et al. (11) that in one large Irish family the gene responsible for ADRP cosegregates with markers tightly linked to the rhodopsin gene on the long arm of chromosome 3 (2).

Here we report that 39 out of 161 unrelated patients with ADRP carry point mutations in the coding sequence of the rhodopsin gene. The mutations produce 13 predicted amino acid substitutions at 12 different positions in the rhodopsin molecule. Related results have been reported by Dryja and Bhattacharya and their colleagues (12-14) in screening for rhodopsin gene mutations in ADRP patients.

MATERIALS AND METHODS

Sample Collection and Processing. Participants were recruited by their ophthalmologists (I.H.M., S.G.J., G.F., J.R.H., and P.G.), optometrist (R.N.), or through the computerized Retinitis Pigmentosa Foundation National Registry (I.J.H.). Control samples were obtained from students at the United States Air Force Academy. DNA was prepared from venous blood by proteinase K digestion, followed by equilibrium centrifugation in a cesium chloride density gradient (1).

PCR Amplification and DGGE. Seven segments of the rhodopsin gene encompassing the entire coding region (1) were amplified by PCR using the following primer pairs [the notation (GC) indicates the sequence CCGC CGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGG added to the 5' end of the oligonucleotide (10)]; exon 1 5' half (1L), TTTG CACATCTTCTGTTG and (GC)TGTGCTGAGCGTGAGGCAGTGAAGGTAAGGAA; exon 1 3' half (1R), ATGCTGGCCTCATGTT and (GC)AAGCGGCAGATCTCCAGACAGCTTCCATGC; exon 2, TGCACCCCTGTTAGCGGTGAG and (GC)AAGACACTCTGGTGTGAGTCTCGA-

Abbreviations: ADRP, autosomal dominant retinitis pigmentosa; DGGE, denaturing gradient gel electrophoresis; amino acid substitutions are referred to by the single-letter amino acid designation of the wild-type residue, followed by its position number in the polypeptide chain and the single-letter amino acid designation of the mutant residue (e.g., P23H refers to the replacement of proline-23 by histidine).

††To whom reprint requests should be addressed.

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CTGGAG; exon 3, GGCAGCACCCTTTGCTGTTC; and (GC)AGATGCATGCTGGGGCTGGACCCCTAGACG; exon 4 3' half (4R), CCCCAGGATCAGCAGGCTGCTGC; exon 3, CATGCATCTG and AGCTAAGCTTATGAGATGGGACCGAAGTTG; exon 4 3' half (4R), CCCCAGGATCAGCAGGCTGCTGC; and (GC)CCCTGCCTGGGAGTAGCTTGCTTGGCAG; exon 5, (GC)GAACGTGCGATCTCAGCACTGTCCTGGCA and GGGCCAAAGCTTGTGGCTGGGGAAGTGT. Thirty rounds of PCR amplification were performed by using a Perkin–Elmer thermocycler. Seven microclones of each reaction mixture was analyzed on a 50–80% denaturing gradient gel (Green Mountain Laboratory Supply, Waltham, MA) as described (10).

Sequence Analysis. Genomic DNA known to contain a variant sequence was amplified with a pair of primers carrying two different restriction enzyme cleavage sites and then subcloned into a plasmid vector. Six or more clones were sequenced from each sample to obtain multiple examples of each sequence alteration. PCR errors were encountered as isolated events at a frequency of one per several thousand nucleotides. In some instances, new bands were excised from a denaturing gradient gel, reamplified, and subcloned. In those cases, DNA templates for sequencing were prepared from pools of several hundred subclones.

Allele-Specific Oligonucleotide Hybridization. Four microclones of a PCR reaction mixture (=100 ng of DNA) was denatured and deposited onto a GeneScreenPlus filter (DuPont) under gentle vacuum. 5'-End-labeled 13-mers identical to and centered about the mutant sequences were annealed in 4× standard saline citrate (SSC)/100 salmon sperm DNA (at 100 μg/ml)/5× Denhardt's solution/0.05% SDS for 5 hr at 23°C. The filters were washed in 0.4× SSC/0.1% SDS at temperatures between 25°C and 40°C to optimize the difference between hybridization to wild-type and mutant sequences.

RESULTS

Screening for Point Mutations by Using DGGE. Based upon the human rhodopsin gene sequence (1), seven pairs of PCR primers were synthesized to amplify the coding regions of the five exons and the adjacent 20–25 base pairs of intron and 5' and 3' noncoding DNA. The largest exons, exons 1 and 4, were amplified in two parts, referred to as L and R for 5' and 3' halves, respectively. One member of each primer pair included a 39-base 5' extension consisting exclusively of G and C (a "GC clamp"). The inclusion of a GC clamp has been shown to increase the sensitivity of DGGE (10).

Blood samples were collected from one affected individual in each of 161 families with ADRP. DGGE of rhodopsin gene PCR products revealed in most instances a single band corresponding to that seen in wild-type controls. New bands were observed in some samples, and these were grouped according to their patterns. Fig. 1 shows the DGGE profile of one sample from each group. The electrophoretic patterns observed for two bands as expected for PCR products derived from heterozygotes: the lower two bands correspond to wild-type and variant homoduplexes and the upper two correspond to the two heteroduplexes.

The nucleotide sequence of each type of variant band was determined. The two most common variants, represented by samples HS440 and HS1001, carry single nucleotide substitutions adenosine 269 → guanosine and cytidine 5321 → adenosine in the 5' and 3' noncoding regions, respectively (see ref. 1 for the numbering system). These variants occur at gene frequencies of 14% and 13%, respectively, in our ADRP population and were also seen in unaffected subjects. They appear to represent polymorphisms in the population. Three infrequent variants, also presumed to represent poly-

![Fig. 1. Examples of denaturing gradient gel patterns of variant and wild-type (WT) PCR products. Ethidium bromide staining patterns are shown as negative images. The two lower bands correspond to homoduplex variant and wild-type PCR products; the two upper bands correspond to heteroduplexes formed between variant and wild-type strands during the final PCR cycle. Subject number and, in parentheses, pedigree designation are indicated above each lane. The exon numbers are indicated above the brackets. Variant bands in DNA from subjects HS440 (exon 1L), HS678 (exons 2 and 4L), HS600 (exon 5), and HS1001 (exon 5) correspond to polymorphisms that appear to be unrelated to retinitis pigmentosa.](http://example.com/figure1.png)
As an adjunct to the pedigree analysis, we compared the frequency of each mutant allele in the ADRP population (n = 161) and in a control population of young adults with normal vision (n = 118). One mutant allele, P23H, is present in 24 of 161 unrelated ADRP subjects and is absent from the 118 control subjects. The other 12 mutations were found either once or twice in the 161 ADRP subjects; they were not seen in the control population (Table 1). Altogether, rhodopsin mutations were observed in 39 of 161 ADRP subjects.

**DISCUSSION**

**Identification of Rhodopsin Mutations.** The results presented here demonstrate that 24% of 161 unrelated ADRP patients carry point mutations in the gene encoding rhodopsin. As the sample population in this study was almost exclusively Caucasian, this value may differ in other population groups. The presence or absence of rhodopsin mutations correlates with the presence or absence of ADRP in 174 of 179 family members tested (including probands). The four individuals in one family who carry mutations but have no visual symptoms may represent examples of incomplete penetrance, delayed onset of the disease, or very mild disease expression. For several alleles the pedigrees are too small for cosegregation to be statistically significant. Analysis of additional families or identification of a biochemical defect in the mutated rhodopsins is needed to confirm a role for these alleles in producing retinitis pigmentosa. The single most common allele, P23H, was found in 15% of the ADRP families. These data are in agreement with those of Dryja et al. (12) who found the P23H mutation in 17 of 148 unrelated ADRP subjects. In the present study no other allele was found in more than two families. For one-quarter of ADRP families, it will now be possible to provide genetic counseling and prenatal diagnosis based on DNA analysis.

It is possible that some mutations escaped detection by the PCR/DDGE screening method employed in this study. Intron and flanking region mutations outside of the amplified segments would not be detected. Deletions or other rearrangements encompassing one of the primer sites would also escape detection because a normal PCR product would be produced from the wild-type chromosome. However, it is unlikely that point mutations residing within the amplified regions would go undetected by DDGE. Sheffield et al. (10) showed that >90% of single base changes produce a PCR product of variant mobility when amplification is performed with a GC clamp primer. Consistent with that observation, only 1 of 18 variant bands observed in the present study comigrated with the wild type under the conditions employed (HS698; exon 1R; see Fig. 1). Because each subject is likely to be heterozygous for the dominant mutation, heteroduplexes carrying a base mismatch are expected to form during the annealing step of the last PCR cycle. In each of the 18 variant patterns, the heteroduplexes show a prominent decrease in mobility compared to the corresponding homoduplexes.

**Heterogeneity of Rhodopsin Mutations.** In this study 13 different mutations were observed at 12 amino acid positions within the rhodopsin coding region. This allelic heterogeneity suggests a corresponding variability in biochemical defects and in the natural history of the associated retinal disease. Factors other than heterogeneity at the rhodopsin locus may also contribute to phenotypic variability. Indeed, two recent studies have found marked variation in natural history among patients with the P23H mutation (15, 16). Additional rhodopsin gene mutations are likely to exist in the human gene pool. They may account for the retinal degeneration or functional impairment in such disorders as
FIG. 3. Pedigrees of ADRP families. A history of night blindness and/or visual field loss were considered diagnostic of retinitis pigmentosa. Most of the affected participants have been independently evaluated by an ophthalmologist. A question mark indicates an uncertain diagnosis. Numbers indicate the family members tested; arrows indicate probands. Families designated by the same letter carry the same mutation (e.g., BI and B2). Subject 11 in family D and subjects 5, 6, and 7 in family L are classified as affected based upon measurements of rod electroretinograms, dark adaptometry, and rhodopsin levels; they report no visual impairment.

Autosomal recessive retinitis pigmentosa, Leber’s congenital amaurosis, or stationary night blindness. It will also be interesting to determine that fraction of simplex retinitis pigmentosa patients who carry new rhodopsin mutations.

The present study indicates that neutral amino acid substitutions in rhodopsin are extremely rare. Of the 322 rhodopsin genes screened, 39 carry mutations responsible for ADRP and none carry an amino acid substitution unrelated to ADRP. This finding rules out rhodopsin gene polymorphism as a source of the reported common variation in the point of maximal sensitivity of human rod vision (17).

Naturally occurring variants have been extremely useful in studies of protein structure and function. The variant rhodopsins responsible for ADRP could be defective in protein folding/stability, intracellular targeting, signal transduction, or as-yet-undefined functions. The availability of tissue culture expression systems for rhodopsin will permit the production of each variant for detailed study (18, 19).

Implications for Large-Scale Genetic Screening of Heterogeneous Disorders. For many inherited disorders, clinical variability suggests a corresponding heterogeneity in underlying genetic defects. One approach to identifying the causal
mutations is to choose as candidates those genes whose products are active in the relevant physiological processes. To overcome the problem of genetic heterogeneity, a large number of unrelated patients can be screened for mutations in the candidate genes. As demonstrated in this study, the combination of PCR and DGGE is well suited for the efficient detection of point mutations in a large patient population. The efficiency of these methods makes feasible the screening of disorders for which a genetic component is known but for which simple Mendelian inheritance has not been demonstrated.

We thank all of the ADRP subjects and their relatives for participating; Ms. Rosalind Palmer and her colleagues at the Wilmer Retinitis Pigmentosa Center for assistance in identifying patients and collecting blood samples; Ms. Pat Catlin, Drs. John Jernigan, and Thomas Loftus and the cadets of the U.S. Air Force Academy for participating; Drs. Charles Weitz, Mark Gray, Monica Traysman, and Haig Kazazian for DGGE advice; Dr. Clark Riley, Ms. Anatoli Collector, Ms. Cynthia Wendling, and Ms. Jodie Franklin for synthetic oligonucleotides; Drs. David Valle, Daniel Nathans, Donald Zack, and Charles Weitz for helpful comments on the manuscript; and Ms. Teri Chase for expert secretarial assistance. This work was supported by the Howard Hughes Medical Institute and the National Retinitis Pigmentosa Foundation.

Table 1. Frequency of rhodopsin mutations in ADRP and control populations

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<th>Mutation</th>
<th>ADRP</th>
<th>Control</th>
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<tr>
<td>T17M</td>
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<tr>
<td>P23H</td>
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</tr>
<tr>
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</table>

The frequency of each rhodopsin gene point mutation in 161 unrelated subjects with ADRP and 118 subjects with normal vision is given. Mutations were detected as in Fig. 4.

Fig. 4. Segregation of rhodopsin mutations in ADRP families by hybridization of mutant allele-specific 13-mers to PCR products from family members indicated in Fig. 3. Asterisks denote individuals with retinitis pigmentosa.