Am. J. Hum. Genet. 53:80–89, 1993

Identification of Novel Rhodopsin Mutations Responsible for Retinitis Pigmentosa: Implications for the Structure and Function of Rhodopsin

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Summary

Ten rhodopsin mutations have been found in a screen of 282 subjects with retinitis pigmentosa (RP), 76 subjects with Leber congenital amaurosis, and 3 subjects with congenital stationary night blindness. Eight of these mutations (gly^{51}-to-ala, val^{104}-to-ile, gly^{106}-to-arg, arg^{135}-to-gly, cys^{140}-to-ser, gly^{188}-to-glu, val^{209}-to-met, and his^{211}-to-arg) produce amino acid substitutions, one (gln^{64}-to-ter) introduces a stop codon, and one changes a guanosine in the intron 4 consensus splice donor sequence to thymidine. Cosegregation of RP with gln^{64}-to-ter, gly^{106}-to-arg, arg^{135}-to-gly, cys^{140}-to-ser, gly^{188}-to-glu, his^{211}-to-arg, and the splice site guanosine-to-thymidine indicates that these mutations are likely to cause retinal disease. Val^{104}-to-ile does not cosegregate and is therefore unlikely to be related to retinal disease. The relevance of gly^{51}-to-ala and val^{209}-to-met remains to be determined. The finding of gln^{64}-to-ter in a family with autosomal dominant RP is in contrast to a recent report of a recessive disease phenotype associated with the rhodopsin mutation glu^{249}-to-ter. In the present screen, all of the mutations that cosegregate with retinal disease were found among patients with RP. The mutations described here bring to 35 the total number of amino acid substitutions identified thus far in rhodopsin that are associated with RP. The distribution of the substitutions along the polypeptide chain is significantly nonrandom: 63% of the substitutions involve those 19% of amino acids that are identical among vertebrate visual pigments sequenced to date.

Introduction

Rhodopsin is the light-absorbing protein in rod photoreceptors that mediates vision in dim light. It is the most abundant protein in the mammalian retina, accumulating to a level of approximately 10^8 molecules per rod photoreceptor (Knowles and Dartnall 1977, pp. 347–423). Over 30 point mutations or small deletions in the rhodopsin gene have been identified in patients with retinitis pigmentosa (RP), an inherited degeneration of the retina that affects 1 person in 4,000 in the United States (Heckenlively 1988). Among RP patients with the same rhodopsin mutation, measurements of rod photoreceptor function show patterns of impairment characteristic of the particular mutation, although there is individual variation in the time course and regional distribution of retinal disease (Berson et al. 1991*a*, 1991*b*; Heckenlively et al. 1991; Jacobson et al. 1991; Fishman et al. 1992*a*, 1992*b*; Kemp et al. 1992; Moore et al. 1992). All but one of the rhodopsin mutations have been identified in patients with autosomal dominant RP (ADRP; Dryja et al. 1990*a*, 1990*b*, 1991; Farrar et al. 1991; Gal et al. 1991; Inglehearn et al. 1991, 1992; Keen et al. 1991; Sheffield et al. 1991; Sung et al.

Received January 19, 1993; revision received March 17, 1993.

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0002-9297/93/5301-0011$02.00

1991*a*; Artlich et al. 1992; Bell et al. 1992; Fishman et al. 1992*a*, 1992*b*; Fujiki et al. 1992). One mutation, a premature stop codon, has been identified in an individual with autosomal recessive RP (ARRP; Rosenfeld et al. 1992). Carriers of that mutation were found to have a severalfold decrease in light sensitivity but did not exhibit a visual field loss indicative of a retinal dystrophy.

An analysis of the biochemical properties of 13 mutant rhodopsins identified in patients with ADRP revealed two broad classes (Sung et al. 1991*b*). Class 1 mutations resemble the wild-type protein in their accumulation to high levels in the plasma membrane of transfected cells and their ability to join in vitro to 11-*cis* retinal to form a photolabile visual pigment. Class 2 mutations accumulate to lower levels, are inefficiently transported from the endoplasmic reticulum to the plasma membrane, and produce little or no photolabile pigment upon incubation in vitro with 11-*cis* retinal. The latter class of mutant rhodopsins are likely to be defective in protein folding and/or stability. Examination of this first set of 13 mutant proteins suggests that members of each class may be distributed in a nonrandom fashion along the polypeptide chain. Class 1 mutations tend to cluster near the carboxy-terminus, whereas many class 2 mutations either introduce a charged residue into the hydrophobic membrane-spanning segments, introduce or remove a proline, or reside near two cysteines that form an essential disulfide bond.

The present paper describes the results of a screen for rhodopsin mutations among 282 subjects with RP, including ADRP, ARRP, multiplex cases in which two or more family members are affected but the pattern of inheritance is uncertain, and simplex cases. The simplex cases presumably represent instances of recessive inheritance, X-linked inheritance ascertained in a male, or a new mutation. We also report the results of screening 76 patients with Leber congenital amaurosis, a heterogeneous group of retinal dystrophies characterized by severe congenital vision loss, and three patients with congenital stationary night blindness.

Material and Methods

Sample Collection and Processing

Participants were recruited through their ophthalmologists or through the National Registry of the Retinitis Pigmentosa Foundation. Control samples were obtained from students at the United States Air Force Academy. Genomic DNA was purified as described by Sung et al. (1991*a*).

PCR Amplification and Denaturing Gradient Gel Electrophoresis (DGGE)

Seven segments of the rhodopsin gene were amplified by PCR using the 14 primer pairs listed in Sung et al. (1991*a*). These seven segments encompass the coding region and approximately 30 bp of intron sequence adjacent to each of the five exons. Exons 1 and 4 were each amplified as two partially overlapping segments; sequences from exons 2, 3, and 5 were each amplified as a single segment. One PCR primer in each pair included a 40-base GC-rich segment (a "GC-clamp") to facilitate detection of mutations by DGGE (Sheffield et al. 1989). PCR products were resolved by DGGE in gels containing a 50%–90% gradient of denaturant as described by Myers et al. (1987).

Sequence Analysis of PCR Products

Those PCR products that produced a variant pattern by DGGE were amplified using a pair of primers carrying two different restriction enzyme cleavage sites and were subcloned into a plasmid vector. Multiple individual subclones were sequenced from each sample to ensure that two or more independent examples of each variant sequence were obtained. The variant sequences were verified in each case by hybridization with an allele-specific oligonucleotide probe as described by Sung et al. (1991*a*). For each oligonucleotide hybridization experiment, approximately 50 ng of PCR product, as determined by agarose gel electrophoresis, was denatured and loaded per slot. Slot blot analysis of control subjects from the United States Air Force Academy included in each case a positive control known to contain the mutation of interest.

Results

Screening Strategy

To efficiently screen for rhodopsin mutations in patients with retinal disease, we collected blood samples from unrelated patients, extracted DNA, amplified the rhodopsin gene exons by PCR, and analyzed the PCR products by DGGE. In an earlier paper we described the use of this strategy to screen for rhodopsin mutations among 161 unrelated probands with ADRP. In the present study we screened for rhodopsin mutations in 53 subjects with ADRP, 41 with ARRP, 56 with multiplex RP, 117 with simplex RP, 15 for whom family data was unavailable, 76 with Leber congenital

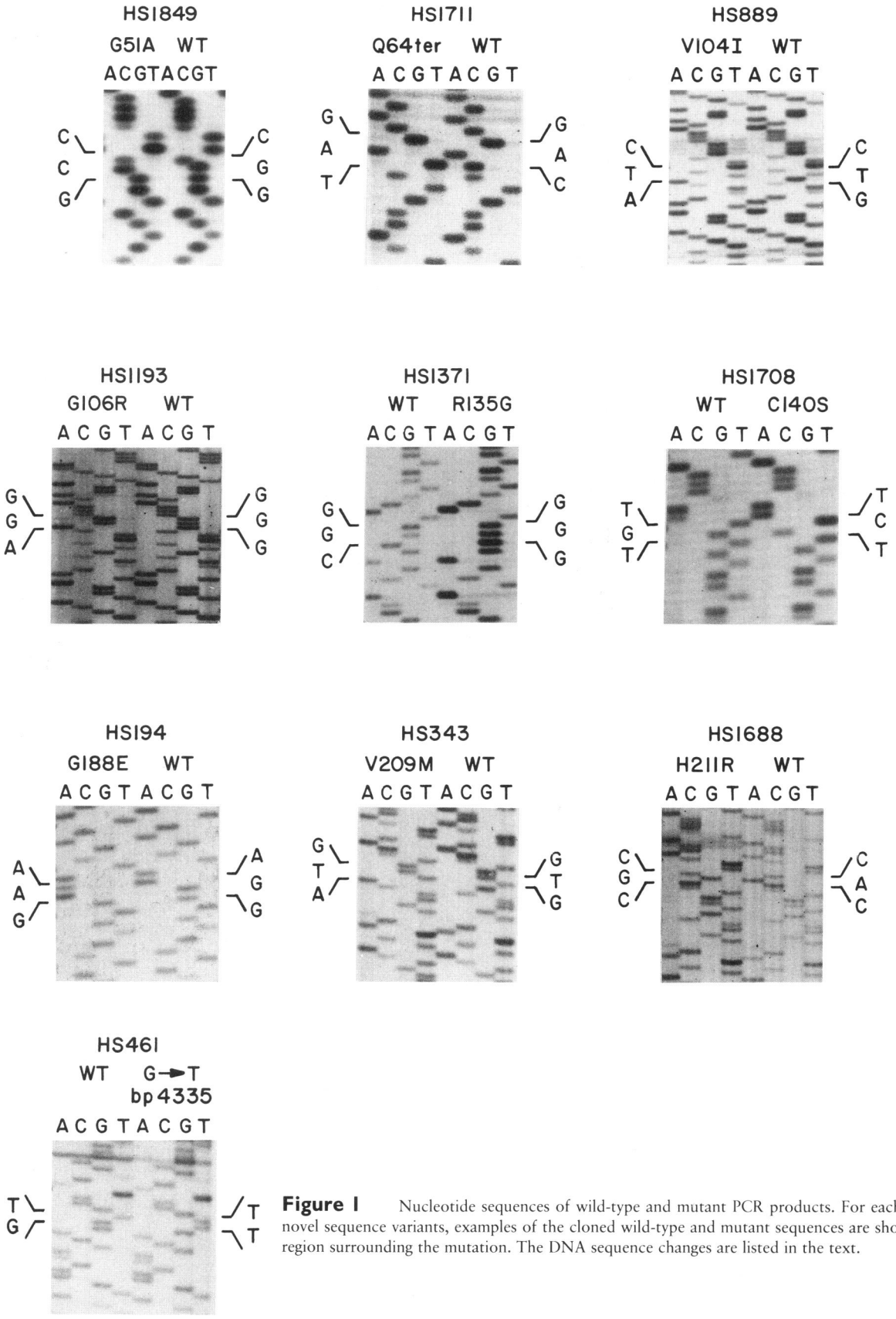

Figure 1 Nucleotide sequences of wild-type and mutant PCR products. For each of the 10 novel sequence variants, examples of the cloned wild-type and mutant sequences are shown for the region surrounding the mutation. The DNA sequence changes are listed in the text.

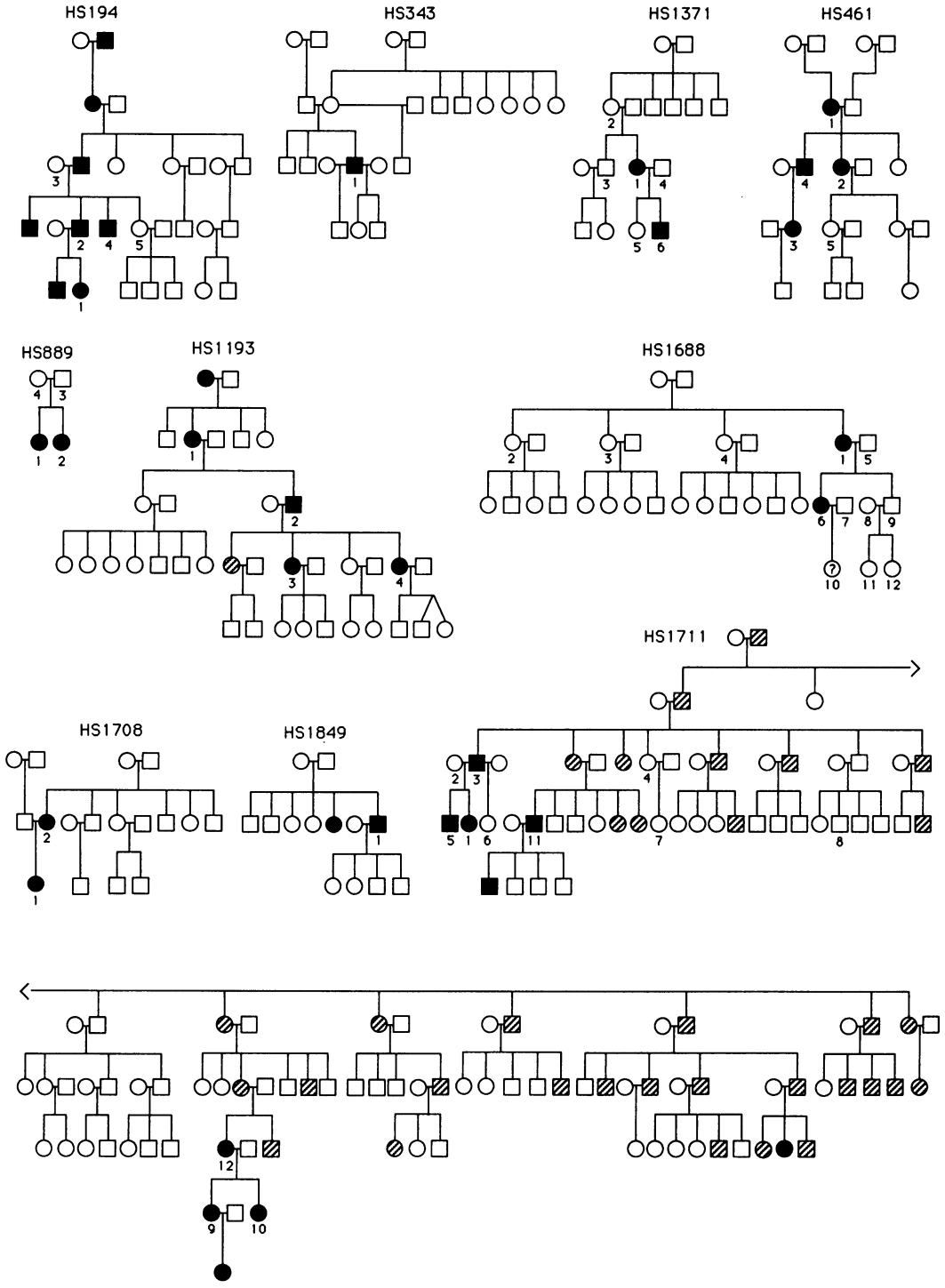

Figure 2 Pedigrees of families in which a rhodopsin gene mutation was identified. Family members from whom a DNA sample was collected are indicated by number; "1" indicates the proband. A history of nightblindness or visual field loss were considered diagnostic of RP. Hatched symbols indicate subjects who are reported to have a visual defect consistent with RP. The question mark indicates a child whose visual function appears grossly normal. All affected individuals whose DNA was tested were examined by an ophthalmologist.

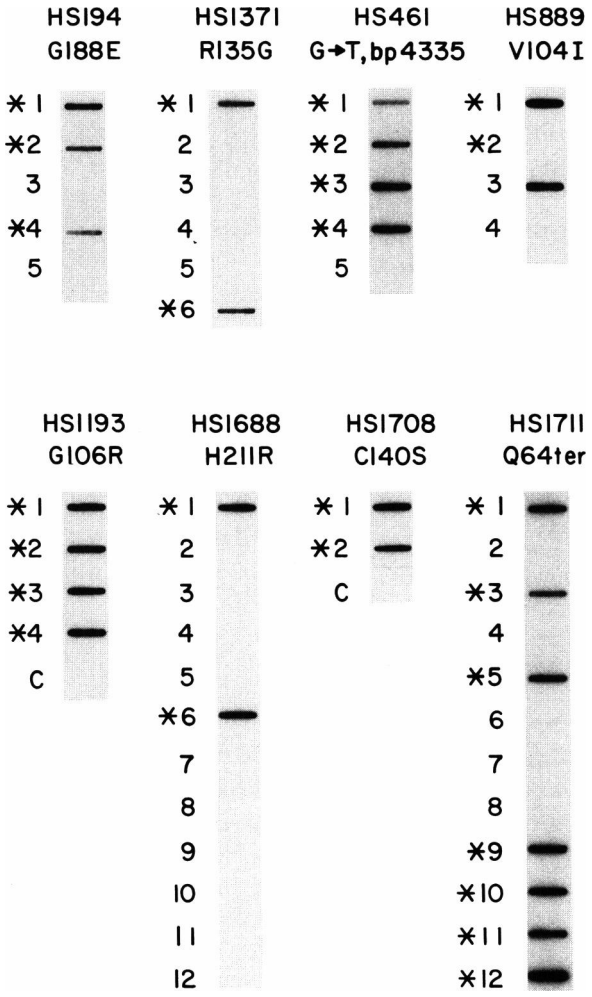

Figure 3 Analysis of coinheritance by hybridization of oligonucleotides specific for the mutant allele to slot blots of PCR products from family members. Numbers refer to the pedigree diagram in fig. 2; asterisks (*) indicate affected individuals; and "C" indicates a control sample that does not carry the mutation.

amaurosis, and 3 with congenital stationary night blindness.

DGGE of the seven rhodopsin gene PCR products from each of the 361 subjects revealed 26 types of band patterns that differed from the wild type. Seven of the band patterns were derived from known sequence variants that do not alter the amino acid sequence (i.e., six single-nucleotide substitutions—adenosine^{269}-to-guanosine, guanosine^{2557}-to-adenosine, cytidine^{3982}-to-thymidine, cytidine^{4289}-to-thymidine, guanosine^{5145}-to-adenosine, cytidine^{5321}-to-adenosine; and one double substitution—guanosine^{5145}-to-adenosine and cytidine^{5321}-to-adenosine) (Dryja et al. 1991; Sung et al. 1991*a*; Rosenfeld et al. 1992; also see Nathans and Hogness 1984 for numbering system). An additional five band patterns were derived from sequence changes that were also presumed to be phenotypically silent—cytidine^{654}-to-thymidine, cytidine^{2515}-to-thymidine, and cytidine^{4289}-to-thymidine, silent substitutions in codons 120, 146, and 297, respectively; cytidine^{4345}-to-thymidine in intron 4; and cytidine^{5311}-to-thymidine in the 3′ untranslated region. Fourteen subjects revealed DGGE band patterns that correspond to four mutations identified in our earlier screen of ADRP samples and that consist of five examples of P23H, four examples of P347L, three examples of R135W, and two examples of R135L (Sung et al. 1991*a*; amino acid substitutions are referred to by the identity of the wild-type residue, abbreviated using the single-letter amino acid designation, followed by the codon number followed by the introduced residue—e.g., arginine^{135}-to-glycine is R135G). Each of these known mutations was present in the heterozygous condition.

The remaining 10 mutations were each found once in the heterozygous condition (fig. 1). These mutations are guanosine^{446}-to-cytidine (G51A) in HS1849, cytidine^{484}-to-thymidine (Q64ter) in HS1711, guanosine^{604}-to-adenosine (V104I) in HS889, guanosine^{610}-to-adenosine (G106R) in HS1193, cytidine^{2480}-to-guanosine (R135G) in HS1371, guanosine^{2496}-to-cytidine (C140S) in HS1708, guanosine^{3845}-to-adeno-

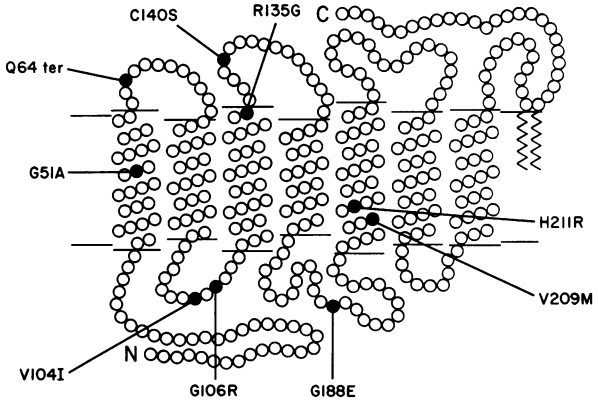

Figure 4 Model of human rhodopsin showing the locations of the nine coding region mutations. The central region of the protein is shown traversing the lipid bilayer. The carboxy-terminus of the protein resides on the cytosolic face of the protein. The zigzag lines represent palmytoylation at cys^{322} and cys^{323}. The splice-site mutation identified in subject HS461 is predicted to affect splicing efficiency at the intron 4 junction located between codons 312 and 313 at the beginning of the cytosolic carboxy-terminal tail.

sine (G188E) in HS194, guanosine^{3907}-to-adenosine (V209M) in HS343, adenosine^{3914}-to-guanosine (H211R) in HS1688, and guanosine^{4335}-to-thymidine (GT to TT at the donor splice junction of intron 4) in HS461. One of the 10, V104I in subject HS889, was found in a family with Leber congenital amaurosis, whereas the remaining nine mutations were found among patients with RP. Mutation G106R in subject HS1193 has been identified in two other screens of RP patients (Fishman et al. 1992*a*; Inglehearn et al. 1992), and the intron 4 splice-site mutation guanosine^{4335}-to-thymidine in subject HS461 has been observed in a 28-year-old subject who does not have RP (Rosenfeld et al. 1992).

Pedigree Analysis and Allele Frequency in a Control Population

To determine whether the 10 novel mutations were associated with retinal disease, they were tested for cosegregation with the disease phenotype in affected families. Figure 2 shows pedigrees, and figure 3 shows the results of hybridization with an allele-specific oligonucleotide to PCR products from those relatives who participated in the study. Examination of the pedigrees indicates that HS194, HS461, HS1193, and HS1711 have an autosomal dominant mode of inheritance (i.e., males and females were affected in three or more generations) and that HS1371, HS1688, and HS1708 are likely to have an autosomal dominant mode of inheritance. Cosegregation with RP is evident in the families of subjects HS194 (G188E), HS461 (guanosine^{4335}-to-thymidine), HS1193 (G106R), HS1371 (R135G), HS1688 (H211R), HS1708 (C140S), and HS1711 (Q64ter), although in some cases the number of individuals examined is too small for the cosegregation to be considered statistically significant. In the family of subject HS889 (V104I), the mutation does not coinherit with retinal disease. Whether the mutation cosegregates with RP in families HS343 (V209M) and HS1849 (G51A) remains to be determined.

As an adjunct to the pedigree analysis, we examined the frequency of each mutation in a control population of 124 young adults with normal vision. As determined by hybridization with allele-specific oligonucleotide probes, none carries the mutations identified here, a result that is consistent with a causal role for these mutations in the genesis of retinal disease.

Discussion

Frequencies of Different Mutations in the RP Population

The genetic screen reported here has identified one splice junction mutation and nine coding region mutations in the rhodopsin gene in patients with retinal disease. The locations along the polypeptide chain of the nine coding region changes are shown in figure 4. Pedigree analysis suggests that seven of these mutations (Q64ter, G106R, R135G, C140S, G188E, H211R, and guanosine^{4335}-to-thymidine) are likely to be causally related to RP and that one (V104I) is likely to be phenotypically silent. The phenotypic significance of the remaining two mutations (G51A and V209M) is unknown. The finding of 21 probands with clinically significant rhodopsin mutations among 53 ADRP patients and 56 multiplex RP patients is consistent with earlier estimates of a 25%–30% frequency of rhodopsin mutations among subjects with ADRP in the United States (Dryja et al. 1991; Sung et al. 1991*a*).

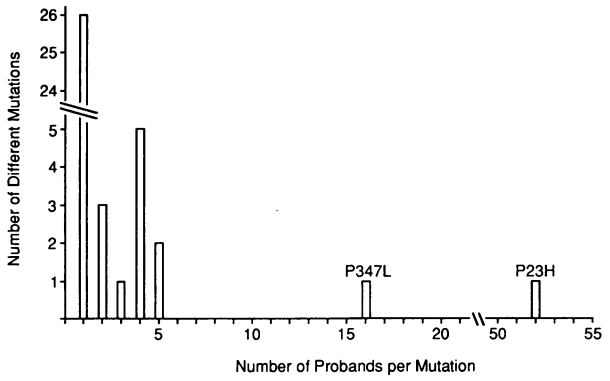

Figure 5 Histogram showing the number of probands with ADRP who carry each of the 40 mutations reported to date (e.g., only a single ADRP proband and his or her family have been found for each of 26 mutations; for references, see text). From the present study, only those seven mutations that cosegregate with RP have been included. Bars representing the P23H and P347L mutations are indicated.

The only rhodopsin mutation identified among 76 patients with Leber congenital amaurosis (V104I in HS889) appears not to be involved in retinal disease, as it is present in an unaffected member of the pedigree and is absent in an affected member. Apparently, mutations in the rhodopsin gene rarely, if ever, lead to retinal disease of the severity seen in Leber congenital amaurosis.

Most of the rhodopsin mutations identified to date in subjects with ADRP have been found in only one or a few families, as illustrated in the histogram in figure 5 (Dryja et al. 1990*a*, 1990*b*, 1991; Farrar et al. 1991; Gal et al. 1991; Inglehearn et al. 1991, 1992; Keen et al. 1991; Sheffield et al. 1991; Sung et al. 1991*a*, 1991*b*; Artlich et al. 1992; Bell et al. 1992; Fishman et al. 1992*a*, 1992*b*; Fujiki et al. 1992). This sample consists

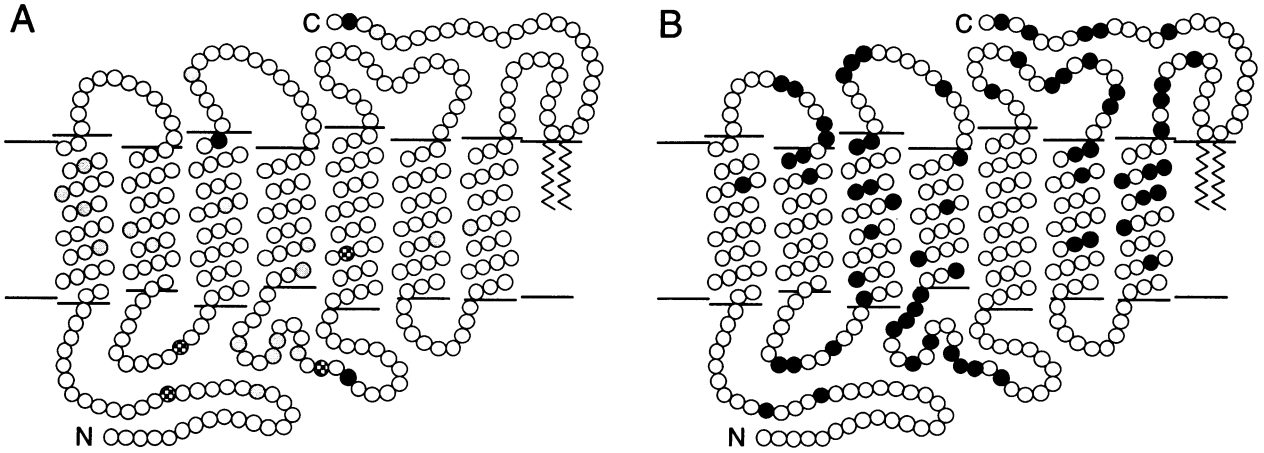

Figure 6 *A*, Locations of all amino acid substitutions in rhodopsin, associated with ADRP, reported to date (for references, see text). From the present study only those seven mutations that cosegregate with RP have been included. Unblackened, light-gray, checkered, or black circles indicate zero, one, two, or three different amino acid substitutions, respectively, identified at that location. *B*, Locations of amino acid residues that are invariant among vertebrate opsins (black circles). Included in this analysis are the human, chicken, and goldfish rod and cone opsins, bovine and lamprey rod opsins, and cavefish long-wavelength cone opsins (for references, see text).

of a total of 475 ADRP subjects from the United States, Britain, Ireland, and Europe and 24 from Japan. All of the subjects with P23H, by far the most common allele, are from the United States (Farrar et al. 1990). The large number of rare mutations suggests that many additional mutations in the rhodopsin gene remain to be discovered.

Phenotypic Variability among Rhodopsin Stop Codon Mutations

The Q64ter mutation (subject HS1711) represents the third example of a stop codon mutation in a patient with RP. One stop codon mutation, Q344ter, was identified in a family with ADRP (Sung et al. 1991*a*). The encoded protein is missing the last five amino acids and resembles the wild type in its subcellular localization in transfected tissue culture cells and in its ability to bind 11-*cis* retinal (Sung et al. 1992*b*). A second stop codon mutation, E249ter, was recently found in the homozygous condition in an individual with ARRP (Rosenfeld et al. 1992). A heterozygous carrier of this mutation showed a threefold decrease in light sensitivity, as measured electroretinographically, but did not exhibit visual field loss. The finding that Q64ter causes RP in the heterozygous condition suggests that synthesis of a rhodopsin fragment consisting of the first 63 amino acids damages the rod cell. Cellular damage could result from disruption of the lipid bilayer structure or from interference with the folding or transport of other proteins.

Mutation of a Conserved Donor Splice Junction Sequence

The mutation in subject HS461 changes the invariant first two nucleotides in intron 4 from GT to TT. Cosegregation of this mutation and RP was observed in the five family members tested. By analogy with the decrease in globin production caused by a GT-to-TT mutation in the first intron of the human beta-globin gene (Orkin and Kazazian 1984), the HS461 mutation would be expected to decrease the efficiency of splicing at this position and may lead to the use of cryptic splice junction donors within either exon 4 or intron 4. The resulting protein is likely to be missing those sequences that normally constitute the carboxy-terminal cytosolic tail. This same mutation was found in the heterozygous state by Rosenfeld et al. (1992) in a 28-year-old subject who does not have RP, suggesting that its pathological effect may either be delayed or variable.

Implications of the Nonrandom Distribution of Amino Acid Substitutions

The 7 mutations reported here which are implicated in the pathogenesis of RP bring to 40 the total number of rhodopsin mutations identified in patients with ADRP. Of these, 35 are single amino acid substitutions. Thirty-one of these mutant proteins have been produced by transfection of tissue culture cells (Sung et al. 1991*b*; C.-H. Sung, C. M. Davenport, and J. Nathans, unpublished data). Mutations near the carboxy-terminus resemble the wild type in yield, plasma membrane localization, and ability to form a photolabile

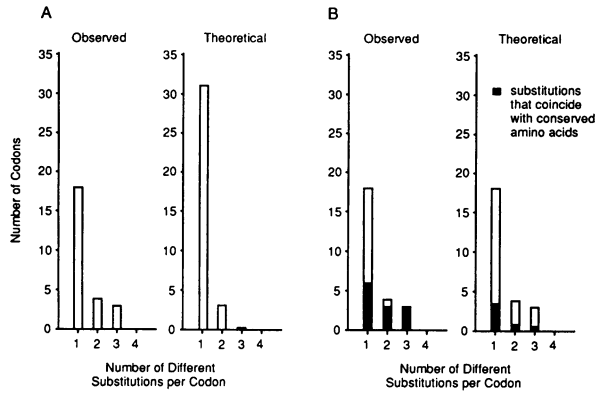

Figure 7 *A, Left,* Number of amino acid substitutions observed per codon for the 35 rhodopsin substitution mutations identified to date in ADRP (fig. 6*A*). *Right,* Expected distribution based on a model in which the 35 amino acid substitutions occur randomly and with equal probability at each of the 348 codons. In this model the probability that a given codon will have exactly n different substitutions is $P(n) = (347/348)^{35-n}(1/348)^n(35!/(35 - n)!n!)$. To compare this distribution with the observed distribution, a scaling factor, x, is introduced so that the total probability density is equivalent to 35 mutations, hence $35 = x(P(1) + 2P(2) + 3P(3) \ldots)$. *B, Left,* Observed distribution of evolutionarily invariant amino acids among the 35 known amino substitutions in ADRP. *Right,* Distribution predicted if the locations of the substitutions and the invariant residues were uncorrelated; i.e., 67/348 = .19 of each bar is filled. The filled portion of each bar in the histogram represents that fraction of the 35 known amino acid substitutions that involve the 67 evolutionarily invariant residues among vertebrate pigments (fig. 6*B*).

pigment on joining to 11-*cis* retinal (class 1 mutations). These are presumed to be defective in some aspect of visual pigment function that is not apparent in a heterologous expression system. By contrast, most of the amino acid substitutions located elsewhere in the protein result in a defect in protein folding and/or stability (class 2 mutations). As predicted from their locations and the significant change that they produce in side-chain properties, substitutions G106R, R135G, G188E, and H211R are likely to be members of class 2 (C.-H. Sung, C. M. Davenport, and J. Nathans, unpublished data).

It would be of interest to determine which regions or amino acids are most important for the correct folding of human rhodopsin. Addressing this question by analyzing the spectrum of naturally occurring ADRP mutations is complicated by the limited range of observed amino acid substitutions due to the structure of the genetic code, by differences in the mutability of different nucleotides, and by sampling error in a patient population of finite size. Despite these complexities, several patterns are emerging that are likely to reflect the importance of particular structural features in determining protein folding and/or stability.

First, of the 13 mutations that occur in the hydrophobic transmembrane segments, 8 involve the introduction of a charged amino acid, and 4 involve the replacement or introduction of a proline. The former type of substitution would be expected to increase the free energy of transfer of this segment from an aqueous to a lipid environment, and the latter type would be expected to either remove or introduce a kink in the alpha helix. Second, 14 mutations are located on the extracellular face of rhodopsin, with a significant clustering in the second extracellular loop. Some of these extracellular substitutions may disrupt the apposition of cysteines 110 and 187 which have been shown in bovine rhodopsin to form an essential disulfide bond (Karnik et al. 1988; Karnik and Khorana 1990). Third, most of the mutations identified to date on the cytosolic face cluster near the carboxy-terminus and accumulate in transfected cells to a level that is indistinguishable from that of the wild type (Sung et al. 1991*b;* C.-H. Sung, C. M. Davenport, and J. Nathans, unpublished data). The only mutations on the cytosolic face that do not cluster near the carboxy-terminus are a deletion of amino acids 68–71 (Keen et al. 1991) and amino acid substitution C140S reported here. The paucity of substitutions in the three cytosolic loops suggests that they are less important for maintaining a correctly folded structure than are the extracellular loops (Doi et al. 1990). Fourth, the 35 different amino acid substitutions identified in patients with ADRP are distributed over only 25 codons, a degree of clustering that is higher than predicted, on the basis of a model in which each codon is equally mutable (figs. 6*A* and 7*A*). And fifth, substitutions are more commonly observed to involve amino acids that are invariant among the visual pigments (figs. 6*B* and 7*B*). Thirteen of the 25 codons that are targets of substitution mutations in patients with ADRP coincide with those 19% (67/348) of codons that are invariant among chicken (Takao et al. 1988; Kuwata et al. 1990; Tokunaga et al. 1990; Okano et al. 1992; Wang et al. 1992), human (Nathans and Hogness 1984; Nathans et al. 1986), and goldfish (Johnson et al. 1993) rod and cone pigments, lamprey (Hisatomi et al. 1991) and bovine (Nathans and Hogness 1983) rhodopsins, and cavefish long-wavelength pigments (Yokoyama and Yokoyama 1990). Within this group of 13 codons are 6 of 7 of the codons where two or three different amino acid substitutions have been found. As a result, 63% (22/35) of the substitutions involve the 19% of residues that are evolutionarily conserved.

Recently, mutations in the vasopressin receptor have been reported in patients with diabetes insipidus (Pan et al. 1992; Rosenthal et al. 1992; van den Ouweland et al. 1992). This represents the first example of mutations in a human G-protein-coupled receptor aside from the visual pigments. It will be of interest to determine whether the pattern of mutations identified in the rhodopsin gene holds generally for defects in other G-protein-coupled receptors.

Acknowledgments

We thank all of the subjects and their relatives for participating; Drs. William Pearce (University of Alberta) and Daniel Finkelstein (Johns Hopkins University) for identifying patients and contributing blood samples; Ms. Pat Catlin, Drs. John Jernigan and Thomas Loftus, and the cadets of the United States Air Force Academy for participating; Dr. Clark Riley, Ms. Anatoli Collector, and Ms. Cynthia Wendling for synthetic oligonucleotides; Dr. William Pearce and two anonymous referees for helpful comments on the manuscript; and Ms. Teri Chase for expert secretarial assistance. This work was supported by the Howard Hughes Medical Institute, the National Retinitis Pigmentosa Foundation, and by an unrestricted grant from Research to Prevent Blindness.

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