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Human Tritanopia Associated with Two Amino Acid Substitutions in the Blue-sensitive Opsin

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Summary

Tritanopia is an autosomal dominant genetic disorder of human vision characterized by a selective deficiency of blue spectral sensitivity. The defect is manifested within the retina and could be caused by a deficiency in function or numbers (or both) of blue-sensitive cone photoreceptors. We have used PCR, denaturing gradient gel electrophoresis, and DNA sequencing of amplified exons to detect in four of nine unrelated tritanopic subjects two different point mutations in the gene encoding the blue-sensitive opsin, each leading to an amino acid substitution. Segregation analysis within pedigrees and hybridization of oligonucleotides specific for each allele to DNA samples from control subjects support the hypothesis that these mutations cause tritanopia. These results complete the genetic evidence for the trichromatic theory of human color vision.

Introduction

Observers with normal color vision can match the color of any arbitrary test light either by mixing three suitably chosen monochromatic lights or by mixing two monochromatic lights and adding a third to the given light (Boynton 1979; Pokorny et al. 1979). This observation provides the basis for the trichromatic theory of color vision, first proposed nearly 200 years ago (Young 1802). It holds that human color discrimination can be accounted for by a set of three receptors, which differ from one another in the wavelength of light that is recognized as an optimal stimulus.

The genetics of human color vision strongly supports the trichromatic theory. There exist three types of inherited color vision deficiency in which subjects require a mixture of only two monochromatic sources to make satisfactory color matches; each type corresponds to a selective defect in one of the three postulated receptor mechanisms (Boynton 1979; Pokorny et al. 1979). Protanopia and deuteranopia are characterized by defects of the red- and green-sensitive mechanisms, respectively. They are among the common X-linked recessive disorders of color vision whose association with rearrangements in the visual pigment gene cluster at Xq.28 identified those genes as encoding the red- and green-sensitive visual pigments (Nathans et al. 1986*a*, 1986*b*).

Tritanopia is characterized by a selective defect of the blue-sensitive mechanism (Wright 1952); its molecular basis has not heretofore been elucidated. It can be acquired as a consequence of various types of ocular disease (Pokorny et al. 1979), suggesting that the blue-sensitive mechanism is vulnerable to physiological derangement. Inherited tritanopia is unique among disorders of color vision in its autosomal dominant transmission (Kalmus 1955). The possibility that different mutant alleles at one locus or that mutations at different loci might be responsible for tritanopia is suggested by the documentation of pedigrees manifesting complete (Went and Pronk 1985) and incomplete

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(Kalmus 1955; Neuhann et al. 1976) penetrance. Although far less common than the X-linked red-green disorders, its incidence may be as high as one in 500 in some populations (van Heel et al. 1980). An abnormal electroretinographic response from tritanopes (Padmos et al. 1978; Miyake et al. 1985) indicates that the defect is manifested within the retina, and the finding that under certain conditions some affected subjects make normal trichromatic color matches (Pokorny et al. 1981) suggests that at least rudiments of a retinal blue-sensitive mechanism may be active.

Material and Methods

PCR Amplification and Denaturing Gradient Gel Electrophoresis (DGGE)

Genomic DNA was obtained from peripheral venous blood as described elsewhere (Sung et al. 1991). All PCR amplifications were carried out in a 100-μl volume, with 1 μg of genomic DNA, 1 μM of each oligonucleotide primer, 100 μM of each deoxynucleoside triphosphate, and 2.5 U *taq* polymerase (Amplitaq, Perkin Elmer Cetus, Norwalk, CT) in 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin. Exons 2–5 were each amplified as a single product, flanked by 25–35 bp of noncoding sequence and containing a 40-bp "GC-clamp" sequence (Sheffield et al. 1989) at the 5′ end. Because its large size could decrease the sensitivity of analysis by DGGE (see below), exon 1 was amplified as two PCR products that overlapped by 20 bp of coding sequence (5′ fragment = exon 1A; 3′ fragment = exon 1B), each containing a GC-clamp sequence at its 5′ end. The first member of each primer pair given below had, as its 5′ end, the following 40-nucleotide GC-clamp sequence: 5′-CGCCCGCCGCGCCCCGCGCCCGCCCCGCCGCCCCCGCCCG. The sequences of the PCR primer pairs were, for exon 1A, 5′(GC-clamp)-TTCACAGAGCCCAAGTGTTTTTAGAGGAGG, 5′-TGCCGCAACTTTTTGTAGCG; for exon 1B, 5′(GC-clamp)-CTTATAGGGTTCCCACTCAATGCCATGGTG, 5′-ACCCCCTCCAGTGGAGTAGC; for exon 2, 5′(GC-clamp)-GATCTGTTTGCCACTCTGCCAGCCAGGCTG, 5′-AGCTCTCTTTCCACCCACAT; for exon 3, 5′(GC-clamp)-AACACAGTCCAGGATCTCAGCTCCCACTG, 5′-GAGCACTCTTCCTCCTTCTCATG; for exon 4, 5′(GC-clamp)-AGCATCCAGAGGGCCAGGAAAAAGAGAGAT, 5′-TAAAAGTCAATGGTGAGAAA; and for exon 5, 5′(GC-clamp)-AGAGAATAAGGTCTTTTTTCCCATACTTCC, 5′-AGGAGTAGAAACTGATGATT. Reaction temperatures were cycled (Perkin Elmer Cetus DNA thermal cycler) as follows: for exons 1A, 1B, 2, and 3, 94°C for 7 min, followed by 30 cycles of 52°C for 30 s, 72°C for 30 s, and 94°C for 30 s, followed by 52°C for 30 s and 72°C for 5 min; for exons 4 and 5, the same, except that the steps at 52°C above were changed to 46°C. DGGE analysis (apparatus from Green Mountain Lab Supply, Waltham, MA) of PCR products was performed as described elsewhere (Sheffield et al. 1989) with 15–20 μl from each 100-μl reaction.

Subcloning and Sequencing Amplified Exons

Exon 1 or exon 3 PCR products were amplified from genomic DNA as above with PCR primers modified to include *Eco*RI (5′ primer) or *Hin*dIII (3′ primer) restriction sites. Products were digested with *Eco*RI and *Hin*dIII, ligated into pUC 118 and/or pUC 119, and bacterial transformants were obtained. Ten to twenty subclones from heterozygotes were each assigned as containing the normal or variant allele by transferring a small portion (1/10–1/5) of each colony into a PCR reaction tube and performing GC-clamp PCR and DGGE analysis alongside PCR products amplified from the genomic DNA of each subject. PCR products from one subclone in about 50 comigrated with neither genomic allele in DGGE, presumably owing to a *Taq* polymerase misincorporation. Single-stranded DNA was produced by M13 infection of selected transformants, and the sequence was determined by the dideoxy method. In each case, two or more subclones were sequenced, and the sequences were confirmed by direct sequencing of bulk PCR products. PCR primers for subcloning were, for exon 1, 5′-CAACGAATTCCCAAGTGTTTTTAGAGGAGG and 5′-GGGCCCAAGCTTACCCCCTCCAGTGGAGTAGC. For exon 3 they were 5′-CAACGAATTCAGGCATCTCAGCTCCCACTG and 5′-GGGCCCAAGCTTGAGCACTCTTCCTTCTCATG.

Hybridization of Oligonucleotide Probes to Control DNA

PCR products were amplified from the genomic DNA of unrelated control subjects of known ancestry as described above. A 15–30-μl aliquot of each PCR product was denatured (0.3 M NaOH), applied through a slot-blotter (Schleicher & Schuell, Keene, NH) to a nitrocellulose filter, and neutralized. Filters were baked under vacuum (80°C, 2 h), incubated at room temperature for 2–24 h (5 × SSC, 1% SDS, 5 ×

Denhardt's solution, 200 μg sonicated salmon sperm DNA/ml), and hybridized for 48 h in the same solution, to which 50–100 ng of ^{32}P 5′ end-labeled oligonucleotide probe specific for the normal allele had been added. Filters were washed in three changes of 0.5 × SSC, 0.1% SDS (25°C, 1 liter, 30 min) prior to exposing X-ray film (– 70°C, 4–24 h). To remove the hybridizing probe, filters were washed as above, but at 70°C; then incubation, hybridization, washing, and film exposure were repeated, this time with the ^{32}P 5′ end-labeled oligonucleotide probe specific for the variant allele. Probes were, for exon 1B, 5′-AGCCTC-CGAAGGA for the normal allele and 5′AGCCTCT-GAAGGA for the variant allele; for exon 3 they were 5′-TGAGGGAGAGAGG for the normal allele and 5′-TGAGGGGGAGAGG for the variant allele.

Clinical and Psychophysical Studies

Diagnostic and clinical studies of members of families A and B (Miyake et al. 1985), C (Higgins et al. 1983), and D (Went and Pronk 1985, fig. 7) have been previously reported. Psychophysical studies of proband E1 included the Farnsworth-Munsell (FM) 100-hue test (Munsell Color, MacBeth Division, Baltimore, MD), La Jolla analytic colorimetry (Boynton and Nagy 1982), Nagel anomaloscopy, and color matching with the F2-Tritan plate. Subject F was studied by means of the FM 100-hue test, electroretinography of rod and cone systems, measurement of spectral sensitivity, and the transient tritanopia test.

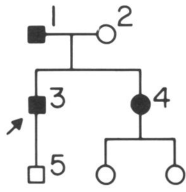
Results

All of the features of inherited tritanopia are consistent with the hypothesis that it is caused by a mutation in the gene encoding the blue-sensitive visual pigment (Nathans et al. 1986*b*) on chromosome 7 (Nathans et al. 1986*a*). To test this hypothesis, we have sought to detect possible mutant alleles of this gene segregating in the unrelated families illustrated in figure 1, *top*. (In all five families the transmission of tritanopia is consistent with its usual autosomal dominant inheritance, but only in families D and E is such transmission unequivocal.) Figure 1, *bottom,* shows the tritanopic errors made by proband E1 and subject F (see below), for whom psychophysical data have not been previously reported.

Southern blots of genomic DNA from one or more affected members of each family, probed with blue-sensitive opsin cDNA hs 37 (Nathans et al. 1986*b*), gave results indistinguishable from those of controls, excluding rearrangements that alter the size of the hybridizing fragment by more than approximately 100 bp (not shown). To detect potential mutations at single-nucleotide resolution, we employed the following strategy. All five exons and their adjacent 25–35-bp flanking regions were amplified from genomic DNA by PCR (Saiki et al. 1985) in a set of six separate reactions, two reactions with overlapping products for exon 1 (1A and 1B) and one reaction each for exons 2–5. A 40-bp sequence containing only G and C (GC-clamp) (Sheffield et al. 1989) was incorporated into each PCR product at one end, and the products were compared with controls of known sequence in DGGE (Fischer and Lerman 1983; Sheffield et al. 1989).

For probands A3 and B2, the exon 1A, 2, 3, 4, and 5 PCR products were indistinguishable in DGGE from those of controls, but the exon 1B PCR product migrated as a single band with mobility different from that of the control and apparently identical in both subjects (fig. 2*a*). For proband C2, the exon 1, 2, 4, and 5 PCR products were indistinguishable in DGGE from those of controls, but the exon 3 PCR product was resolved in two alleles, one with mobility indistinguishable from that of the control and one with variant mobility (fig. 2*b*). For proband D1, all PCR products were indistinguishable from those of controls in DGGE and by single-strand conformational polymorphism electrophoresis (Orita et al. 1989). For proband E1, the exon 1, 2, 4, and 5 PCR products were indistinguishable in DGGE from those of controls, but the exon 3 PCR product was resolved into two alleles, one

Figure 1 *Top,* selected members of five families in which tritanopia is segregating. Families A and B are from Japan, C and E are American of northern European descent, and D is Dutch. *Bottom,* FM 100-hue test results for E1 (*upper graph*) and subject F (*lower graph*) (see text). In addition to errors on the FM 100-hue test, proband E1 made tritanopic errors on the F2-Tritan plate and made tritanopic color matches with the La Jolla analytic colorimeter. Nagel anomaloscope results were normal. E3 and the mother of E1 and E3 made tritanopic errors on both the FM 100-hue test and the F2-Tritan plate. Other family members have been tested only with the F2-Tritan plate; their diagnoses should be considered tentative. In addition to making tritanopic errors on the FM 100-hue test, subject F showed apparent absence of blue spectral sensitivity in the spectral sensitivity function and the transient tritanopia test; electroretinographic responses of rods and long wavelength–sensitive cones were normal, whereas response of blue-sensitive cones were undetectable. There was no evidence of progressive retinal dysfunction on ophthalmoscopic examination.



Family A
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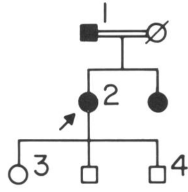

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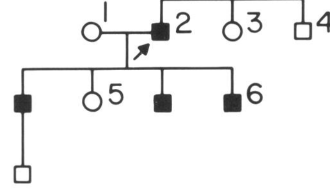

Family C
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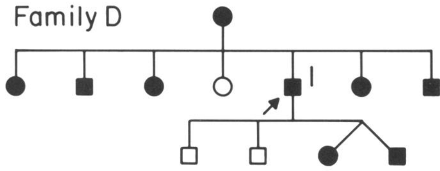

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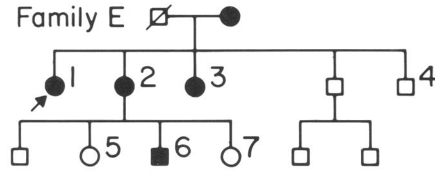

Family E
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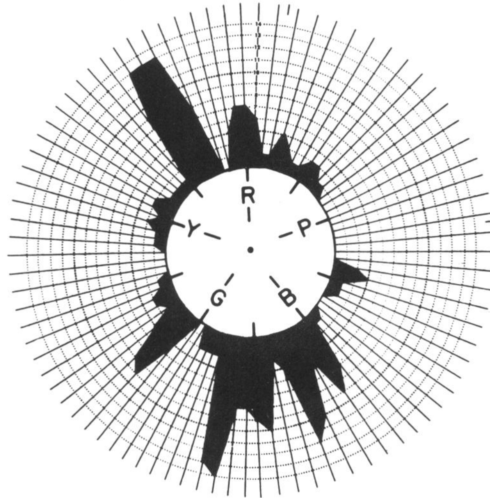

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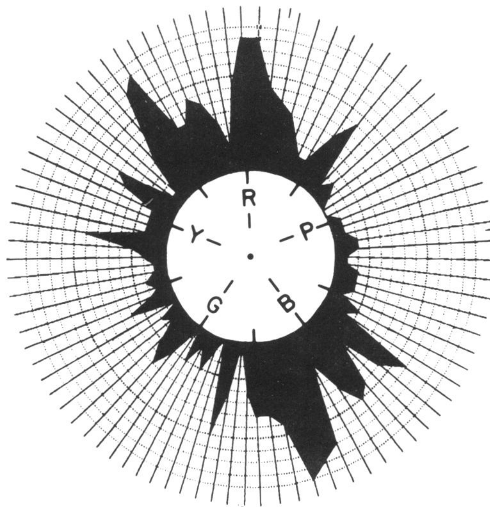

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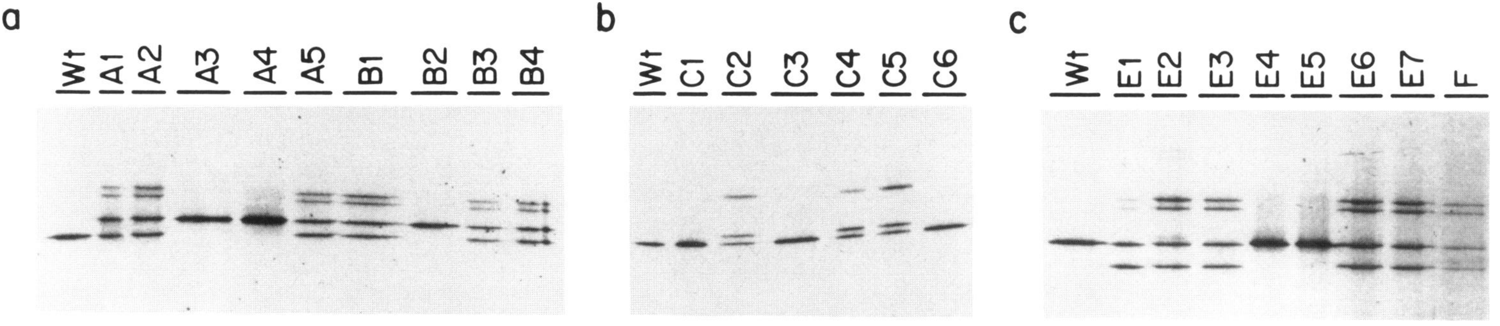

Figure 2 DGGE profile of PCR products from wild-type control and members of families A, B, C, and E. *a*, Exon 1B, families A and B (20%–80% denaturant); *b*, exon 3, family C (40%–80% denaturant); *c*, exon 3, family E and subject F (40%–80% denaturant).

with mobility indistinguishable in DGGE from that of the control and one with variant mobility (fig. 2*c*). The variant allele is clearly different from that of proband C2 (fig. 2*b*). The pair of minor bands migrating more slowly than the pair of major bands from heterozygotes is composed of mismatched duplexes (one strand from each of the two alleles) that arise during PCR by random reannealing of single strands (See Nagamine et al. 1989).

In addition, we analyzed DNA from four unrelated tritanopes for whom there is no known family history of tritanopia (and for whom, therefore, acquired tritanopia cannot be excluded). For three, the Southern blot pattern and the mobility of all PCR products in DGGE could not be distinguished from those of controls; one of these subjects was described in Wooten and Wald (1973). For the fourth (subject F), all PCR products were indistinguishable from those of controls by DGGE except for the exon 3 product, which was resolved into two alleles, one with normal mobility and one with variant mobility apparently identical to that of proband E1 (fig. 2*c*).

Next we determined the nucleotide sequences of the normal and variant alleles from the probands and from subject F. The nucleotide sequence of the variant exon 1 allele from proband A3 differed from that of the control only by the transition of G^{644} to A (fig. 3*a;* see Nathans et al. [1986*b*] for numbering system), leading to the replacement of Gly^{79} by Arg (G79R) in the proposed second transmembrane domain of the blue-sensitive opsin (fig. 3*d*). As suggested by DGGE (fig. 2*a*), the nucleotide sequence of the variant allele from proband B2 was the same as that from A3. In addition, the nucleotide sequence of all five exons and intron-exon junctions of a genomic clone derived from B2 revealed that the only difference from the control sequence was the G^{644}-to-A transition in exon 1.

The nucleotide sequence of the variant exon 3 allele from proband C2 differed from the normal allele only by the deletion of a single nucleotide, the G in position 5 of intron 3 (fig. 3*b*). This deletion causes positions 5 and 6 of intron 3 to differ from the donor site consensus sequence for splicing of pre-mRNA (Treisman et al. 1983; Kazazian and Antonarakis 1988).

The nucleotide sequence of the variant exon 3 allele from proband E1 differed from that of the normal allele only by the transition of C^{1049} to T, leading to the replacement of Ser^{214} by Pro (S214P) in the proposed fifth transmembrane domain of the blue-sensitive opsin (fig. 3*d*). As suggested by DGGE (fig. 2*c*), the nucleotide sequence of the variant allele from subject F was the same as that from E1.

The pattern of bands in figure 2*a* indicates that A3, A4, and B2 are homozygous and that A1, A2, A5, B1, B3, and B4 are heterozygous for G79R; oligonucleotide hybridization confirmed the genotype assignments for B2 and B4 (fig. 4*a*). Thus, all affected subjects carry the G79R allele; homozygotes are all affected (A3, A4, and B2), and heterozygotes can be affected (A1 and B1) or unaffected (A2, A5, B3, and B4). These results are consistent with the G79R allele conferring tritanopia via autosomal dominant inheritance with low penetrance.

The pattern of bands in figure 2*b* indicates that C1, C3, and C6 are homozygous for the normal allele and that C2, C4, and C5 are heterozygous for the intron 3 G-deletion allele. To exclude a selective failure of PCR primer binding to an undetected variant allele in affected subject C6, two additional sets of PCR primers that amplified across the exon 3–intron 3 junction were used; in each case, known heterozygotes were confirmed by DGGE, while C6 showed only a band representing the normal allele (not shown). The genomic DNA from C6 was shown to be derived from an offspring of subjects C1 and C2 by detecting parentally derived alleles at four unlinked loci highly polymorphic for dinucleotide repeats (Weber and May 1989) and at nine hypervariable sites in the major histocompatibility complex locus. The fact that affected subject C6 does not detectably carry the variant allele indicates that the intron 3 G-deletion allele is unrelated to tritanopia and is therefore only coincidentally present in the proband.

The pattern of bands in figure 2*c* indicates that E1, E2, E3, E6, E7, and subject F are heterozygous for S214P and that E4 and E5 are homozygous for the normal allele; oligonucleotide hybridization confirmed the genotype assignments for the members of family E (fig. 4*b*). Thus, all affected subjects are heterozygotes for S214P (E1, E2, E3, E6, and F), whereas one unaffected subject is heterozygous (E7) and the others are homozygous for the normal allele (E4 and E5). These results are consistent with the S214P allele conferring tritanopia via autosomal dominant inheritance with high penetrance.

To test whether the G79R or S214P variants merely represent neutral mutations present by chance in families segregating tritanopia, we used oligonucleotide probes specific for the normal and variant alleles to assess the prevalence of each in human populations of ancestry similar to each family. No examples of the variant alleles were detected in PCR products amplified from the genomic DNA of unrelated control subjects (fig. 4). The G79R variant occurs in two of the

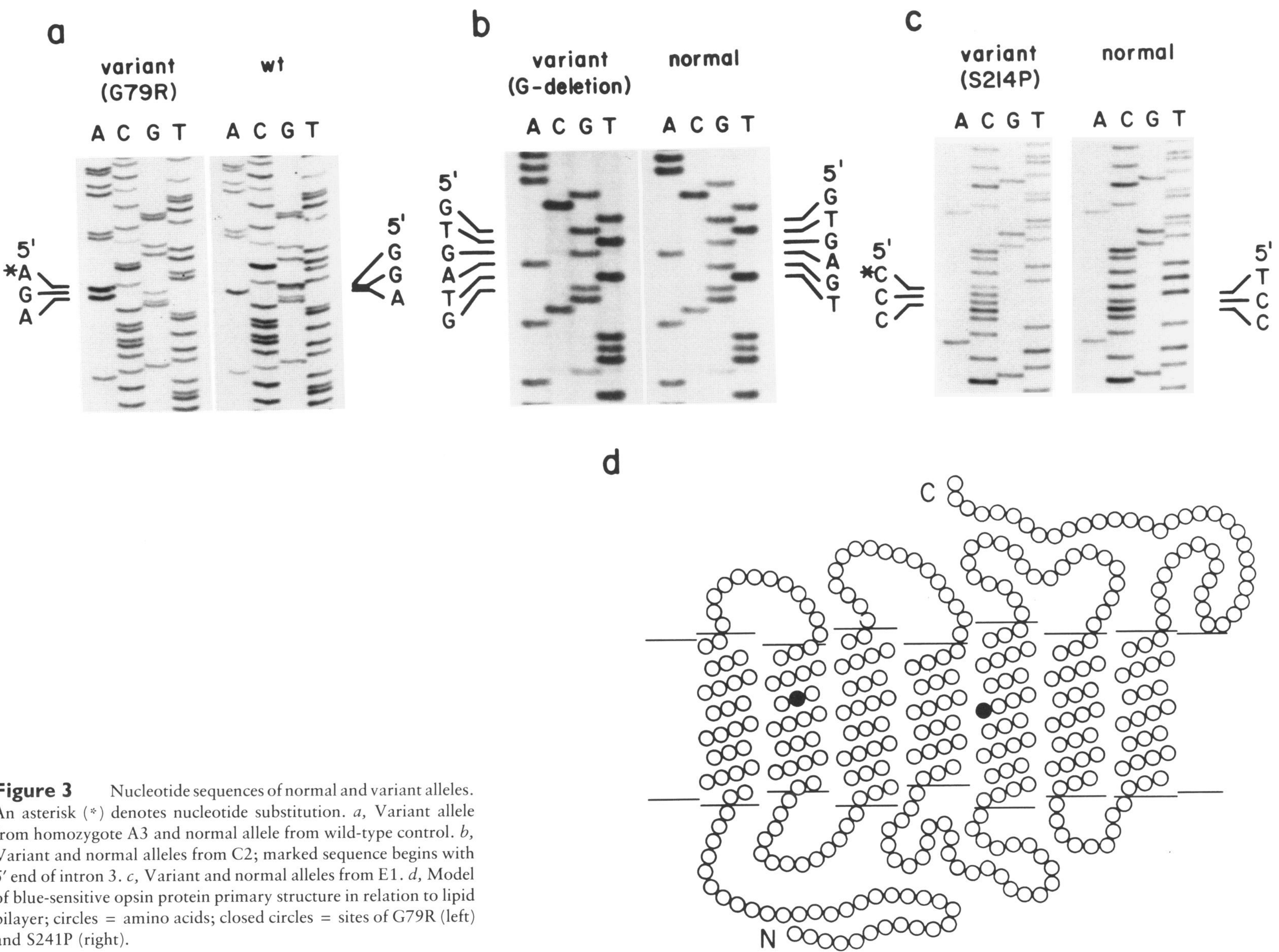

Figure 3 Nucleotide sequences of normal and variant alleles. An asterisk (*) denotes nucleotide substitution. *a*, Variant allele from homozygote A3 and normal allele from wild-type control. *b*, Variant and normal alleles from C2; marked sequence begins with 5′ end of intron 3. *c*, Variant and normal alleles from E1. *d*, Model of blue-sensitive opsin protein primary structure in relation to lipid bilayer; circles = amino acids; closed circles = sites of G79R (left) and S241P (right).

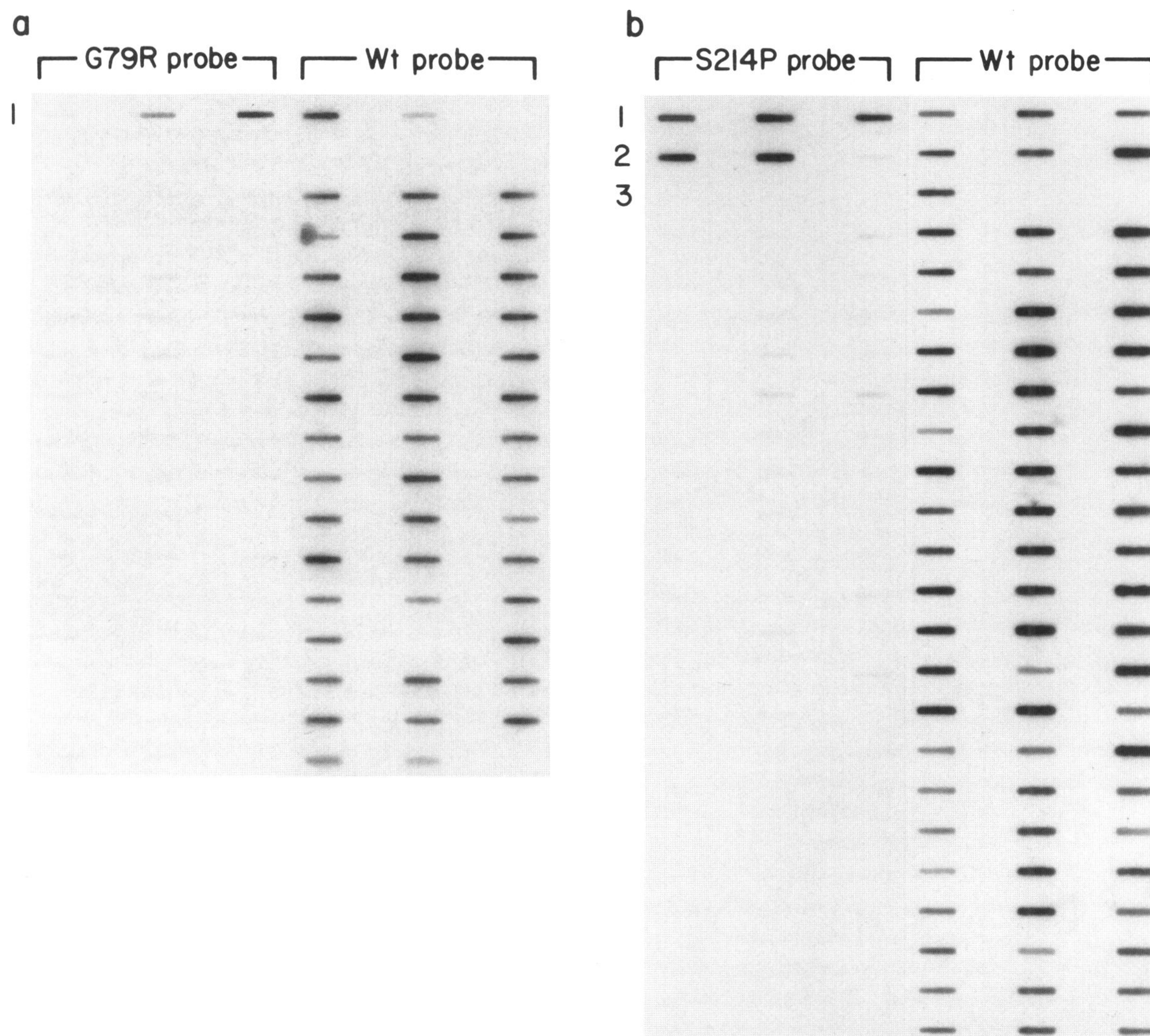

Figure 4 Hybridization of oligonucleotide probes specific for G79R and S214P alleles to PCR products from selected pedigree members and unrelated control subjects. Each slot blot was probed with the oligonucleotide specific for the wild-type sequence, stripped of probe by washing, and then reprobed with the oligonucleotide specific for the variant sequence. *a,* Exon 1B. Row 1, wild-type control (left), B4 (middle), B2 (right); all other rows, control subjects of Japanese ancestry. *b,* Exon 3. Row 1, E1 (left), E2 (middle), E3 (right); row 2, E6 (left), E7 (middle), E5 (right); row 3, E4 (left); all other rows, control subjects of northern European ancestry.

five unrelated probands, as compared with zero of 43 control subjects (χ^2 = 17.8; P < .0001) (fig. 4*a*). If we also include those tritanopes with no known family history, then this variant occurs in two of nine unrelated tritanopes (χ^2 = 9.77; P = .0018). In conjunction with the pattern of familial segregation (see above), we conclude that G79R is very likely the mutation causing tritanopia in members of families A and B.

The S214P variant occurs in one of five unrelated probands, as compared with zero of 63 controls (χ^2 = 13.4; P = .00025) (fig. 4*b*). If we also include those tritanopes with no known family history, then this variant occurs in two of nine unrelated tritanopes (χ^2 = 14.3; P = .00016). In addition, no variant bands were detected by DGGE analysis of exon 3 PCR products amplified from 84 controls. In conjunction with the pattern of familial segregation (see above), we conclude that S214P is very likely the mutation causing tritanopia in members of family E and in subject F.

The intron 3 G-deletion allele detected in members of family C must be a rare sequence variant—it was not detected in any of 63 control subjects by oligonucleotide hybridization or by DGGE analysis of exon 3 PCR products amplified from the DNA of 84 control subjects.

Discussion

When all of our data are taken into account, the association of tritanopia with two amino acid substitutions in the blue-sensitive opsin is very strong—G79R or S214P occur in three of five unrelated probands (or four of nine tritanopes if those without a family history are included), as compared with zero of 43 and zero of 84 controls, respectively ($P < .00001$). These results provide direct genetic evidence linking the blue-sensitive opsin gene with blue spectral sensitivity, confirming that its original identification (Nathans et al. 1986*a*, 1986*b*) was correct.

The failure to detect a variant allele in proband D1 or a variant in family C that cosegregated with tritanopia does not exclude the blue-sensitive opsin gene as the target of a mutation. In addition to any sequence changes in the PCR products that escaped detection, we would not have found a point mutation in the upstream flanking region or within introns outside of the regions of PCR amplification.

The dominant inheritance of the G79R and S214P mutations suggests that the aberrant gene products actively interfere with the viability or fidelity of blue-sensitive cone photoreceptors, but the mechanism in each case is as yet unknown. Of special interest are the three affected subjects homozygous for G79R—it is not yet known whether they differ as a group from affected heterozygotes in psychophysical or electroretinographic manifestations. Nor is it known to what extent, if any, genetic background, environment, or diagnostic variability contributes to the low penetrance manifested by G79R. The substitution of Arg, a bulky, positively-charged residue, for Gly, a small, nonpolar residue, within a predicted transmembrane domain might be expected to perturb the folding, processing, or stability of the blue-sensitive opsin. Supporting this notion is the finding that substituting positively charged for nonpolar residues in predicted transmembrane domains of rhodopsin has led in some cases to a complete loss of spectral activity (Sakmar et al. 1989).

It is likely that the S214P mutation also perturbs the folding, processing, stability, or structure of the blue-sensitive opsin. There are several precedents for the loss of protein function following replacement of a residue within an alpha-helix by proline (Pakula and Sauer 1989), and, when it normally occurs there, proline is known in some cases to introduce a 20° kink in alpha-helical rods (Kendrew et al. 1960; Schulz et al. 1974).

Unlike the mutations in the rhodopsin gene associated with autosomal dominant retinitis pigmentosa (ADRP) (Dryja et al. 1990*a*, 1990*b*; Sung et al. 1991), those described here do not cause progressive retinal degeneration. It is not yet known whether this difference in clinical course reflects an inherent difference in the mechanism of mutations causing ADRP and tritanopia, a difference in the physiology of rod and cone photoreceptors, or simply the much greater number of rods in the human retina.

Acknowledgments

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