Trichromatic color vision with only two spectrally distinct photopigments

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Protanomaly is a common, X-linked abnormality of color vision. Like people with normal color vision, protanomalous observers are trichromatic, but their ability to discriminate colors in the red–green part of the spectrum is reduced because the photopigments that mediate discrimination in this range are abnormally similar. Whereas normal subjects have pigments whose wavelengths of peak sensitivity differ by about 30 nm, the peak wavelengths for protanomalous observers are thought to differ by only a few nanometers. We found, however, that although this difference occurred in some protanomalous subjects, others had pigments whose peak wavelengths were identical. Genetic and psychophysical results from the latter class indicated that limited red–green discrimination can be achieved with pigments that have the same peak wavelength sensitivity and that differ only in optical density. A single amino acid substitution was correlated with trichromacy in these subjects, suggesting that differences in pigment sequence may regulate the optical density of the cone.

Human color vision depends on three classes of cone photoreceptors[1–3] whose outputs are processed by two parallel neural subsystems[4]. One subsystem is phylogenetically ancient and compares the outputs of the short-wavelength-sensitive (S) cones with those that absorb in the middle-to-long wavelengths. The second system compares outputs of the middle- (M) and long-wavelength (L) cones to provide color discrimination in the red to green region of the spectrum. The latter appeared recently in mammalian evolution and is found only in man and other primates. L and M cones are distinguished from each other because they contain different photopigments. A gene duplication apparently gave rise to separate L and M photopigment genes within the timeframe of primate evolution. Having had little time to diverge, the L and M genes are ~98% homologous, and they are arranged in a tandem array on the X chromosome[5]. This arrangement is unstable; the high homology and close proximity of the genes promotes frequent recombination events including gene duplications, deletions and the production of hybrid or chimeric genes. Presumably as the result of this type of genetic activity, there is considerable diversity in the genes for 'red–green' color vision in present-day humans[5]. The number and ratio of the L and M genes varies across individuals, as do the individual gene sequences. The sequence variation produces spectral variants within the normal L and M classes of pigments, which are responsible for subtle variations in color vision in the normal population[6,7]. Presumably, gene rearrangements are also responsible for defects in red–green color vision, which occur at a very high frequency in humans[8].

A common form of red–green color vision deficiency is protanomalous trichromacy (protanomaly). Individuals with protanomaly are missing normal L photopigment; this loss usually results from the absence of all L genes. Protanomalous trichromats are able to make limited color discriminations in the red–green region of the spectrum, but this ability is greatly reduced compared to normal. In normal color vision, red–green discrimination is mediated by comparing the relative rates of photon capture by the L and M cones. The L and M cone absorption spectra overlap, but their peaks (λ_{max}) are well separated (**Fig. 1a**). Two lights that differ in wavelength produce different photon-catch rates in L cones relative to M cones, and the resulting relative difference in responses of the two cone classes is the basis for color discrimination. If the difference between the absorption spectra of the cone classes is substantially reduced, then a much larger change in wavelength is required to produce equivalent changes in relative photon-catch rates, and color-discrimination ability is reduced. Even though protanomalous trichromats are missing L genes, their color vision capabilities imply that they must retain at least two M genes expressed in separate subpopulations of cones. In protanomaly, the M-cone subtypes differ subtly in spectral absorption properties, providing the basis for a much-reduced form of red–green color vision. The spectral difference between M-cone subtypes in protanomaly is usually conceived as a small difference in spectral peak (λ_{max}; **Fig. 1b**.)

Among L and M photopigments, substitutions at only seven amino acid positions have been identified as responsible for differences in λ_{max}[2,9–12]. Two positions within exon 5 of the genes are responsible for the large shift separating M from L photopigment classes[2,9–13]. The other five positions produce smaller shifts (7 nm)[2,6,7,9], which may represent the subtle pigment differences that underlie anomalous color vision[2,9,14]. One of the seven, serine for tyrosine at position 116 (S116Y), produces a small shift in L pigments but has no effect on λ_{max} in pigments

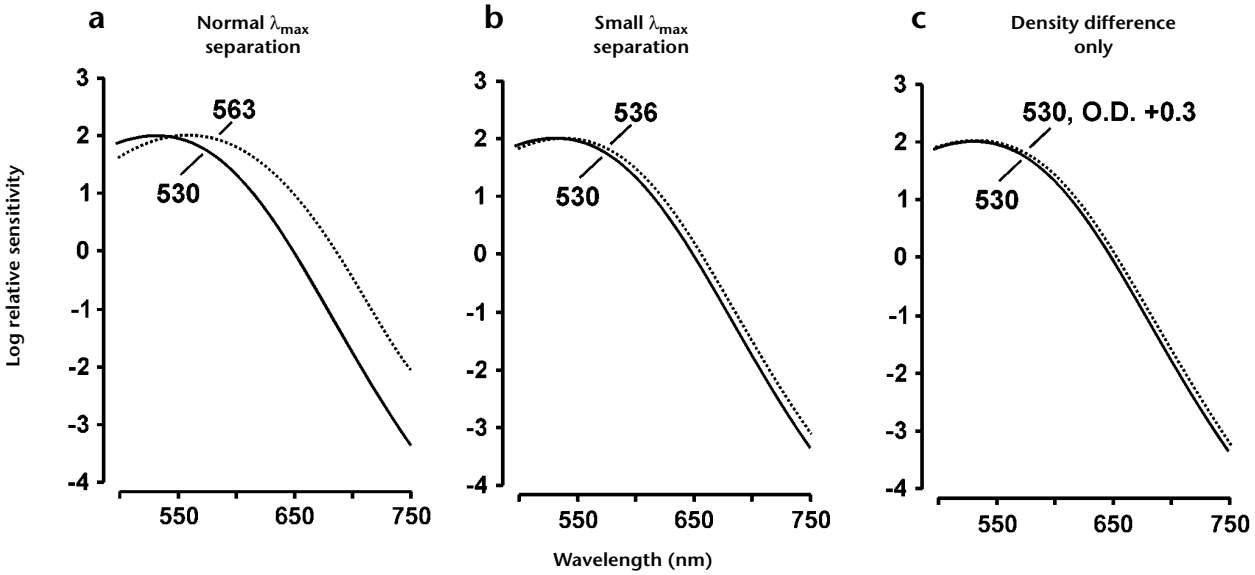

Fig. 1. Theoretical spectral sensitivity curves for L and M cones in normal trichromats and M-cone subtypes in protanomalous trichromats. These represent theoretical action spectra or absorption spectra for the cones, assuming the light travels axially through the outer segment. (**a**) Relative spectral sensitivities of normal M and L pigments with ~30 nm difference in wavelength of peak sensitivity. (**b**) Relative spectral sensitivities of cones with theoretical protanomalous pigments separated by 6 nm in spectral peak wavelength. (**c**) Relative spectral sensitivities of protanomalous pigments that differ only in optical density (no difference in peak wavelength). The effect of increasing optical density is due to increased 'self-screening'. An increase in optical density broadens the relative spectral sensitivity curve. The absorption curve with the higher optical density reflects higher sensitivity to wavelengths on both sides of the spectral peak. However, for wavelengths near the peak and in the long wavelengths, the optical density difference qualitatively mimics the difference produced by a spectral shift.

in which the carboxy terminal is derived from an M pigment[2,9,15], as is the case for all the X-encoded pigments of protanomalous trichromats reported here.

Because protanomaly arises by rearrangements within the normal tandem array of pigment genes[5,16–18], which usually involves the loss of all L-pigment genes, the residual red–green color vision depends on the remaining M-pigment genes. When the array is reduced to a single gene, the result is a more severe form of color vision defect, called protanopia. Protanopes have dichromatic (as opposed to trichromatic) color vision and are completely unable to make color discriminations in the red-to-green region of the spectrum. However, like protanomalous trichromats, some protanopes have multiple M genes[5]. It is proposed that, when multiple genes remain, presence or absence of red–green color vision depends on differences among the encoded pigments at the identified spectrally active amino acid residues[2,9]. Pigments identical at all of the active residues have identical λ_{max}. It is supposed that, if the multiple X-chromosome pigment genes for a person encode the identical amino acids at all spectral-tuning sites, then the person must be a dichromat—in this discussion, a protanope. In the present study, this was found not to be true. A subset of protanomalous trichromats did not have differences at any of the positions known to shift the λ_{max} of M pigments. The question becomes, if the encoded pigments do not differ in spectral peak, then how do they differ to produce the basis for red–green color vision? The experiments reported here suggest that red–green color vision in these individuals is based on a difference in the effective optical density of the M-cone subtypes. Amino acid differences encoded by exon 2 of the gene were found among the M pigments of all protanomalous trichromats, but not in any protanopes, and are thus identified as ones that may be involved in controlling the optical density of the cone photopigment.

Results

We examined the nucleotide sequences of the X-linked pigment genes looking for differences within each protanomalous trichromat that might underlie the spectral difference responsible for his red–green color vision. Surprisingly, the protanomalous trichromats fell into two classes according to the deduced amino acid sequences. One class was as predicted from the prevailing theory (numbers 10–13, **Fig. 2**). These protanomalous trichromats each had genes differing at one or more of the codons that determine the spectral peak. In these men, the peak separations on which color vision could be based were predicted to vary from as little as 2 nm to as much as 9 nm. A separation as small as 2 nm can still provide some color discrimination[18].

The second class of protanomalous trichromats had no differences at any of the positions known to shift λ_{max} in M pigments (numbers 16–19, **Fig. 2**). However, each had two genes that differed in exon 2. These men all shared one genetic characteristic: they had X-encoded opsins that differed by threonine versus isoleucine at position 65 (T65I). Two of the men had additional differences at position 111 and 116.

If the encoded pigments do not differ in spectral peak, then how do they differ to produce the basis for red–green color vision? A possibility is suggested by earlier measurements of spectral sensitivity in the protanopes[15]. In those experiments, the protanopes numbered 1–7 (**Fig. 2**) were measured to have the same spectral peak sensitivity. The spectral sensitivities of subjects 1–6 were nearly identical in every aspect; however, one protanope (number 7, subject 0018, **Fig. 2**), who differed from the others in the replacement of T65I, I111V and S116Y, had a spectral-sensitivity curve that was very slightly broader. A broadening of the spectral absorption curve would occur if the exon 2-encoded differences changed the effective optical density of the cone pigment (**Fig. 1c**), as might

happen, for example, if a substitution altered the stability of the molecule or the efficiency with which it absorbs light. A spectral sensitivity difference among the cones arising from differences only in optical density (and not in λ_{max}) is sufficient, in theory, to provide the basis for color vision[18–20].

If a protanomalous trichromat has two pigments that differ only in optical density, then specific measurable aspects of his color vision should be distinct from those of a protanomalous trichromat with two pigments separated in λ_{max} by even as little as 1–2 nm. The former should be able to discriminate middle from long wavelengths at modest light levels, but not immediately following exposure to a bright light, which bleaches a high percentage of pigment. Bleaching sharply reduces the effective optical density of each pigment to a low level, regardless of the initial optical density, so the two pigments' relative spectral sensitivities become virtually identical. A strong bleach, therefore, should cause a complete loss of color discrimination in the red–green range if a protanomalous trichromat has pigments that share a single λ_{max}. Protanomalous trichromats with pigments having distinct wavelengths of λ_{max}, on the other hand, should maintain color discrimination at all light levels.

This was investigated in psychophysical examination of four additional subjects (9, 14, 15, 20, **Fig. 2**) whose genetic results were unknown at the time of psychophysical testing. Subsequent genetic analysis revealed that two of these observers (numbers 14 and 15, **Fig. 2**) have genes for pigments distinct in λ_{max}. Color-matching measurements from one of these observers (number 14, **Fig. 3a**) are shown by a plot of the Rayleigh[21] color-match range (vertical axis) as a function of retinal adapting illumination (horizontal axis). (Results from subject 15 were similar.) In Rayleigh matching, the subject was given a mixture of two primary lights, one with a wavelength of 547 nm (green to a normal observer) and another with a wavelength of 640 nm (red to a normal observer), and was asked to adjust their proportion in the mixture to exactly match the appearance of a monochromatic comparison light of 589 nm (yellow to a normal). Proportions of the 547 nm plus 640 nm mixture chosen to match 589 nm were determined and quantitatively specified as the proportion of 640 nm light in the mixture field, which could vary from 0 (pure 547 nm light) to 1 (pure 640 nm light). At the usual, modest light level used for testing (left-most point, **Fig. 3a**), simple protanomalous trichromats perceive a match only when the mixture proportion falls within a narrow range. Subject 14 maintained a nar-

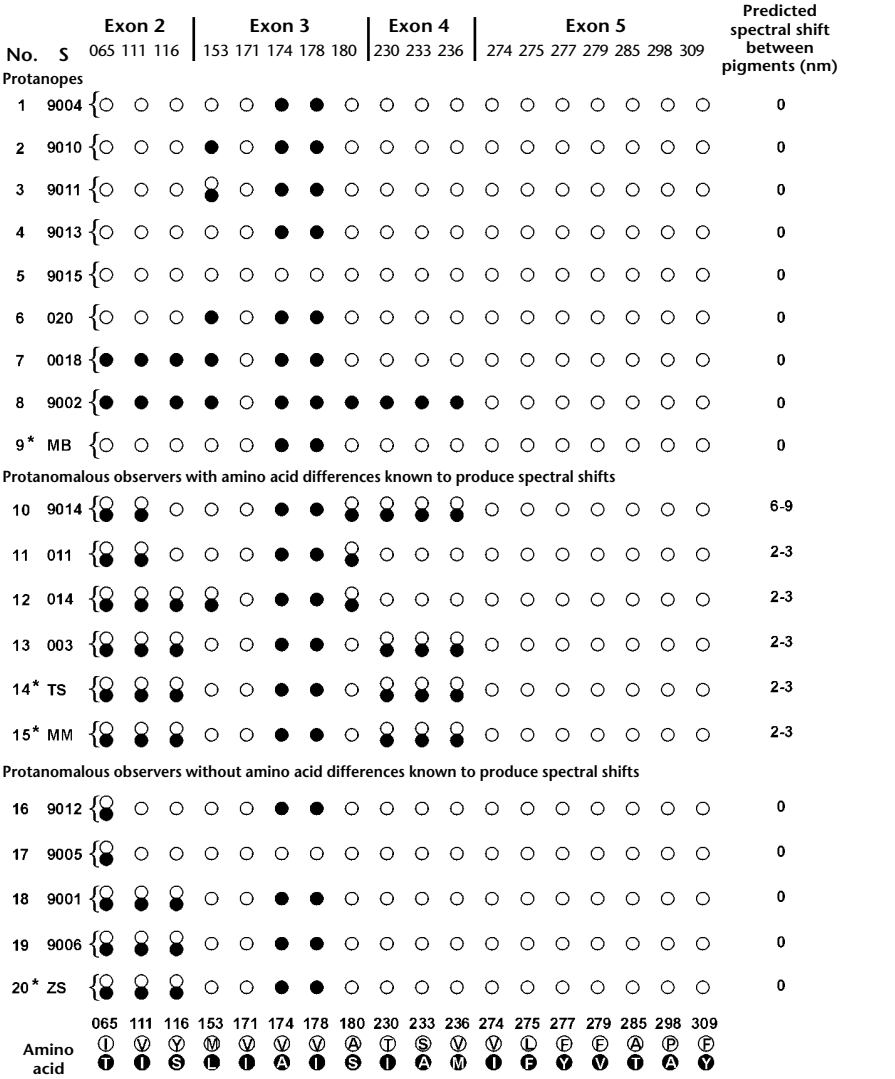

Fig. 2. Schematic representation of the X-encoded pigments in protanopes (dichromats) and protanomalous trichromats. A solid or open circle represents the identity of each of the 18 dimorphic amino acids among the M and L pigments of humans. A key to the amino acid identities is shown along the bottom of the figure. The approximate difference in λ_{max} that would be expected to distinguish the subjects' pigments is shown in the right-most column. The spectral separations were estimated from measurements of hybrid pigments *in vitro*[2,9]. The authors of an *in-vitro* study[9] do not explicitly report the identities of amino acids at sites 171, 174 and 178, but indicate that they are the same as those previously reported[5]. Thus, the 'wild-type' M pigment[9] had a deduced amino acid sequence identical to protanope (number 1, 9004). In all locations where a single circle appears, there was only one sequence for that codon among the person's genes. Presence of both filled and unfilled circles indicates that the person had multiple genes with a difference at that codon. Theoretically, all protanomalous trichromats need more than one M gene to encode spectrally different pigments to support red-green color vision; this is what was observed. Most of the protanopes also had more than one M gene. However, M genes that differed in sequence were observed only in subject 9011. Only three dichromats had a single pigment gene on the X-chromosome (9013, 0018, 9002). An asterisk (*) indicates the four subjects who were tested in the high luminance and extended Rayleigh match conditions; their Rayleigh match data is presented in Fig. 3.

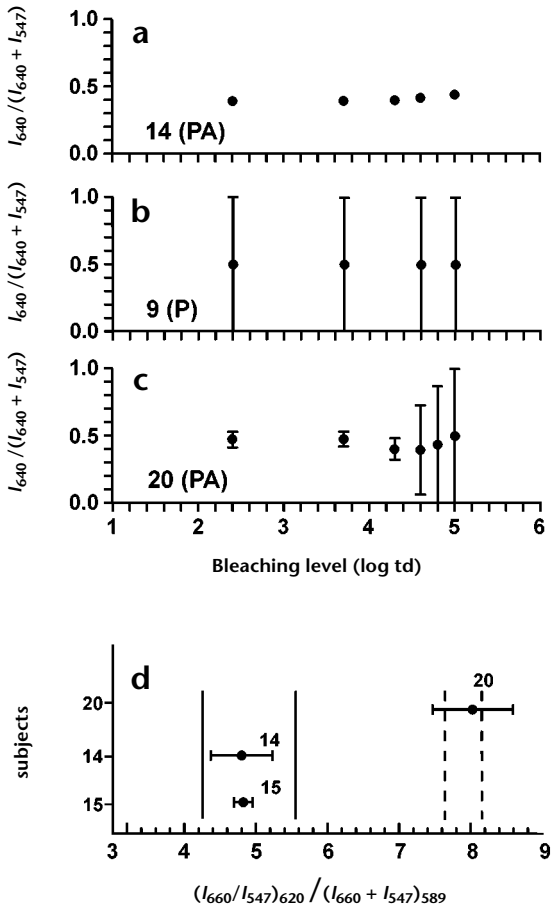

Fig. 3. Rayleigh color-match data. (**a**–**c**). Proportion of 640 nm light in the 547 nm plus 640 nm mixture matched to 589 nm (vertical axis), as a function of bleaching light level (horizontal axis). Bars indicate range of mixture proportions judged to match to 589 nm, except where the range was smaller than the circle plotted at its midpoint (**a**). Measurements are shown (**a**) for a protanomalous trichromat (number 14, **Fig. 2**) with a polymorphism resulting in pigments that differ in λ_{max}, (**b**) for a protanope (dichromat) and (**c**) for a protanomalous trichromat with no polymorphism that affects pigment λ_{max} (number 20, **Fig. 2**). Mixture primary units are in protan mode. (**d**) Extended Rayleigh-equation measurements from three protanomalous trichromats (numbers 14, 15 and 20, **Fig. 2**). Each measurement was repeated in three separate sessions; the circle and error bars show the mean and s.e., respectively. The value for the extended Rayleigh equation is a ratio of two color-matching measurements, and thus is meaningful only when the range of each constituent match is narrow, as was the case here when retinal illumination was below bleaching levels. Extended Rayleigh measurements are unaffected by transmissivity of lens or ocular media[22]. Separate ranges of theoretical values are bounded by solid and dashed vertical lines (see text).

row match range at all levels of adapting light (**Fig. 3a**), indicating that the relative spectral sensitivities of pigments mediating his matches were never identical. The results of the observers with X-chromosomal genes encoding pigments with unequal λ_{max} (numbers 14 and 15) were very different from those of a protanope (dichromat; number 9 in **Fig. 2**), who had no color discrimination in the red-to-green region of the spectrum and matched pure 547 or pure 640 nm light (or any mixture of them) to 589 nm at every adapting level (**Fig. 3b**).

Figure 3c gives color-matching measurements for a protanomalous trichromat whose pigment genes had no polymorphisms known to alter λ_{max} (number 20, **Fig. 2**). His Rayleigh range was narrow at the standard light level (left-most point) but gradually increased when adapting illumination was raised above 4.0 log trolands (td), the level at which significant photopigment is bleached. At 5.0 log td, which bleaches about 85% of pigment, he was unable to discriminate 547 or 640 nm light from 589 nm. At this point, he behaved as a dichromat (**Fig. 3b**), exactly as expected if he had two pigments with different initial optical densities but the same λ_{max}.

An independent psychophysical observation corroborated the conclusion that observer number 20 had two pigments that differed only in optical density. The extended Rayleigh equation[22] uses 547 nm plus 660 nm primaries in two separate color matches, one to the usual 589 nm and one to a different wavelength (620 nm is used here). A ratio of the mixture-primary measurements from the two matches $(I_{660}/I_{547})_{620}/(I_{660}/I_{547})_{589}$, is sensitive to whether an observer has two pigments that differ in λ_{max} or, alternatively, has two pigments with the same λ_{max} and a difference only in optical density. Extended Rayleigh measurements from the same three protanomalous trichromats (numbers 14, 15, 20) show that observer number 20, whose pigment genes were expected to have the same λ_{max}, had a much higher value for this ratio than the others (**Fig. 3d**). A precise theoretical value can be derived for this measure, given each pigment's λ_{max} and density[19,20]. If a protanomalous trichromat's two pigments differ in λ_{max} by 1 nm or more, then the theoretical value for any combination of λ_{max} wavelengths falls within the bounds indicated by the solid vertical lines. The measurements from observers with genes for pigments separated in λ_{max} were well within this range, as expected, but the measurement for the other protanomalous trichromat (number 20) was not. When two pigments share the same peak wavelength, higher theoretical values are expected. Dashed vertical lines bound the range of values for any plausible wavelength of the λ_{max} and any optical densities that differ by at least 0.1 (a smaller optical density difference would result in dichromacy rather than trichromacy). The value measured for observer number 20 is within this distinct predicted range for pigments that differ in optical density but not in λ_{max}.

Discussion

The protanomalous trichromats fell into two classes according to deduced amino acid sequences. One class (numbers 10–15, **Fig. 2**) had genes encoding pigments predicted to differ in λ_{max}, whereas the other class had genes encoding pigments predicted to be identical in λ_{max} (numbers 16–20, **Fig. 2**). Members of the second class each had genes for pigments differing at one or more amino acid positions encoded in exon 2. The difference at position 65, which they all shared, and additionally, perhaps, I111V and S116Y, seem to be the basis for red–green color vision in the second class of protanomalous observer. This conclusion is supported by the fact that these changes were the only differences among the X-encoded pigments in this class of protanomalous trichromat, whereas none of nine protanopic dichromats (numbers 1–9, **Fig. 2**) had two genes that differed in exon 2, even though most of them had more than one X-linked pigment gene. The probability of obtaining this perfect correspondence—five protanomalous trichromats with exon 2-encoded dimorphisms and nine protanopes without them—by chance is low ($p < 0.005$).

The psychophysical experiments indicate that color vision in the second class of protanomalous trichromat is based on two classes of M cone that differ in effective optical density but not in

the λ_{max} of their pigments. The change in spectral sensitivity that occurs with increasing optical density is due to increased 'self-screening'. Consider what happens as light travels the length of the cone outer segment. As the light enters the cone, wavelengths near the spectral peak are absorbed most strongly, so less light in that spectral region is passed on for absorption by pigment more distal in the light path. This self-screening is progressively less effective for wavelengths away from the absorption maximum and, thus, absorption is more cumulative in the spectral limbs. The effect is that an increase in optical density broadens the relative spectral sensitivity function for the cone. The results reported here suggest that exon 2-encoded differences changed the effective optical density of the cone. There are a limited number of possibilities for how this may occur. A change in the effective optical density of the cone could be produced by a change in the length of the cone outer segment, by a change in the concentration of the pigment within the cone or by a change in the efficiency with which individual pigment molecules absorb light. A change in the light-absorbing efficiency of the individual pigment molecules would be the most direct mechanism by which amino acid changes could influence the optical density of the cone. For the other two possibilities, it is difficult to imagine how a change in the pigment could increase optical density; however, it is easy to imagine how unfavorable mutations could reduce it. For example, a reduction of the molecule's stability in the membrane that disrupted the integrity of the outer segment might shorten it. Similarly, a mutation that interfered with photopigment biogenesis might reduce the concentration of photopigment in the membrane or the outer segment length.

Aside from effects on λ_{max}, little is known about how cone opsin structure controls various cone pigment properties. Here, identified mutations did not affect λ_{max}, yet pigment spectral sensitivity was altered to produce the striking behavioral difference between anomalous trichromacy and dichromacy. We suggest that amino acid substitutions in the pigment gene control both the λ_{max} and the effective optical density. When color vision depends on subtle differences between two pigments, as in color vision defects, relating genotype to phenotype requires consideration of genetic polymorphisms that might affect optical density as well as those that shift λ_{max}.

Methods

Genetic analysis. Exons 2, 3, 4 and 5 of the X-linked visual-pigment genes were amplified. Exons 3, 4 and 5 were amplified using primers and conditions described elsewhere[14], except that here, the primers were tagged with the M13 forward or reverse primer sequences for dye-primer sequencing. To amplify exon 2, the forward primer was 5′tgtaaaacgacggccagtCTCGAATTCGGTGCTGCAGCCGAGCTCC and the reverse primer was 5′caggaaacagctatgaccCTCGAATTCGAGCCTGGGCCCCGACTGGC, where the lower-case letters correspond to the M13 forward and reverse primer-tag sequences. Each PCR product was sequenced directly in both directions using AmpliTaq FS (Perkin-Elmer) as recommended by the manufacturer and the ABI Prism 310 automated sequencer. The genetic analyses were performed without knowledge of the psychophysical results, and the psychophysical analyses were done without knowledge of the genetic outcomes. The procedures in this study were in accordance with guidelines approved by the Institutional Review Boards. Informed consent was obtained from all subjects.

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