Genetic Basis of Photopigment Variations in Human Dichromats

MAUREEN NEITZ,* JAY NEITZ,*† GERALD H. JACOBS‡

The spectral sensitivities of the X-encoded pigments in dichromats were studied using the electroretinogram. The action spectra measured for these subjects correspond to four distinctly different X-encoded visual pigments, two different middle-wave pigments spectrally separated by 7 nm and two different long-wave pigments separated by 5 nm. Amino acid sequences were deduced from examination of the genes encoding the pigments. Pairwise comparisons of the opsin structures and pigment spectra confirm and clarify earlier conclusions. Substitutions in exon 5 of the genes produce the spectral difference that separates human X-encoded pigments into middle- and long-wave classes. Polymorphisms in exons 2–4 produce subtypes of pigments that fall within those major classes. Substitution of half of a middle-wave gene with long-wave sequence (exons 1–3) does not shift the middle-wave spectrum. Combined substitutions at positions 230, 233 and 180 produce a 7 nm shift in the middle-wave pigment spectrum. Two subtypes of long-wave pigments that differ in the presence of serine or alanine at position 180 and occur in dichromats, deuteranomalous trichromats, and color normals are spectrally separated by 5–7 nm.

Cones Photopigment Electroretinogram Photopigment genes Dichromacy Color vision

INTRODUCTION

Human color vision is based on input from three classes of cone photoreceptor, each maximally sensitive to a different region of the spectrum (short-wave, middle-wave and long-wave sensitive). The visual pigment molecules responsible for the spectral properties of the cones are heptahelicals with seven transmembrane segments. The long-wave and middle-wave cone photopigments are encoded by genes that lie in a head-to-tail tandem array on the X-chromosome; each gene has six exons (Nathans, Thomas & Hogness, 1986). The first and sixth exons encode tails of the heptahelicals that are not imbedded in the membrane and appear to be identical between the long- and middle-wave genes. Exons 2–5 encode the transmembrane portion of the heptahelical. Amino acid differences in this region are involved in tuning the spectra of the pigments. Among the X-linked pigments the largest spectral shifts are produced by amino acid substitutions at two closely separated positions, 277 and 285, encoded by exon 5 of the genes. Substitution of hydroxyl-bearing amino acids for non-polar amino acids at both positions produces a

net red-shift of as large as 25–26 nm (Asenjo, Rim & Oprian, 1994; Chan, Lee & Sakmar, 1992; Merbs & Nathans, 1993; Neitz, Neitz & Jacobs, 1991). Other amino acid differences encoded by exons 2–4 of the genes also can produce spectral shifts, but these are small compared to the shift produced by exon 5 differences. Thus, human X-encoded pigments can be thought of as falling into two classes, middle-wave and long-wave (Neitz & Neitz, 1994). These are well separated in spectral peak because of differences encoded by exon 5. Polymorphisms in exons 2–4 of the genes that encode each pigment class produce subtypes of middle-wave and long-wave pigments that occur among observers with normal color vision, among anomalous trichromats, and among dichromats.

Red–green dichromats base their color vision on a short-wave pigment paired with a single X-encoded pigment; either middle-wave (protanopes) or long-wave (deuteranopes). The spectral sensitivities of the X-linked pigments in dichromats have been studied using the electroretinogram (ERG). These give insight into the nature of the variety of spectral differences that occur among human pigments and that underlie variations in normal color vision and color vision anomalies. Comparisons of the deduced amino acid sequences of the pigments expressed in dichromats reveal how naturally occurring amino acid substitutions affect pigment spectral sensitivities. The results of these *in vivo* experiments can be compared with those obtained from mutant pigments expressed and characterized *in vitro*.

*Departments of Ophthalmology and Cellular Biology, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226, U.S.A.
†To whom all correspondence should be addressed [*Fax* 414 266 8496; *Email* jneitz@post.its.mcw.edu].
‡Department of Psychology, University of California, Santa Barbara, CA 93106, U.S.A.

TABLE 1. PCR primers

Primer	Location	Strand	Sequence	Specificity	Nucleotide position
1	Intron 1	+	5'CTCGAATTCGGTGCTGCAGCCGAGCTCC		
2	Intron 2	−	5'CTCGAATTCGAGCCTGGGCCCCGACTGGC		
3	Intron 2	+	5'TTCCCCTTTGCTTTGGCTCAAAGC		
4	Intron 3	−	5'GACCCTGCCCACTCCATCTTGC		
5	Intron 3	+	5'**CTCGAATT**CCTGGGTCACCCCACCCTGCA		
6	Intron 4	−	5'**CTCTGTC**GACTCATTTGAGGGCAGAGCAGC		
7	Intron 4	+	5'TCCAACCCCCGACTCACTATC		
8	Intron 5	−	5'ACGGTATTTTGATGTGGATCTGCT		
9	Exon 4	+	5'CATCCACTCGCTATCATCATG	Long-wave	1181–1202
10	Exon 4	+	5'ACCCCACTCAGCATCATCGTG	Middle-wave	1182–1202
11	Exon 5	−	5'CAGCAGAATGCCAGGACCATC	Middle-wave	1336–1310

METHODS

Subjects

The subjects were young (age range 18–25 years) males with dichromatic color vision. Subjects were initially screened according to their performance on the AO–HRR pseudoisochromatic plates. They were further characterized by their performance in color matches obtained with a Maxwellian-view optical system (Neitz & Jacobs, 1990). The primaries and comparison light were presented in temporal alternation as a 3–10 foveally centered annulus. The subjects were categorized as dichromatic by failures to discriminate red–green mixtures (546 + 670 nm) from the comparison light (589 nm) in the Rayleigh match.

Electrophysiology

The ERG was recorded from the eye of each subject. ERG flicker photometry was performed as described elsewhere (Jacobs & Neitz, 1987; Neitz & Jacobs, 1984; Neitz, Neitz & Jacobs, 1989; Neitz *et al.*, 1991). Lights from a Maxwellian view optical system produced a 53 deg field of view. To determine spectral sensitivities, the quantal intensity of a rapidly flickering monochromatic light was adjusted to produce a response that matched in amplitude the response to an interleaved flickering achromatic reference light. The reference and test lights each had a pulse rate of 31.25 Hz, producing a pulse rate in the final combined beam of 62.5 Hz. Sensitivity measurements were made at 10 nm steps across the spectrum. Complete spectral sensitivity functions were measured twice for each subject. ERG sensitivity values were corrected for preretinal absorption by the lens (Wyszecki & Stiles, 1982). The wavelength of peak sensitivity was determined by translation of a standard

visual pigment absorption curve (Dawis, 1981) on a log-wavenumber axis (Baylor, Nunn & Schnapf, 1987; Mansfield, 1985) to obtain the best fit.

Genomic DNA isolation, polymerase chain reaction (PCR), and DNA sequence analysis

Genomic DNA was isolated either from whole blood or semen samples obtained from each subject. The PCR was used to amplify segments of the X-linked visual pigment genes. Primer sequences are shown in Table 1. Primers corresponding to intron sequences are located 30–50 bp from the intron–exon junctions. The nucleotide numbers are given for exon-specific primers, the numbering system is that of Nathans *et al.* (1986). For primers 5 and 6, the bold letters indicate linkers incorporated into the primers which are not part of the visual pigment gene sequences. The amplification strategy and PCR cycling parameters are shown in Table 2. PCRs contained, in a final reaction volume of 40 μl: 50 mM Tris–HCl, pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$; 200 μM each of dGTP, dATP, dCTP and dTTP; 200 nmol of each primer; 1.5 units of Taq DNA polymerase; and 100 ng total human genomic DNA. The cycling parameters indicated in Table 2 were carried out for 35 cycles; these were preceded by a single cycle of 94°C for 5 min and followed by a single cycle of 72°C for 10 min. Asymmetric PCR was carried out as for regular PCR except that the nucleotide concentrations were reduced to 20 μM, one of the primers was reduced to 4 nmol, and the cycling parameters indicated in Table 2 were carried out for 40 cycles.

For deuteranope subjects D_1 and D_2, three different gene segments were amplified, ligated into a plasmid vector, and the resulting recombinant plasmids were subjected to DNA sequence analysis. The amplified

TABLE 2. PCR amplification strategy and cycling parameters

| Primer pair	Region amplified	Cycling parameters
1 & 2	Exon 2	94°C 30 sec, 72°C 1 min
3 & 4	Exon 3	94°C 30 sec, 65°C 30 sec; 72°C 1 min
3 & 6	Exon 3–intron 3–exon 4	94°C 30 sec, 65°C 30 sec; 72°C 1 min
5 & 8	Exon 4–intron 4–exon 5	94°C 30 sec, 65°C 30 sec; 72°C 1 min
7 & 8	Exon 5	94°C 30 sec, 65°C 30 sec; 72°C 1 min
9 & 11	Exon 4–intron 4–exon 5, long–middle fusion genes	94°C 30 sec, 61°C 30 sec; 72°C 1 min
10 & 11	Exon 4–intron 4–exon 5, middle-wave genes	94°C 30 sec, 61°C 30 sec; 72°C 1 min
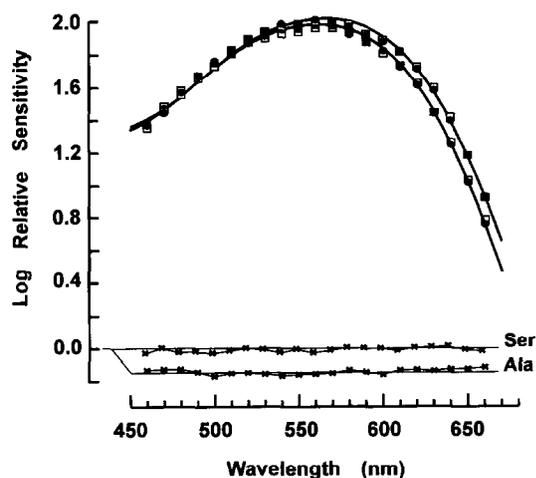
FIGURE 1. Flicker-photometric ERG spectral-sensitivity functions obtained from four deuteranopes. Two are best fit by a pigment absorption curve (—) with a spectral peak at 558 nm and the other two are best fit by a pigment curve (—) with a spectral peak at 563 nm. The two different deuteranopes (D_1 and D_3; □ vs ●) with the longer spectral peak have serine at position 180 of their long-wave opsin. The two with the shorter spectral peak (D_2 and D_4; □ vs ●) have alanine at that position. The differences between pairs of observers with the same amino acid at position 180 are plotted below the spectral sensitivities. The differences are small and do not change systematically with wavelength.

segments were exon 2, exon 3–intron 3–exon 4, and exon 4–intron 4–exon 5 (Table 2). For protanope subjects P_1 and P_3–P_8, and deuteranope subjects D_1–D_4, exon 3 of the X-linked visual pigment genes was amplified with primers 3 and 4 (Tables 1 and 2) using asymmetric PCR. The nucleotide sequence of the resulting single-strand DNA was determined. For subjects P_1, D_1, and D_2, the DNA sequence of exon 5 of the X-linked visual pigment genes was confirmed by direct sequence analysis of asymmetric PCR products. Exon 5 was amplified with primers 7 and 8 (Tables 1 and 2). DNA sequence analysis was done with the Sequence Version 2.0 kit (United States Biochemical).

RESULTS

The spectral sensitivity functions measured using ERG flicker photometry for four deuteranopes, subjects D_1–D_4, are shown in Fig. 1. Two distinctly different spectral sensitivity functions were found. Subjects D_1 and D_3 have spectral sensitivities that match a pigment with peak sensitivity at 563 nm, while the other two subjects, D_2 and

D_4, have spectral sensitivities corresponding to a pigment that peaks 5 nm shorter at 558 nm.

Complete DNA sequences of exons 2–5 were determined for the X-linked pigment genes in two deuteranope subjects, D_1 and D_2. The genes in these subjects encode pigments that differ in spectral peak (λ_{max}) by 5 nm. The nucleotide sequence differences between the genes, and the encoded amino acid differences, are shown in Table 3. The pigments differ at five amino acid positions, and are encoded by genes that differ at eight nucleotide positions. The differences between the genes from subjects D_1 and D_2 correspond to polymorphisms found among long-wave genes that occur in the color normal population (Nathans et al., 1986; Neitz, Neitz & Jacobs, 1993; Winderickx, Battisti, Hibibya, Motulsky & Deeb, 1993; Winderickx, Lindsey, Sanocki, Teller, Motulsky & Deeb, 1992; Neitz, Neitz & Grishok, 1995). Only non-homologous amino acid differences are expected to be potential candidates for causing a spectral difference between pigments. The only non-conserved amino acid difference between these two deuteranope pigments is the serine (Ser) for alanine (Ala) difference at position 180 (Table 3). This substitution has been shown previously to produce a red shift (Asenjo et al., 1994; Chan et al., 1992; Merbs & Nathans, 1992a; Neitz et al., 1991, 1993; Sanocki, Lindsey, Winderickx, Teller, Deeb & Motulsky, 1993; Winderickx et al., 1992).

The exon 3 sequences of the X-linked pigment genes in two additional deuteranopes, subjects D_3 and D_4, were examined by direct DNA sequence analysis of asymmetric PCR products to determine the amino acid specified at position 180 in each of the pigments. Subject D_3 has a gene encoding a pigment with λ_{max} = 563 nm, and it specifies serine at position 180. Subject D_4 has a gene encoding a pigment with λ_{max} = 558 nm, and it specifies alanine at position 180. The Ser[180]Ala polymorphism is the only non-conserved, exon 3 encoded amino acid substitution observed among the four deuteranope pigments. No polymorphic sites other than those indicated in Table 3 for subjects D_1 and D_2 were observed in exon 3 of the pigment genes from subjects D_3 and D_4.

The spectral sensitivity functions measured using ERG flicker photometry for eight protanopes are shown in Fig. 2. Spectral sensitivity functions for seven of the eight protanopes were virtually identical. One protanope, subject P_1, has a pigment spectrum that differs

TABLE 3. Nucleotide and amino acid differences between two deuteranope pigments

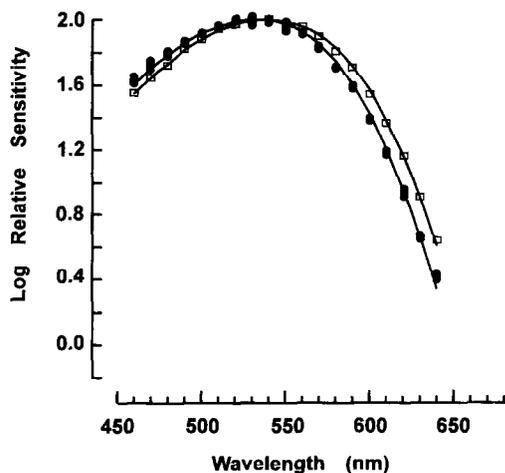| Exon	λ_{max} Nucleotide position	563 D_1	558 D_2	Amino acid position	Substituted amino acids
2	765	A	G	95	Ile/Val
3	947	A	G		
	951	A	C	153	Met/Leu
	959	C	G		
	1015	T	C	174	Ala/Val
	1032	T	G	180	Ser/Val
4	1200	G	A	236	Val/Met
5	1322	A	G		

FIGURE 2. Flicker-photometric spectral-sensitivity functions obtained from eight protanopes. Seven (●) are similar and are best fit by a pigment absorption curve (—) with a spectral peak at 530 nm. The eighth (□) is best fit by a pigment curve with peak at 537 nm (—).

significantly from the others. The continuous lines are for the best-fitting visual pigment absorption curves. The spectral sensitivities for the other seven protanopes, (subjects P_2–P_8) were best fit by a pigment with spectral peak at 530 nm. The spectral sensitivity of protanope P_1 is shifted to the longer wavelengths and fits a pigment curve with a peak at 537 nm.

Complete DNA sequences of exons 2–5 were determined for the pigment gene in subject P_1 encoding the 537 nm pigment. The sequence of a gene encoding a protanope pigment with a λ_{max} of 530 nm (subject P_2) was reported previously (Neitz *et al.*, 1989). Table 4 summarizes the nucleotide differences between the pigment genes from subjects P_1 and P_2, and the encoded amino acid differences. Both genes are hybrids, having upstream sequences usually found in long-wave genes and downstream sequences of middle-wave genes. Pigment P_2 is encoded by a gene with exons 2 and 3 derived from a long-wave gene. Pigment P_1 is encoded by a gene with exons 2–4 corresponding to long-wave gene sequences. These two protanope pigment genes differ at eight nucleotide positions, and the encoded pigments differ at four amino acid positions. Three non-homologous amino acid substitutions occur between the pigments, and these are at positions 230, 233, and 180. As mentioned above, position 180 is known to be spectrally active. Spectral

shifts have also been measured as the result of substitutions at amino acid positions 230 and 233 (Asenjo *et al.*, 1994; Merbs & Nathans, 1992b).

The nucleotide sequence of exon 3 was examined in the six other protanopes (P_3–P_8), each with a pigment having a spectral peak at 530 nm. All specify alanine at position 180. Among these six pigments, two other exon 3 encoded amino acid positions (153 and 174) were observed to be polymorphic. The same polymorphisms are observed in the color normal population (Winderickx *et al.*, 1993; Neitz *et al.*, 1995). Neither appears to influence the spectral peak of the pigment.

Protanopes can be considered to have a 5′long–3′middle-wave hybrid gene. The hybrid genes are thought to be the product of an unequal crossover that deletes long-wave genes occurring in the most upstream positions in the array. The loss of long-wave genes causes protan color defects. In order to estimate the location of the crossover that produced the fusion gene, the DNA sequences of exon 2 were examined directly and the exon 4 sequences were examined indirectly in six protanope subjects (P_3–P_8). In exon 2, there are three nucleotide positions, 65, 111, and 116, which encode amino acid differences that occur between the middle- and long-wave pigments characterized by Nathans *et al.* (1986). The six protanope pigments are identical at all three positions and have the amino acids found in Nathans' middle-wave pigments.

To determine the identity of exon 4, genomic DNA segments from each of the six subjects (P_3–P_8) were PCR amplified using two different sequence specific exon 4 primers. One primer (primer 9, Table 1) corresponds to the sequence usually found in exon 4 of long-wave genes; the other (primer 10, Table 1) corresponds to the sequence usually found in middle-wave genes. In each reaction, a long- or middle-wave selective exon 4 primer was paired with a middle-wave specific exon 5 primer (primer 11, Table 1). Using genomic DNA from each protanope, amplification products were obtained only with the primer pair 10 and 11 (Table 2). This suggests that each subject has a gene with middle-wave exon 4 and middle-wave exon 5. No PCR products were obtained using the long-wave specific exon 4 primer (primer pair 9 and 11, Table 2). The results of sequence-specific amplification and DNA sequencing suggest that these six protanopes (P_3–P_8) all have an X-linked pigment gene (or

TABLE 4. Nucleotide and amino acid differences between two deuteranope pigments

Exon	λ_{max} Nucleotide position	537 P_1	530 P_2	Amino acid position	Substituted amino acids
3	956	T	G		
	1032	T	G	180	Ser/Ala
4	1183	T	C	230	Ile/Thr
	1191	G	A	233	Ala/Ser
	1192	C	G	233	
	1193	T	C	233	
	1200	A	G	236	Met/Val
5	1343	C	A		

TABLE 5. Gene structures and spectral peaks of five different cone pigments

Gene structures	Subject	ERG (this study)	Asenjo et al. (1994)	Merbs and Nathans (1992b)
			Spectral peak (nm)	
Ala[180]	P₃	530 (protanope)	532	530
Ala[180]	P₂	530 (protanope)	532	529
Ser[180]	P₁	537 (protanope)	538	536
Ala[180]	D₂	558 (deuteranope)	556	552
Ser[180]	D₁	563 (deuteranope)	563	557

genes) with the sequences of exons 2–5 of a prototypical middle-wave gene. The crossover that deleted the long-wave gene from these subjects must have occurred upstream of exon 2.

DISCUSSION

In this study of eight protanopes and four deuteranopes we find four distinctly different spectral sensitivities. These correspond to two different long-wave pigment subtypes and two middle-wave pigment subtypes. The middle-wave pigment found in seven of eight protanopes has a spectral peak of 530 nm. The eighth has a pigment that peaks at 537 nm. Deuteranopes were measured to have either a 558 nm pigment or a 563 nm pigment.

The X-linked pigment genes are highly polymorphic. An illustration can be seen in the sequence differences between two deuteranopes who have long-wave genes that differ at eight nucleotide positions (Table 3). Three of these are silent substitutions, i.e. they do not produce amino acid differences, and four of the five amino acid substitutions are homologous exchanges. On theoretical grounds, it has been expected that only non-homologous exchanges, particularly substitution of hydroxyl-bearing for non-polar amino acids, would influence the pigment spectra. Among the middle- and long-wave pigments this intuition has been born out by experiment (Asenjo et al., 1994; Neitz et al., 1991). The two deuteranopes differ by only one non-homologous substitution, Ser[180]Ala. If we consider only non-homologous substitutions, among the 12 dichromats we find five different classes of opsin structures. The three middle-wave opsin structures are: (1) pigments encoded by intact middle-wave genes; (2) a pigment encoded by a hybrid gene with exons 2 and 3 from a long-wave gene and Ala[180]; and (3) a pigment encoded by a hybrid gene with exons 2–4 from a long-wave gene, with Ser[180]. The two different long-wave opsins are simpler in their differences. They differ by the amino acid (Ser or Ala) encoded at position 180. The other amino acid differences between them are ones that are observed among the long-wave pigments in the color

normal population (Winderickx et al., 1993; Neitz et al., 1995).

The spectral peaks of the five different type of opsins are summarized in Table 5. These are compared with the absorption spectra for similar opsin structures measured in vitro. The spectral peaks measured in vitro are remarkably close to those obtained from the ERG measurements. Particularly similar are the ERG measurements and those from the recent mutagenesis study of Asenjo et al. (1994). Their in vitro pigment measurements do not differ from the corresponding dichromat by more than 2 nm. The middle-wave pigments measured in vitro by Merbs and Nathans (1992b) closely predict the results in protanopes, however, their two long-wave pigment measurements yield peaks that are about 6 nm shorter than either the deuteranopes examined in this study or the in vitro measurements of the analogous pigments made by Asenjo et al. (1994).

Pairwise comparisons of these deduced opsin structures and the spectral sensitivities of the pigments they produce show the effects of amino acid substitutions on the spectral sensitivities of the human pigments. These are summarized in Table 6. Protanope P₂ has a hybrid middle-wave gene in which exons 2 and 3 derive from a long-wave gene. This pigment has the same spectral peak as the pigments in the six protanopes (P₃–P₈) which are encoded by genes with exons 2–5 of a prototypical middle-wave gene. Consistent with our earlier conclusion (Neitz et al., 1989), this indicates that substitution of exons 2 and 3 of a middle-wave gene with the corresponding long-wave sequence does not detectably shift the middle-wave spectrum. Comparison of two protanopes, P₁ and P₂, whose middle-wave genes contain different amounts of long-wave sequence indicates that the non-homologous substitutions at amino acid positions 230 and 233, encoded by exon 4 together with the substitution of serine for alanine at position 180 specified by exon 3, produce a 7 nm shift in the middle-wave pigment. Comparison of the pigments in subjects P₁ and D₁ gives the magnitude of the shift (26 nm) produced by exchange of long- for middle-wave exon 5.

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Finally, comparison of the two types of deuteranopes, e.g. D_1 and D_2, indicates the magnitude of the spectral shift (5 nm) produced by the substitution of serine for alanine at position 180 of the long-wave pigments. All of these are closely predicted from the *in vitro* measurements of similar pigment structures (Asenjo *et al.*, 1994; Merbs & Nathans, 1992b). The largest discrepancy is for the effect of exon 5 encoded substitutions measured by Merbs and Nathans (1992b); this reflects the shorter peak they measure for the long-wave pigments *in vitro*.

It has long been recognized that individual differences in the spectral sensitivities of dichromats may arise from factors other than shifts in the absorption spectra of the pigments (Alpern & Pugh, 1977). These factors include individual differences in the density of lens pigment and differences in the effective optical densities of the cone visual pigments. Each factor that influences spectral sensitivity produces a distinct pattern of sensitivity changes as a function of wavelength. Figure 3 shows, plotted on a much expanded scale, the difference in spectral sensitivity between the two types of deuteranope pigments [Fig. 3(a)] and the difference between the two different types of protanope pigments [Fig. 3(b)]. The solid curves in each panel are the predicted differences between two pigments that are shifted in peak. There is a close correspondence between the curves predicted by a pure spectral shift and the differences between the dichromatic types. Shown for comparison are the differences predicted by a change in optical density (U-shaped dashed curves). Increases in optical density broaden the curve but do not change the location of the spectral peak. To generate the predicted optical density difference shown, two pigment absorption curves with the same spectral peak but different optical densities were subtracted. The optical density of one curve was increased until the difference best fit the long-wave side of the dichromat spectral sensitivity difference. All the curves generated by changing optical density produce similarly poor descriptions of the differences between the dichromat. Individual differences in lens density will have their major influence in the short wavelengths. Lens density variations also give a poor account of the

differences between the dichromats (Fig. 3, lower dashed line). These comparisons suggest that the measured differences in spectrum produced by exchanges at amino acid positions 180, 230, and 233 in protanopes, and the exchange at position 180 in deuteranopes are dominated by a pure shift in the absorption spectra of the pigments.

The spectral peak of the hybrid middle-wave pigment in protanope P_2, which has exons 2 and 3 derived from a long-wave gene, is measured to be the same as that for the protanopes with prototypical middle-wave genes. It seems surprising that the large difference in opsin structure that results from this genetic difference would have no effect on pigment spectral sensitivity. The results from the two types of protanopes with the same λ_{max} are compared in Fig. 4. These curves are remarkably similar, but there is a hint of a small difference. The protanope with the fusion gene (P_2) has a slightly higher sensitivity in the limbs compared to near the peak. This could be accounted for if the pigment of subject P_2 has a slightly higher optical density. The difference between the two types of protanopes is plotted (on an expanded scale, 2 ×) below the sensitivity curves (Fig. 4). The overlaid smooth curve plots the optical density difference (0.10) that best fits the spectral difference. The averaged, individual relative sensitivity values for the six protanopes P_3–P_8 plotted in Fig. 4 are quite reliable. These mean sensitivity values have standard deviations that are, on average, 0.012 log unit and they have 95% confidence limits that are, on average, 0.009 log unit. The test–retest reliabilities for individual subjects exceed these interobserver reliabilities. For protanope P_2, compared to the others, the sensitivity differences near the extremes of the limbs are on the order of 0.04 log unit higher than those near the peak. The sizes of the differences relative to the interobserver variability of subjects P_3–P_8 (above) and the fact the differences vary somewhat systematically with wavelength (e.g. compared with those in Fig. 1) suggests a real spectral difference between the pigments of these two types of protanopes. It seems possible that some substitutions in the opsins may influence the optical densities of the cones by affecting, for example, the stability of the pigment molecule or the efficiency with which it absorbs light.

TABLE 6. Genetic differences and differences in spectral peak among five cone pigment pairs

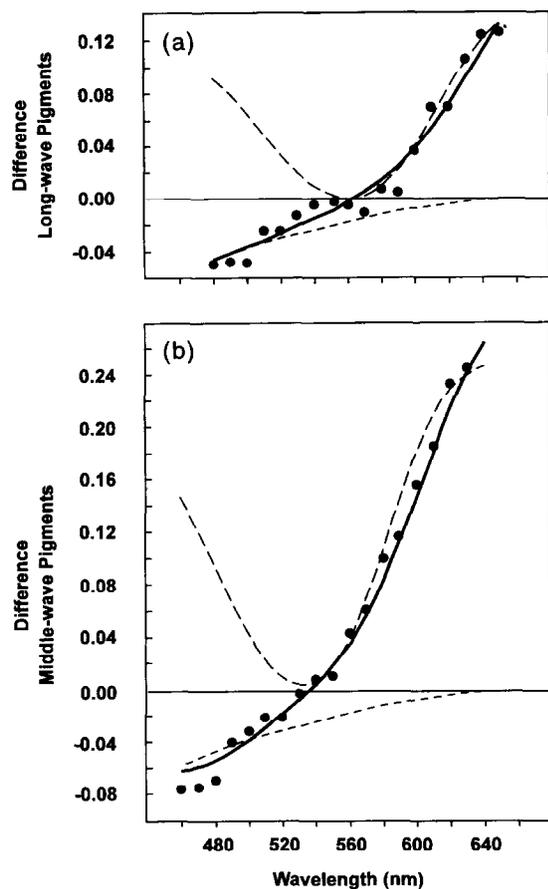| Gene structures	ERG (this study)	Asenjo *et al.* (1994)	Merbs and Nathans (1992b)
		Spectral shift (nm)	
P_3 Ala[180] → P_2 Ala[180]	0	0	−1
P_2 Ala[180] → P_1 Ser[180]	7	6	7
P_1 Ser[180] → D_1 Ser[180]	26	25	21
D_2 Ala[180] → D_1 Ser[180]	5	7	5

FIGURE 3. Spectral differences between subtypes of deuteranopes and subtypes of protanopes. (a) The result of subtracting the averaged ERG spectral-sensitivity functions of deuteranopes D_2 and D_4 from the averaged functions of deuteranopes D_1 and D_3. The — line is the difference in spectral sensitivity that is predicted from the subtraction of two long-wave pigment absorption curves that are spectrally separated by 5 nm. Shown for comparison, are the differences predicted by the subtraction of two long-wave pigments with the same spectral peak but different optical densities (U-shaped - - - curve) and the spectral differences predicted for two observers with different lens pigment densities (lower - - - curve). (b) The result of subtracting the averaged ERG spectral-sensitivity function of protanopes P_2–P_8 from protanope P_1. The — line is the difference in spectral sensitivity that is predicted from the subtraction of two middle-wave pigment absorption curves that are spectrally separated by 7 nm. The - - - curves are the differences predicted from changing pigment optical density or lens density as above.

The spectra of macaque photoreceptors measured either by microspectrophotometry (Mansfield, 1985) or from electrical recordings using suction electrodes (Baylor *et al.*, 1987) assume a common shape when plotted on a log-wavenumber axis. The spectral shapes of the protanope and deuteranope spectral sensitivities are also similar when translated on a wavenumber axis (Fig. 5). However, the long-wave (deuteranope) spectrum is slightly broader than the middle-wave (protanope) spectrum. This difference is not observed in measurements of isolated photoreceptors when the light is passed traversely through the outer segment and the effective optical density is accordingly minimal. The broader curve of the deuteranopes can be explained by higher effective optical density of the

long-wave cones than middle-wave cones as has been suggested earlier (Burns & Elsner, 1993; Smith, Pokorny & Starr, 1976). The optical density difference required to account for the difference plotted in Fig. 5 is on the order of about 0.10. The validity of these small measured differences is similar to that discussed with respect to Fig. 4 (above).

This study reinforces the earlier conclusion that amino acid substitutions in exon 5 are responsible for the spectral shift between middle- and long-wave cone pigments. Substitution of middle-wave for long-wave exon 5 produces a 25–26 nm shift in spectra that separates middle- and long-wave pigment classes. Exons 2–4 of the pigment genes have polymorphisms that can produce subtypes of pigments within each of the major classes. The results reported here are consistent with *in vitro* measurements in finding that substitution of long-wave exons 2 and 3 in the middle-wave gene does not produce a measurable shift in pigment spectrum. Of the spectral differences produced by polymorphisms in exons 2–4, the shift produced by substitution of serine for alanine at position 180 is of particular interest. The amino acid at this position is polymorphic among the long-wave pigments and the middle-wave pigments of color normal observers. Individual differences at this one position can account for 89% of the variance in color matches of males with normal color vision (Neitz *et al.*, 1993). Polymorphism at this position can also be found within the long-wave genes of individual deuteranomalous trichromats. Thus, in some deuteranomalous observers, it
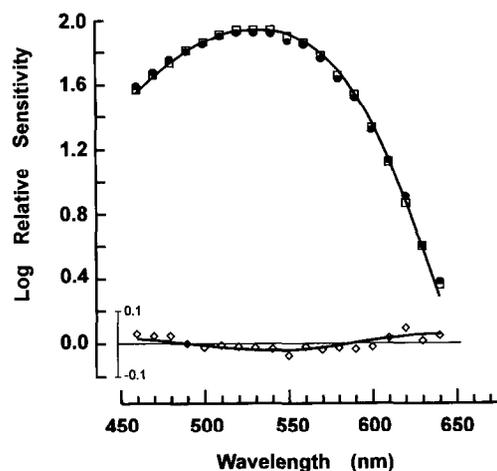

FIGURE 4. Spectral-sensitivity differences between protanopes who differ in exons 2 and 3 of their X-linked photopigment genes. ●, ERG spectral-sensitivity function of protanope P_2, who has a middle-wave pigment gene with exons 2 and 3 derived from a long-wave gene. □, Averaged spectral-sensitivity functions of protanopes P_3–P_8 who have prototypical middle-wave genes. The difference between the two types of protanope is shown (◇) plotted on an expanded scale (2 ×) at the bottom of the figure. The difference does not suggest any systematic shift in spectral peak between the two types of protanope, however, the curve for protanope P_2 does appear to be slightly broader than the others. The lower smooth curve is the differences predicted by the subtraction of two middle-wave pigments with the same spectral peak but different optical densities (optical density difference = 0.10).
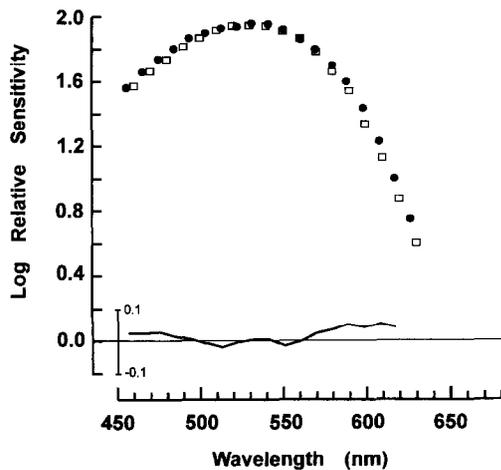
FIGURE 5. Comparison of the spectral shapes of protanope and deuteranope ERG spectral-sensitivity functions. ●, Averaged spectral-sensitivity functions of deuteranopes D_2 and D_4; they have long-wave genes with Ala[180] and spectral sensitivities that peak at 558 nm. □, Averaged spectral sensitivities of protanopes, P_3–P_8; they have prototypical middle-wave genes and spectral sensitivities that peak at 530 nm. The curve for the deuteranopes has been shifted toward the shorter wavelengths on a log-wavenumber axis by 28 nm so its peak superimposes with that of the protanopes. The residual difference between the deuteranope long-wave pigments and the protanope middle-wave pigments is plotted below the curves on an expanded scale (2 ×). Although they have similar shapes, the long-wave curve is slightly broader.

is likely that this difference alone accounts for the spectral separation between pigments on which they base red–green color discrimination. Results from deuteranopes presented here agree with previous *in vitro* and *in vivo* measurements that substitution of Ser[180] for Ala[180] produces a 5–7 nm shift in long-wave pigment spectrum (Asenjo *et al.*, 1994; Merbs & Nathans, 1992a; Neitz *et al.*, 1991).

In this study, the two protanope middle-wave pigments that differ at position 180 also have substitutions of non-polar for hydroxyl-bearing amino acids at positions 230 and 233. The serine for alanine substitution at position 180 alone produces a 5–7 nm shift in the long-wave pigments (Table 5). If the substitution at 180 produced a shift of exactly the same magnitude between the two middle-wave pigments that are separated by 7 nm, then the additional shift produced by substitutions at positions 230 and 233 in the protanope pigment would be 0–2 nm. Earlier, this kind of logic lead us to suggest that substitutions at these positions do not produce a significant spectral shift (Neitz *et al.*, 1991). More recent *in vitro* studies measure small but significant spectral shifts when substitution at these positions are introduced. The close correspondence between the shifts measured in the ERG spectral sensitivity and the *in vitro* measurements indicate that the *in vitro* measures are usually very accurate predictors (within about 2 nm) of what happens *in vivo*. This would indicate that the substitutions at positions 230 and 233 are, together, responsible for 2–4 nm of the spectral shift observed between the 530 nm protanope and the one with spectral peak at 537 nm.

Among 12 dichromats we have found four discretely different X-encoded cone pigments. Ser/Ala[180] is the predominant spectrally active polymorphism observed in human long-wave and middle-wave pigments. Thus, the two types of long-wave pigments observed in the deuteranopes in this study represent the two major subtypes of long-wave pigments in the normal population. However, long-wave pigments in normal males can have substitutions at other spectrally active positions (e.g. positions 230 and 233). Such substitutions would produce more minor spectral variants of the long-wave pigments in addition to those measured here. The 537 nm middle-wave pigment is encoded by a gene that has not been observed in the normal population (Winderickx *et al.*, 1993; Neitz *et al.*, 1995). If such genes do exist in normals they are probably rare variants. However, it is not uncommon to find middle-wave genes that have serine at position 180. The *in vitro* measurement suggests that middle-wave genes encoding Ser[180] produce a variant of the middle-wave pigment with a spectral peak intermediate to the 530 and 537 nm pigments measured here.

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