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Genotype-Phenotype Relationships in Human Red/Green Color-Vision Defects: Molecular and Psychophysical Studies

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Summary

The relationship between the molecular structure of the X-linked red and green visual pigment genes and color-vision phenotype as ascertained by anomaloscopy was studied in 64 color-defective males. The great majority of red-green defects were associated with either the deletion of the green-pigment gene or the formation of 5′ red-green hybrid genes or 5′ green-red hybrid genes. A rapid PCR-based method allowed detection of hybrid genes, including those undetectable by Southern blot analysis, as well as more precise localization of the fusion points in hybrid genes. Protan color-vision defects appeared always associated with 5′ red-green hybrid genes. Carriers of single red-green hybrid genes with fusion in introns 1–4 were protanopes. However, carriers of hybrid genes with red-green fusions in introns 2, 3, or 4 in the presence of additional normal green genes manifested as either protanopes or protanomalous trichromats, with the majority being protanomalous. Deutan defects were associated with green-pigment gene deletions, with 5′ green-red hybrid genes, or, rarely, with 5′ green-red-green hybrid genes. Complete green-pigment gene deletions or green-red fusions in intron 1 were usually associated with deuteranopia, although we unexpectedly found three carriers of a single red-pigment gene without any green-pigment genes to be deuteranomalous trichromats. All but one of the other deuteranomalous subjects had green-red hybrid genes with intron 1, 2, 3, or 4 fusions, as well as several normal green-pigment genes. The one exception had a grossly normal gene array, presumably with a more subtle mutation. Amino acid differences in exon 5 largely determine whether a hybrid gene will be more redlike or more greenlike in phenotype. Various discrepancies as to severity (dichromacy or trichromacy) remain unexplained but may arise because of variability of expression, postreceptoral variation, or both. When phenotypic color-vision defects exist, the kind of defect (protan or deutan) can be predicted by molecular analysis. Red-green hybrid genes are probably always associated with protan color-vision defects, while the presence of green-red hybrid genes may not always manifest phenotypically with color-vision defects. Four subjects who were found to have 5′ green-red hybrid genes in addition to normal red- and green-pigment genes had normal color vision as determined by anomaloscopy. These were discovered among a group of 129 Caucasian males who had been recruited as volunteers for a vision study. We hypothesize that green-red hybrid genes in a more distal (3′) position of a gene array that includes one or more normal green genes may not be expressed sufficiently to measurably affect color vision.

Introduction

Normal human color vision is mediated by the presence of three kinds of cones, containing three different visual pigments: blue (short), green (middle), and red (long-wavelength sensitive) (Cornsweet 1970; Wyszecki and Stiles 1982). The terms "green" and "red" visual pigments, rather than the more technically correct terms of "middle-" and "long-wavelength-sensitive" pigments, will be used to facilitate communication.

Hereditary red-green color defects have been known for more than 200 years (Scott 1779), and X-chromo-

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some linkage was correctly postulated by Wilson in 1911 (Wilson 1991). Approximately 8% of the Caucasian male population are affected (for a review, see Drummond-Borg et al. 1989). Individuals with color-vision defects affecting the red visual pigment are known as protans, while those with green visual pigment abnormalities are known as deutans. Persons who lack the red or green pigment completely are known as protanopes (P) or deuteranopes (D), respectively, and are dichromats; that is, they have two instead of the normal three pigments (red, green, and blue). Anomalous trichromats are individuals who have three pigments but whose red (in the case of protanomalous trichromacy [PA]) or green (in the case of deuteranomalous trichromacy [DA]) pigment absorption spectrum is atypical (i.e., shifted along the wavelength axis) (for review, see Pinckers et al. 1979).

The various types of red-green color defects can be readily categorized by a Rayleigh (1881) match, which requires that subjects match an admixture of red ($\sim$650 nm) and green ($\sim$540 nm) primary colors to a yellow ($\sim$589 nm) standard (Wyszecki and Stiles 1982). Dichromats have only one functional pigment in this spectral range and thus can match the yellow standard with any mixture of red and green, including red or green alone. Normals and anomalous trichromats have two pigments in this spectral range and thus can make at least some color discriminations in this range.

The molecular nature of normal color-vision pigments and the molecular basis of X-linked color-vision defects were first delineated by Nathans et al. (1986*b*), who cloned and sequenced the genes encoding the apoproteins of the red and green visual pigments. They showed that males with normal color vision have one red-pigment gene and one or more green-pigment genes, and they demonstrated that the highly homologous red- and green-pigment genes consist of six exons. The red-pigment gene is "upstream" (5′) to the green-pigment gene(s) in head(5′)-to-tail(3′) tandem arrays (Vollrath et al. 1988; Feil et al. 1990). On the basis of studies in 25 red-green color-deficient males they concluded that color-vision defects result from homologous recombination between the red- and green-pigment genes, leading either to deletions of green-pigment genes or to full-length hybrid or fusion genes consisting of portions of both red- and green-pigment genes (Nathans et al. 1986*a*).

We have studied the red and green visual pigment gene arrangements in 64 males who were diagnosed as red-green color defective on the basis of 2-degree Rayleigh matches by Nagel anomaloscopy. Our goal was to examine the degree to which genotype and phenotype were correlated in red-green color-deficient subjects, with particular reference to the nature and severity of the color-vision defect. To this end, we developed techniques to detect the site of fusion of the red- and green-pigment portions in hybrid genes.

We also determined whether the presence of a fusion gene was always associated with red-green color-vision deficiency. Drummond-Borg et al. (1989) and Jorgensen et al. (1990) examined the molecular arrangements of green- and red-pigment genes in Caucasian and African-American males. The frequency of hybrid genes in these subjects was higher than the expected prevalence of phenotypic color-vision defects. This result suggested that not all hybrid genes are associated with color-vision defects; however, the subjects of these earlier studies could not be examined for color-vision defects. In the present study 129 males were examined (all of them by molecular analysis of red- and green-pigment gene arrays and 65 of them by anomaloscopy) in order to determine whether the presence of a fusion gene is always associated with red-green color-vision deficiency.

Subjects and Methods

Subjects

The 64 red-green color-defective subjects were male Caucasian volunteers (except for subject 2071 [with PA] and subject 1933 [with DA], who were Asian) recruited through advertisements in classes, the school newspaper at the University of Washington, and local newspapers specifically asking for color-defective individuals. Another group of 129 Caucasian males were recruited through other advertisements asking for volunteers for a vision study. The red- and green-pigment gene arrays of these 129 volunteers were screened for presence of hybrid genes by Southern blot hybridization. Sixty-five of these 129 subjects were available for anomaloscopic testing. A signed consent form describing the relevant procedures involved was obtained prior to psychophysical testing and collection of peripheral blood samples for DNA isolation.

Psychophysics

Subjects were tested in each eye by a Nagel model 1 anomaloscope. The instrument provided a nominal 2-degree bipartite color-matching field of about 50-td mean retinal illuminance. The spectral calibration of

the instrument had been modified previously, and the Rayleigh equation for this instrument involved matches of an admixture of 541-nm (green) and 644-nm (red) lights to a 591-nm (yellow) standard. The approximate normal settings on this instrument were determined by testing a sample of subjects known to have normal color vision (laboratory personnel) and were found to be 37 units on the red-green-mixture scale and to be 16 on the yellow-intensity scale.

Subjects were tested using a protocol similar to that described by Linksz (1964; also see Birch et al. 1979). Subjects first adapted for 2 min to a lighted neutral screen on the front panel of the anomaloscope. The experimenter controlled the setting of the red-green mixture knob in each trial, and the subject was asked to adjust the yellow-intensity knob to determine whether the red-green mixture and standard fields could be made equivalent in appearance. Guided by subjects' reports of a match or mismatch between the two fields, the experimenter adaptively varied (*a*) the red-green setting from trial to trial and determined the endpoints of the range of red-green settings over which the subject matched the appearance of the two hemifields and (*b*) the yellow-intensity settings associated with each match. Approximate test time was 15 min/eye. Some subjects were retested at a later date (see below).

Subjects were assigned to five categories on the basis of the anomaloscopic examination: color normal (N), protanope (P), protanomalous (PA), deuteranope (D), and deuteranomalous (DA). Subjects with normal color vision for whom results are shown were classified readily, since all had midmatch points (means of endpoints of match range) within ± 2 units of 37 on the red-green scale, had match ranges of 3 units or less, and required the expected intensity of the yellow standard field. Subjects were assigned to the protanope (P) category if they accepted all mixtures of red and green (matching range 73 units) but required systematically less yellow radiance as the amount of red in the mixture field was increased. Subjects were assigned to the deuteranope (D) category if they matched all mixtures of red and green to the yellow standard of nearly constant intensity. The assignment of subjects to the DA or PA categories was made on the basis of a matching range shifted significantly to the red (in the case of PA) or green (in the case of DA) side of normal (see fig. 1). With some subjects (e.g., 2116 and 2185), however, the width of the range of a subject's matches did not permit unequivocal assignment to the PA or DA categories. In such cases, assignment was based on the amounts of yellow standard required for the matches.

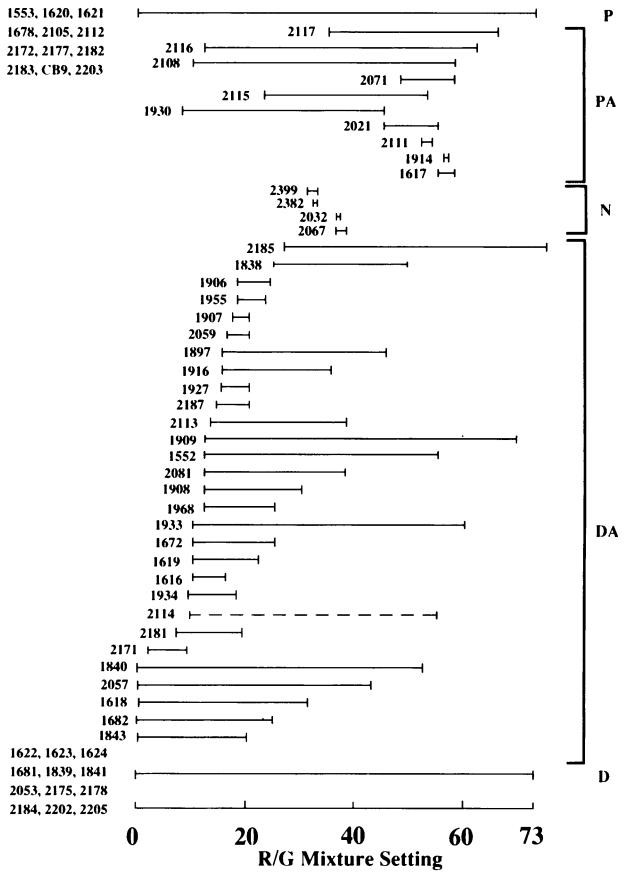

Figure 1 Rayleigh match ranges of protan, deutan, and normal subjects. Each horizontal bar represents the range of red-green mixtures that the subject could not distinguish from the standard yellow light. Subject identification numbers are shown next to each bar. Subjects 2032, 2067, 2382, and 2399, who had normal color vision, carried 5′ green-red fusion genes (fig. 6). Color-vision test results for subjects CB8 and CB9 have been reported elsewhere (as subjects II-1 and III-2, respectively, in Thuline et al. 1969). Match range information on subject CB8 was not available. Subject 2114 had unreliable color match reports; the dotted line represents an approximation of his match range. This subject showed a deutan error axis on the FM100 hue test (error score 265).

DNA Preparation and Southern Blot Analysis

DNA was isolated from leukocytes of venous blood by lysis in proteinase K–SDS, followed by phenol extraction and ethanol precipitation on an Applied Biosystems (Foster City, CA) model 340 A nucleic acid extractor, according to the manufacturer's protocol. Southern blot analysis of restriction fragments generated by digesting genomic DNA with either *Rsa*I or a combination of *Eco*RI and *Bam*HI, together with

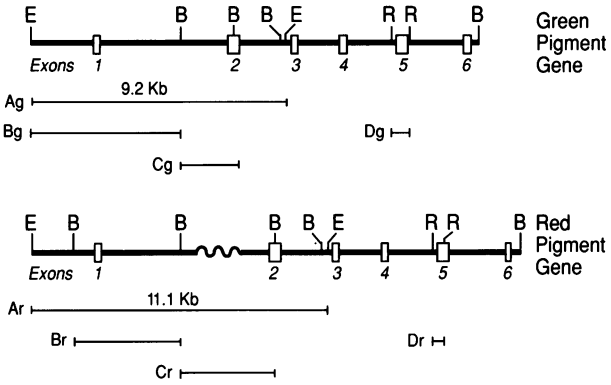

Figure 2 Restriction map of the X-linked red- and green-pigment genes. E = *Eco*RI; B = *Bam*HI; and R = *Rsa*I. The restriction fragments of the red gene (A_r, B_r, C_r, and D_r) differ, in length, from the corresponding fragments of the green gene (A_g, B_g, C_g, and D_g) because of both the longer (by 1.9 kb; *wavy line*) intron 1 of the red-pigment gene and the absence of an *Rsa*I restriction site in exon 5 of the green-pigment gene. The red- and green-specific fragments were detected on Southern blots of genomic DNA digested with either *Eco*RI and *Bam*HI or with *Rsa*I, using a 350-bp cDNA probe encompassing exon 1 and part of exon 2, to detect the A, B, and C fragments, and using a 400-bp genomic DNA probe from the 3′ end of the fourth intron of the green-pigment gene, to detect the D fragments (Nathans et al. 1986*a*).

interpretation of blot patterns, has been described in detail elsewhere (Nathans et al. 1986*a*; Drummond-Borg et al. 1989). The DNA fragments generated from the 5′, middle, and 3′ regions of the red- and green-pigment genes are shown in figure 2. Based on absence of certain DNA fragments and on the ratios between fragments derived from the green- and red-pigment genes, the gross structure of gene arrays could be deduced, including that of hybrid genes. The precise location of fusion points in hybrid genes, however, could not be determined by this technique. For example, if a 5′ green-red 3′ hybrid gene is composed of B_g, C_g, and D_r fragments, then the point of fusion could be anywhere between the 3′ end of intron 1 and the 5′ end of exon 5 (fig. 2). DNA probes for the color-vision-pigment genes were provided by J. Nathans (Johns Hopkins University, Baltimore) and have been described elsewhere (Nathans et al. 1986*a*).

PCR Amplification and Sequencing

The primers and conditions used for in vitro DNA amplification (Saiki et al. 1988) by PCR of various segments of the red- and green-pigment genes are given in table 1. In general, 30 cycles each of 1 min at 94°C,

Table 1

Primers Used in PCR Amplification and Sequencing

	DNA Sequence (5′–3′)	Exon	Position
General primers:[a]			
1A	CTTTCCATAGCCATGGCCCAG	1	30
1B	TCTGGTGGAGTTGCTGTTGGT	1	152
2A	CACATCGCTCCCAGATGGGT	2	177
2B	ACACAGGGAGACGGTGTAGC	2	449
3A	ATCACAGGTCTCTGGTCTCTG	3	453
3B	CTGCTCCAACCAAAGATGGGC	3	619
4A	TACTGGCCCCACGGCCTGAAG	4	621
4B	CGCTCGGATGGCCAGCCACAC	4	785
5A	GTGGCAAAGCAGCAGAAAGAG	5	786
5B	CTGCCGGTTCATAAAGACATAG	5	1025
6A	TTTCGAAACTGCATCTTGCAG	6	1026
6B	GCAGTGAAAGCCTCTGTGACT	6	1265
		Specificity / Exon	
Gene-specific primers:[b]			
41-R	GCTGCATCACCCCACTCAG	Red / 4	721
41-G	GCTGCATCATCCCACTCGC	Green / 4	721
51-R	GACGCAGTACGCAAAGATC	Red / 5	868
51-G	GAAGCAGAATGCCAGGACC	Green / 5	868

[a] Used in amplification of both red- and green-pigment gene sequences.
[b] Used in amplification of either green- or red-pigment sequences.

1 min at 64°C, and 1–3 min at 72°C were employed using a Perkin Elmer Cetus (Norwalk, CT) Gene-Amp kit and DNA thermal cycler. Oligonucleotide primers were synthesized on an Applied Biosystems model 380 DNA synthesizer. The amplified fragments were either cloned into the plasmid vector pGEM 3 (Promega, Madison, WI) (for arrays that comprised more than one gene) or used as templates to generate single stranded DNA (for arrays that contained only one gene) and sequenced by the dideoxy chain-termination method (Sanger 1977) using the Sequenase kit (U.S. Biochemical Corp., Cleveland) and alpha-^{32}P-dCTP (New England Nuclear, Boston) (specific activity 3,000 Ci/mmol). To obtain single-stranded DNA, 1 μl of the double-stranded DNA product of the first round of amplification was used as a template in a second 30-cycle PCR amplification reaction containing 60 pmol of one of the primers and only 0.6 pmol of the second primer. The amplified single-stranded DNA was purified by filtration through an Amicon 30 spin column (Amicon, Danvers, MA) before use as a template in the sequencing reaction.

Single-strand conformation polymorphism (SSCP) analysis (Orita et al. 1989) was also used to examine sequences of red- and green-pigment gene exons after PCR amplification with gene-specific or general primers. This technique allowed separation and identification of individual red- and green-pigment exon sequences and their respective variants even when present as mixtures in PCR-amplified products. A detailed protocol of SSCP analysis applied to the red- and green-pigment genes have been presented elsewhere (Winderickx et al. 1992).

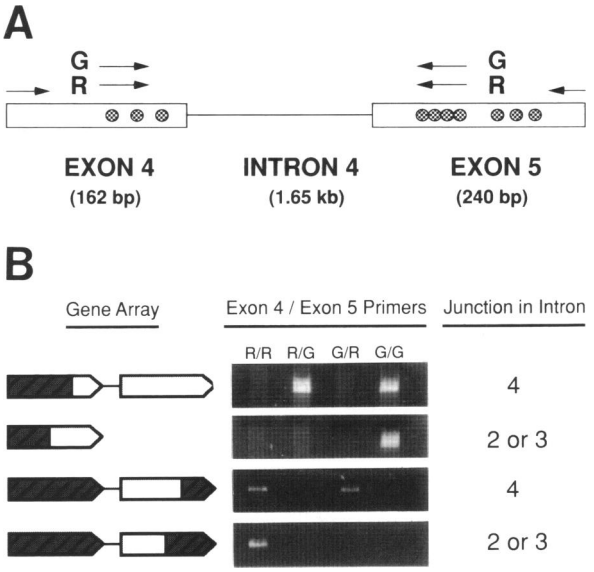

Figure 3 Gene-specific amplification across intron 4. *A*, Diagram (not to scale) showing the position of primers used for amplification across intron 4 of the red-, green-, and hybrid-pigment genes. Arrows marked "G" and "R" represent green-specific and red-specific primers, respectively; and unmarked arrows represent nonspecific primers. Circles represent amino acid residues that differ between the red- and green-pigment genes in exons 4 and 5. *B*, Ethidium bromide–stained DNA fragments amplified using the indicated pairs of gene-specific primers and separated on a 1% agarose gel (*middle panel*). Genomic DNA samples isolated from subjects having the diagrammed red-green gene arrays were used as templates in amplification. Solid hatched arrows represent red-pigment gene sequences; and unmarked arrows represent green-pigment gene sequences (*left panel*). The intron of junction in hybrid genes is also shown (*right panel*). The intron of fusion was confirmed by sequencing of exons 4 and 5 of fragments amplified by the nonspecific primers as described in Subjects and Methods.

Determination of Fusion Points in Hybrid Genes

More precise localization of the fusion points in hybrid genes was performed by gene-specific PCR amplification and sequencing. On the basis of nucleotide differences between the red- and green-pigment genes in exons 4 and 5, two sets of gene-specific PCR oligonucleotide primers (fig. 3*A* and table 1) were used to identify fusion points in intron 4. Figure 3*B* shows amplification products produced by using all possible combinations of these primers on a variety of genomic DNA templates. The results show that the primers could clearly discriminate between red and green gene sequences, and they appropriately identified 5′ green-red 3′ and 5′ red-green 3′ fusions that most probably occurred in intron 4. The fusion most likely occurred in intron 4, since its length is more than 10 times that of the coding sequences of exons 4 and 5 that are included in the amplified segment. For example, a 5′ red-green 3′ hybrid gene in which the fusion point lies in intron 4 would be expected to yield a fragment of 1.8 kb when amplified using primers 41-R and 51-G (fig. 3*B*, top panel). The specificity of amplification using these primers was further tested by direct sequencing of the coding regions of fragments that encompass exon 4, intron 4, and exon 5 (1.8 kb), all amplified using primers 4A and 5B (fig. 3*A* and table 1), which are *not* gene specific. Sequencing of the amplified products derived from 10 individuals (6 were shown to have a fusion junction in intron 4, and 4 did not) confirmed both the specificity of amplification by the gene-specific primers 41-R and 51-G (table 1) and the validity of this simple and rapid method of detecting crossover events that had occurred in intron 4.

Fusion points in introns 2 and 3 could not be distinguished from each other, for lack of known green- and red-specific sequences in exon 3. It was also not possible to distinguish between fusions that occurred between the *Bam*HI site in intron 1 and exon 2 and those that have occurred in intron 2, since the former was undetectable by Southern blot analysis (no new fragment would be generated; fig. 2) and since no gene-specific PCR primers are yet available to use in amplifying red- and green-pigment exons 2 and 3. Fusions that occurred 5′ of the *Bam*HI site in intron 1 were detected on Southern blots, since they result in the formation of a different-sized *Bam*HI fragment (fig. 2).

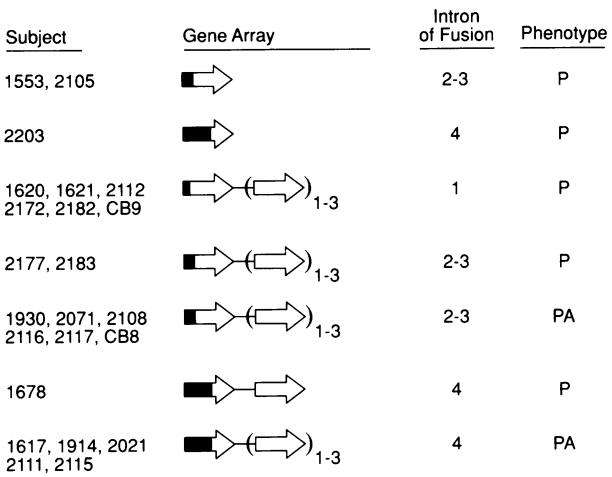

Figure 4 X-linked color-vision-pigment gene arrays in protan subjects. Blackened and unblackened arrows represent red- and green-pigment genes, respectively. Hybrid genes consist of both red- and green-pigment gene sequences, as illustrated. The subscript number (1–3) next to the green genes refers to the number of green-pigment genes present; i.e., one to three normal green-pigment genes were observed. The color-vision phenotypes (as determined by anomaloscopy) are indicated as P (protanopia) and PA (protanomaly). Subjects 2021, 2108, 2111, 2115, 2116, 2117, 2172, 2182, 2183, CB8, and CB9 had one green-pigment gene; subjects 1617, 1620, and 1621 had two green-pigment genes; and subjects 1914, 1930, 2071, 2112, and 2177 had three green-pigment genes. The DNA of subjects was analyzed by Southern blot analysis (table 2) and PCR amplification using the gene-specific primers as described in fig. 3. The assignments of intron of fusion for subjects 1553, 1617, 1678, 1914, 2105, and 2183 were based on results of the above analyses and were confirmed by sequencing as described in Subjects and Methods. Finally, all other assignments were confirmed by SSCP analysis using gene-specific amplification primers as described elsewhere (Winderickx et al. 1992). The intron in which fusion had occurred in hybrid genes is indicated. The uncertainty in assigning the fusion point to intron 2 or intron 3 is due to the polymorphism in the sequence of exon 3, so that some sequences are shared by the red- and green-pigment genes. Subjects with the same phenotype and intron of fusion were grouped. The color-vision phenotypes (as determined by anomaloscopy) are indicated.

Results

Psychophysical Characteristics

The anomaloscopy results for 67 subjects (63 red-green color-defective subjects and 4 color-normal subjects who had fusion genes; see below) with abnormal gene arrays are depicted in figure 1. The matching ranges are shown in units of the red-green-mixture scale (0–73) of the Nagel model I anomaloscope. The matching ranges shown in figure 1 are average results from the two eyes of each observer. The corresponding settings of the standard yellow radiance for a match are not shown. CB8 and CB9 (see fig. 4) had been characterized in other studies (i.e., as subjects II-1 and III-2, respectively, in Thuline et al. 1969; also see Drummond-Borg et al. [1988]).

The distribution of subjects into the four classes of red-green color deficiency—i.e., P, PA, D, and DA—was similar to that found in various studies on color-vision defects among Caucasians (Drummond-Borg et al. 1989). About 50% of all color-vision defects are expected to be DA, while roughly equal proportions of D, PA, and P constitute the remainder. In our study, the proportions of subjects who tested as having DA, D, PA, and P were .44, .19, .17, and .19, respectively. The similarity to the expected frequencies indicates that the less severe types of color-vision defects (such as PA and DA) were known to our volunteers, since they had responded to advertisements seeking color-defective individuals.

Protan Subjects

Twenty-three (36%) of 64 color-deficient subjects tested were protans. A summary of the results obtained from these subjects is shown in figure 4. The figure shows the various combinations of gene array type (deduced from data shown in table 2), intron of fusion, and type of protan defect (P or PA) found by anomaloscopy.

The gene arrays of all protan subjects were characterized by the presence of a 5′ red-green 3′ hybrid gene in place of a normal red-pigment gene. Furthermore, the intron of fusion was upstream of exon 5 in all cases, thus supporting the idea that exon 5 is important in establishing the spectral characteristics of the normal red visual pigment.

In three subjects (1553, 2105, and 2203) we could identify only a single 5′ red-green hybrid pigment gene.

Table 2

Green-Pigment/Red-Pigment Gene Fragment Ratios in Color-Vision-Defective and Normal Subjects

Category	B_g/B_r	C_g/C_r	D_g/D_r[a]
Protans:			
Protanopes:			
1620	2.50 [2]	1.83 [2]	
1621	2.30 [2]	1.93 [2]	
1678	1.06 [1]	1.14 [1]	
2112	3.20 [3]	∞	
2172	.99 [1]	∞	
2177	2.35 [2]	3.03 [3]	
2182	.81 [1]	∞	
2183	.94 [1]	.87 [1]	
CB9	1.04 [1]	∞	
Protanomalous subjects:			
1617	2.30 [2]	1.80 [2]	
1914	3.00 [3]	2.80 [3]	
1930	2.67 [3]	2.85 [3]	
2021	1.13 [1]	1.10 [1]	
2071	3.20 [3]	5.10 [3]	
2108	1.53 [1]	1.45 [1]	
2111	1.29 [1]	1.56 [1]	
2115	1.14 [1]	.71 [1]	
2116	1.15 [1]	1.36 [1]	
2117	1.31 [1]	1.47 [1]	
CB8	1.53 [1]	.75 [1]	
Deutans:			
Deuteranope:			
1681	3.10 [3]	2.70 [30]	.90 [1]
Deuteranomalous subjects:			
1552	3.60 [3]	2.41 [3]	1.43 [1]
1616	1.72 [2]	2.00 [2]	.76 [.5]
1618	2.80 [3]	1.00 [1]	.75 [1]
1619	2.90 [3]	1.00 [1]	.89 [1]
1672	4.18 [4]	4.86 [5]	.72 [1]
1682	1.16 [1]	.97 [1]	0
1838	1.00 [1]	1.20 [1]	2.09 [2]
1840	4.85 [5]	3.80 [4]	.45 [.5]
1843	4.50 [4]	3.00 [3]	1.50 [1.5]
1906	5.22 [5]	3.60 [4]	.65 [1]
1907	1.00 [1]	1.00 [1]	0
1908	4.26 [3]	3.22 [3]	0
1909	2.70 [3]	2.71 [3]	2.75 [3]
1916	2.90 [3]	3.00 [3]	1.68 [2]
1927	1.72 [2]	1.73 [2]	1.75 [2]
1934	1.40 [1]	1.10 [1]	0
1955	1.67 [1]	1.13 [1]	0
1968	2.51 [2]	2.20 [2]	0
2057	2.20 [2]	1.89 [2]	1.05 [1]
2059	3.18 [2]	.87 [1]	1.28 [1]
2081	2.81 [3]	3.14 [3]	.44 [.5]
2113	3.33 [3]	5.70 [5]	1.06 [1]
2114	2.07 [2]	2.08 [2]	.79 [1]
2171	3.54 [3]	3.37 [3]	.51 [.5]
2181	5.25 [5]	6.98 [6]	3.26 [3]
2187	4.30 [4]	2.27 [2]	2.03 [2]
Normal subjects with hybrid genes:			
2032	2.22 [2]	2.57 [2]	.55 [.5]
2067	2.23 [2]	.75 [.5]	.44 [.5]
2382	5.40 [4]	4.03 [3]	.43 [.5]
2399	5.67 [3]	1.17 [1]	1.27 [.5]
Normal subjects' G:R's (N[b])			
1:1 (17)	1.03 (±.16)	.97 (±.17)	1.34 (±.19)
2:1 (25)	2.18 (±.29)	2.02 (±.34)	2.33 (±.66)
3:1 (12)	3.00 (±.61)	2.61 (±.41)	3.08 (±.35)
4:1 (5)	4.12 (±.31)	3.52 (±.69)	4.44 (±.51)

(*continued*)

Table 2 (continued)

NOTE.—Ratios between red- and green-pigment gene fragments (explained in fig. 1) were determined by densitometric tracing autoradiographs of Southern blots. Numbers in square brackets are the designated ratios; and numbers in parentheses are SD values. These ratios were used to define the gene array patterns of the protans and deutans, described in figs. 4 and 5, respectively. Subjects who have only one gene are not shown. Normal color vision was ascertained by anomaloscopy.

[a] For all protan subjects, this value is infinity, since they lack the D_r fragment.

[b] Number of subjects.

Presumably, the single hybrid gene encoded a photopigment that was sufficiently "greenlike" in its spectral properties that the subjects performed as if missing the red-pigment gene—i.e., as protanopes—on anomaloscopy. This was true regardless of whether the fusion points were in intron 2 or 3 (subjects 1553 and 2105) or in intron 4 (subject 2203).

In the remaining 20 protan subjects, the presence of one or more normal green-pigment genes in addition to the red-green hybrid gene was detected. Six of these subjects had fusion points in intron 1, and all tested as protanopes. This result is expected, since exon 1 is identical in the normal red- and green-pigment genes, and therefore these red-green hybrid genes effectively encode a green photopigment. Eight subjects had a fusion site in intron 2 or intron 3. Six of these eight were protanomalous and two were protanopes. Because the sequence of exon 3 of the red-pigment gene is not always different from that of the green-pigment gene (Nathans et al. 1986*b*), we were unable to determine whether the point of fusion was in intron 2 or 3.

The remaining six protan subjects had a fusion site in intron 4 of the red-pigment gene, and the pattern of results was similar to that found in subjects with intron 2 or 3 fusion. One of the six was a protanope, while the other five tested as protanomalous.

In summary, the finding of a 5′ red-green hybrid gene at the position of the normal red-pigment gene was sufficient to identify protan subjects. All six individuals with intron 1 fusions were protanopes, as expected. Subjects carrying single red-green hybrid genes with fusion points in introns 2–4 without additional green-pigment genes also tested as protanopes. Gene arrays with red-green fusion genes in introns 2–4 with additional normal green-pigment genes usually tested as protanomalous. However, 3 of 14 in this class of gene arrays were protanopes.

Table 2 shows the relationship between the point of fusion in hybrid genes and the severity of color deficiency as measured by the Rayleigh match range. The match range indicates the range of settings of the anomaloscope red-green mixture over which the subject can match the mixture to the yellow standard. A relatively small match width indicates relatively good color discrimination and therefore a relatively mild color deficiency (anomaly). A match range of 73 indicates that the subject could match all mixtures of red and green (including red or green alone) to the yellow standard and that the subject is therefore a dichromat (P or D). The severity of color deficiency of our protan subjects was not well predicted by the intron of fusion of the hybrid gene. The distribution of match ranges was quite similar for subjects with either intron 2 or 3 fusions or intron 4 fusions.

Deutan Subjects

A summary of the results obtained from subjects classified as deutans by anomaloscopy is shown in figure 5. Forty-one (64%) of 64 red-green color-deficient subjects fell into this category. All subjects had a normal red-pigment gene, and all but one subject (subject 1909) had major gene rearrangements that could be detected by Southern analysis (table 2) and/or gene-specific amplification across intron 4.

Gene deletions.—Molecular analysis on 13 of the 41 deutan subjects indicated a complete absence of green-pigment gene material, as revealed by Southern blot analysis and by PCR amplification and sequencing of all exons. Sequences coding for the red-pigment gene only were found in all 13 subjects. Ten of the 13 subjects with a single, presumably normal red-pigment gene were classified by anomaloscopy as deuteranopes, a finding that is expected, since these individuals lacked the capacity to synthesize the green visual pigment.

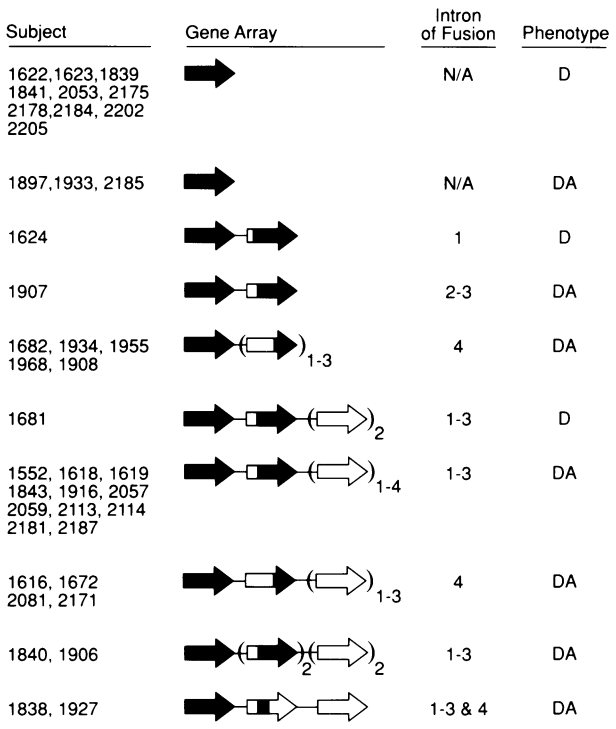

Figure 5 X-linked color-vision-pigment gene arrays in deutan subjects. Explanations concerning gene arrays are given in the legend to fig. 4. N/A = not applicable. Subjects 1616, 2057, and 2114 had one green-pigment gene; subjects 1552, 1618, 1619, 1916, 2059, 2081, 2113, and 2171 had two green-pigment genes; and subjects 1672, 1843, 2181, and 2187 had three or four green-pigment genes. Subjects 1682, 1934, and 1955 had one hybrid gene; subject 1968 had two hybrid genes; and subject 1908 had three hybrid genes. The coding sequence (six exons) of the red-pigment gene of the 13 subjects who had only that gene in their arrays was determined by PCR amplification and direct sequencing using the general oligonucleotide primers shown in table 1. Assignments of intron of fusion were made on the basis of Southern blot analysis (table 2) and gene-specific PCR amplification. For subjects 1624, 1907, 1908, 1927, 1934, and 1938, gene-specific amplification results were confirmed by sequencing clones of fragments containing exons 4 and 5 of the hybrid genes produced by amplification with the general primers of exons 4 and 5 (table 1 and fig. 3) as described in Subjects and Methods. A single deuteranomalous subject with a grossly normal gene array is not included (Winderickx et al., in press).

It is surprising that 3 of the 13 tested as severe deuteranomalous with larger than average matching ranges. The red-pigment gene sequence in each of these three deuteranomalous subjects was identical to that of one or more of the deuteranopes. In order to

rule out the presence of an additional pigment gene in these three deuteranomalous subjects we reduced the stringency of the conditions employed in Southern hybridization experiments. The results (not shown) failed to reveal any evidence of an additional pigment gene. The pattern of a single red-pigment gene in a deuteranomalous subject has also been observed in two subjects in previous studies (Nathans et al. 1986*a*; Neitz et al. 1987).

Simple hybrid genes.—Twenty-five of our 41 deutan subjects had gene arrays characterized by the presence of 5′ green-red hybrid genes in addition to the normal red-pigment gene. Seven of these 25 subjects had one or more green-red hybrid genes but no normal green-pigment genes, while the remaining 18 subjects had one or more hybrid genes and one or more normal green-pigment genes. Although the hybrid genes are always shown immediately 3′ of the normal red-pigment genes in figure 5, their actual position in relation to other green-pigment genes is unknown. Thus, they could be preceded in the array by one or more normal green-pigment genes.

There were seven subjects with one normal red-pigment gene and one or more green-red hybrid genes but no normal green-pigment genes. One subject (subject 1624) with a fusion gene in intron 1 was a deuteranope as might be expected, since the fusion resulted in the formation of a second redlike-pigment gene. The remaining six subjects had fusions in intron 2 or 3 (subject 1907) or in intron 4 (subjects 1682, 1908, 1934, 1955, and 1968), and all tested as deuteranomalous.

Eighteen of the deutan subjects had one or more normal green-pigment genes in addition to a normal red-pigment gene and one or more green-red hybrid genes (16 subjects had one hybrid gene, and 2 subjects had two hybrid genes). All of these subjects tested as deuteranomalous, with the exception of a single deuteranope (subject 1681) whose hybrid gene had a fusion point somewhere between introns 1 and 3. The presence of more than one hybrid gene in an array does not seem to be associated with a more severe color-vision defect. It is noteworthy that all of these 18 subjects had one or two normal green-pigment genes and therefore had the genetic capacity to synthesize normal green pigment. Yet none did synthesize normal green pigment, at least in quantities detectable by Nagel anomaloscopy under conditions employed in this study.

Double-fusion genes.—In two deuteranomalous subjects (subjects 1838 and 1927), the middle portion of a green-pigment gene was replaced by red-pigment gene sequences. This type of hybrid gene seems to have been the product of either gene conversion or double crossover events. The hybrid genes in these two individuals were detectable by gene-specific amplification and nucleotide sequencing, but not by the conventional Southern blotting method (Nathans et al. 1986*a*). The red-pigment gene sequences in these hybrids include exon 4 and possibly exons 2 and 3. Thus, although most of our results for both protan and deutan observers suggest that a crossover upstream of exon 5 is sufficient to produce a red-green color-vision defect, the results on the double-fusion gene subjects indicate clearly that amino acid residues that differentiate red pigment from green pigments and that are encoded by this region which includes exons 2–4 are also involved in determining spectral sensitivity.

In summary, except for one deuteranomalous subject (not shown in fig. 5), deutan color-vision defects were found to be associated with either gene deletion, a simple hybrid gene, or a double-fusion gene. The anomaloscopic findings were compatible with the formulation that DA usually is produced by a green-red (or more complex) hybrid gene, while D is frequently associated with deletion of all green-pigment genes (in the case of subject 1681, with fusion between introns 1 and 3, we assume that the fusion occurred in intron 1). However, a few subjects with green-pigment gene deletions (subjects 1897, 1933, and 2185) unexpectedly tested as deuteranomalous.

Table 3 shows the relationship between the point of fusion in hybrid genes and the severity of color

Table 3

Rayleigh Match Range as a Function of Fusion Point

	No. of Protan Subjects with Fusion in Intron[b]			No. of Deutan Subjects with Fusion in Intron(s)[b]	
Match Range[a]	1	2 or 3	4	1–3	4
0–20	0	2	4	8	7
21–40	0	2	1	2	2
41–60	0	2	0	4	0
61–72	0	0	0	0	0
73	6	4	2	2	0

[a] Range of red-green mixture settings judged to match the standard yellow light. Values were derived from fig. 1.

[b] Points of fusion were derived from figs. 4 and 5.

deficiency in the deutan subjects, as measured by the Rayleigh match range. As was the case with protan subjects, the severity of color deficiency was not well correlated with the point of fusion in the hybrid genes. The distribution of match ranges was quite similar for subjects with either intron 1–3 fusions or intron 4 fusions.

Green-Red Fusion Genes in Color-Normal Individuals

We had previously observed that the frequency of hybrid color-vision-pigment genes among Caucasians—and, more so, among African-Americans—is greater than that of phenotypic color-vision defects (Drummond-Borg et al. 1989; Jorgensen et al. 1990). We hypothesized that some individuals with hybrid genes have normal color vision. We therefore recruited two groups (totaling 129 individuals) of Caucasian males as volunteers for a vision study. The first group of subjects (52) was ascertained to have normal color vision, by anomaloscopy. Their red-green arrays were then analyzed by Southern blot hybridization (table 2) and by PCR amplification across intron 4 (fig. 3). Two were found to have 5′ green-red hybrid genes in addition to normal red- and green-pigment genes (subjects 2382 and 2399 in fig. 6). These results indicate that approximately 4% (2 of 52) of these Caucasian males with normal color vision have hybrid 5′ green-red pigment genes. This number is likely to be an *underestimate* because not all hybrid genes are detectable by these methods.

We also analyzed DNA specimens from another 77 subjects (whose color-vision status was unknown prior to analysis) for major gene rearrangements, by Southern blot analysis. Seven were found to have gene arrays with 5′ green-red hybrid genes, and 70 had apparently normal gene arrays. Anomaloscopic examination of the seven with hybrid genes revealed five to be deuteranomalous and two (subjects 2067 and 2032 in fig. 6) to have normal color vision. If we assume that the 70 persons with apparently normal patterns on Southern blots had normal color vision, then the frequency of abnormal gene arrays among males with normal color vision in 2 of 72, or 2.8%, in this subsample. Hybrid genes detectable by PCR only may have been overlooked in this group of subjects.

The points of fusion in the hybrid genes of the four individuals with normal color vision were determined by gene-specific PCR amplification (and were confirmed by sequencing exons 4 and 5 after amplification with nonspecific primers) to be in either intron 1, introns 1–3, or intron 4 (fig. 6). The relative position of the hybrid genes vis-à-vis the normal green-pigment genes in these arrays, however, is unknown. On the basis of preliminary ongoing studies, we postulate that pigment genes located downstream of the first two pigment genes in an array are not expressed. Figure 6 illustrates the position of the hybrid genes in this manner.

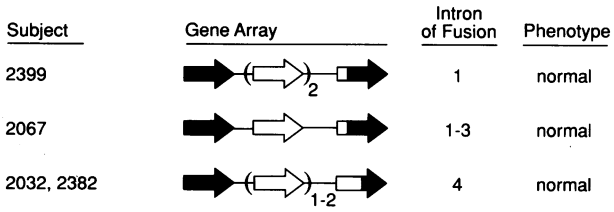

Figure 6 Green-red hybrid genes in four subjects with normal color vision. Gene arrays in four subjects who anomaloscopically tested as having normal color vision but who were shown to carry 5′ green-red hybrid genes are shown. Explanations of arrays are as given in the legend to fig. 4, except that the hybrid genes in arrays are positioned 3′ to the normal green-pigment genes. This is in accord with our hypothesis that genes that occupy this position are not expressed and therefore do not lead to deficiencies in color vision. Subject 2032 had two normal green-pigment genes, and subject 2382 had one normal green-pigment gene.

Discussion

The principal goal of this study was to compare the molecular patterns of color-vision genes in a large population with psychophysically determined normal and abnormal red-green color vision. We have examined the color vision and the molecular structure of the X-linked red-green color-vision gene arrays in 64 male subjects with color-vision defects. Using a combination of Southern blot analysis and the method of detection of hybrid genes by gene-specific PCR amplification, we were able to detect all hybrid genes and determine the approximate location of points of fusion in the hybrid genes of these color-deficient subjects. These results aid both in providing a molecular explanation for differences in the absorption spectra of normal and anomalous photopigments and in explaining corresponding differences in color-vision phenotypes.

In our study of 64 color-deficient subjects, gene arrays in which the normal red-pigment gene was replaced by a 5′ red-green 3′ hybrid gene were found in 23 protans, and arrays that were either missing the green-pigment gene, or that contained 5′ green-red 3′ hybrid genes were observed in 40 deutans. These results are in general agreement with those of Nathans et al. (1986*a*). The gene array of the single remaining

deuteranomalous subject had no detectable major gene rearrangements, indicating that a more subtle mutation underlies this defect. Indeed, a Cys-to-Arg substitution at position 203 of all three of this subject's green-pigment genes was detected (Winderickx et al., in press).

Thus, with only one exception in 64 subjects, abnormal red-green color vision as measured by Nagel anomaloscopy was associated with major rearrangements at the X-linked red-pigment/green-pigment gene complex. As suggested earlier (Nathans et al. 1986*a*), these rearrangements are presumably the result of either homologous recombination or gene conversion between the red- and green-pigment genes. These events have introduced into the population relatively common variants of the red and green pigments that differ in amino acid sequence and presumably in absorption spectra, and hence result in abnormal color vision.

The visual pigments constitute a family of homologous membrane-bound receptor proteins to which the chromophore 11-*cis* retinaldehyde is covalently linked. They contain seven predominantly hydrophobic transmembrane alpha-helical segments that form a cavity containing the chromophore (see reviews by Applebury and Hargrave 1986; Nathans 1987). In the case of rhodopsin, the chromophore lies parallel to the plane of the membrane bilayer (Chabre et al. 1982; Thomas and Stryer 1982). The red and green color-vision pigments differ in sequence in, at most, 15 of the 364 amino acid residues. These differences are located in exons 2–5 (Nathans et al. 1986*b*). The presence of polar OH-bearing residues, versus nonpolar residues, constitutes the difference at 7 (residues 65, 180, 230, 233, 277, 285, and 309) of these 15 positions (fig. 7). The other differences do not involve significant changes in polarity. It has been proposed that the spectral difference between the red and green pigments is due to the net effect of interaction between the OH-bearing side chains and the chromophore (Kosower 1988). All such groups are located within the transmembrane pocket and are believed to be positioned within one turn of the helix of the location of the retinylidine group (Kosower 1988). In support of this hypothesis, Neitz et al. (1991) reported that differences at three amino acid positions (Ser_{180}, Phe_{277}, and Tyr_{285}) determine the spectral differences among the middle- to long-wavelength visual pigments of South American monkeys as well as of humans.

In the protan series, three subjects had only one

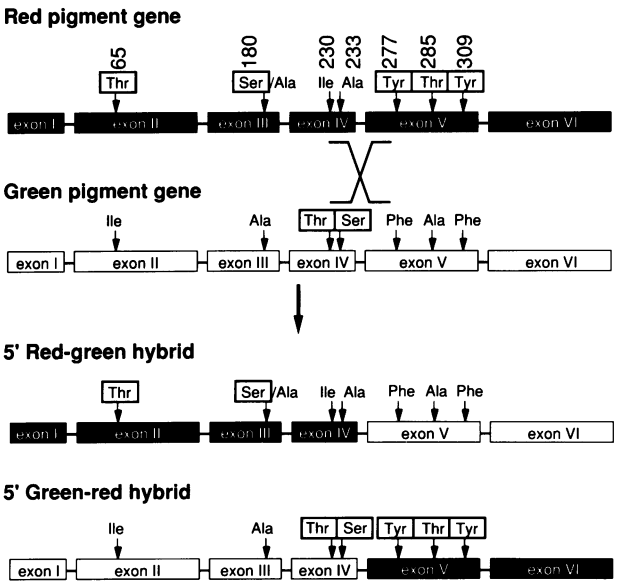

Figure 7 Distribution of hydroxyl-bearing amino acids in normal and hybrid genes. Shown are diagrams (not to scale) of the normal red-pigment (blackened boxes) and green-pigment (unblackened boxes) genes, together with 5′ red-green and 5′ green-red hybrid genes resulting from a crossover in intron 4. Amino acid differences, between the red- and green-pigments genes, that involve hydroxyl-bearing groups (boxed), together with residue numbers, are indicated above exons. Note the Ser/Ala polymorphism at position 180 of the red-pigment gene, which was shown to underlie variation in color matching among male subjects with normal color vision. (Neitz et al. 1991; Winderickx et al. 1992). The 5′ red-green hybrid is as observed in subject 2203 in the protan series (fig. 4), and the 5′ green-red hybrid is as observed in subject 1682 in the deutan series (fig. 5).

gene, a 5′ red-green 3′ hybrid (fig. 4); in one (subject 2203) the fusion occurred in intron 4, and in the other two (subjects 1553 and 2105) it occurred in either intron 2 or intron 3. This result indicates that replacement of the three OH-bearing amino acids in exon 5 of the red-pigment gene by the corresponding nonpolar residues of the green-pigment gene (fig. 7) was sufficient to encode a pigment that is spectrally either identical or closely similar to the normal green pigment and that will result in P rather than in D. In fact, a 5′ red-green 3′ hybrid gene in a protanope (similar to our subjects 1553 and 2105) has been shown, by electroretinography, to encode a photopigment that was indistinguishable from the normal green pigment (Neitz et al. 1989). The fusion point in this subject was found, by sequencing, to be in intron 3. As expected, the six protans (fig. 1) who have a hybrid gene with intron 1 fusions in addition to one or two normal

green-pigment genes also tested as protanopes, since the red- and green-pigment genes have the same exon 1. In fact, on the basis of the aforementioned results, all protans listed in figure 4 would be expected to test as protanope. Yet, subjects who have apparently identical hybrid genes and additional normal green-pigment genes can test, by the psychophysical technique employed in this study, as either protanope or protanomalous. In fact, the majority tested as protanomalous subjects. Polymorphisms in amino acid sequence of the red- and green-pigment genes (Nathans et al. 1986*a;* Neitz et al. 1991), particularly the Ser/Ala at position 180 in exon 3 of the red-pigment gene (fig. 7), have been shown to underlie variations in color matching in a population of color-normal males (Winderickx et al. 1992) and are unlikely to underlie the above discrepancies in genotype-phenotype relationships. Thus, the coding sequences of both the hybrid and normal green-pigment genes of subject 1678 with P were identical to those of the corresponding genes of subject 2115 with PA. Similarly, the coding sequences of two protanopes (subjects 2183 and 2177) were identical to those of four protanomalous subjects (subjects 2116, 1930, 2108, and 2117).

In the deutan series, 13 subjects had only one gene, the normal red-pigment gene (fig. 5). Ten of these subjects tested as deuteranopes, as expected. Unexpectedly, three of these individuals tested as deuteranomalous, suggesting the existence of some greenlike pigment. The sequence of all exons of all 13 subjects was determined, and each individual had only one red-pigment gene sequence. The coding sequence of the red-pigment gene in the three deuteranomalous subjects was identical to that of one or another of the deuteranopes, indicating that differences in amino acid sequence could not underlie these phenotypic subclasses. Furthermore, low-stringency Southern blot hybridization revealed only one red-pigment gene in the genome of the three deuteranomalous subjects.

As in the protan series, exchange of amino acids of exon 5 was sufficient to convert the green-pigment gene to one that encodes a pigment more redlike in its absorption spectrum. With only one exception (subject 1681), subjects possessing 5′ green-red 3′ hybrid genes together with normal red- and green-pigment genes tested as deuteranomalous. It is most likely that subject 1681 (a deuteranope) has a hybrid gene with a fusion point in intron 1, whereas in the deuteranomalous subjects the fusion points were in introns 2–4. Furthermore, the observation that subject 1681 tested as a dichromat most strongly supports emerging observations (J. Winderickx, L. Battisti, A. G. Motulsky, and S. S. Deeb, unpublished data) that some genes in the arrays are not expressed, since a mixture of normal green and hybrid pigments, if fully expressed, would be expected to be associated with trichromacy.

We observed a novel type of hybrid gene in two of the deuteranomalous subjects (subjects 1838 and 1927); 5′ green-red-green 3′, in which an internal region was exchanged (as a result of either a double crossover or a gene conversion) between the green- and red-pigment genes. This finding suggests that amino acid differences in exons 2–4 are also involved in determining spectral sensitivities of the photopigments.

The results of our studies and those of Nathans et al. (1986*a*) therefore indicate that individuals could generally be classified as either protan or deutan by molecular study of the genotype. Red-green fusion genes are associated with protan defects. As expected, intron 1 fusions were proptanopes, but intron 2–4 fusions could be either protanomalous or protanopes, with the majority being protanomalous subjects. All deutans had either deletion of the green-pigment gene or green-red (or more complex) hybrid genes. An absent green-pigment gene occasionally expressed as DA and not as D, while green-red hybrids due to fusion in intron 1 were D. All other phenotypically defective subjects with green-red hybrid genes tested as deuteranomalous. Although certain trends were evident, the genotype was not strictly correlated with the phenotype (dichromacy vs. anomalous trichromacy) within the protan and deutan series. Furthermore, there was no correlation between the point of fusion in hybrid genes of either protan or deutan subjects and the severity of color-vision deficiency as measured by the Rayleigh match range (table 3).

There have been proposals that the structure of a hybrid gene, alone, may not be sufficient to predict the severity of red-green anomaly in anomalous trichromats. Hurvich (1972) has advanced the hypothesis that the degree of severity in anomalous trichromacy involves not only the absorption spectra of red and green pigments but also depends on postreceptoral neural factors. A similar conclusion has been reached by Nagy and Purl (1987). In support of this hypothesis is the finding that the spectral difference between the red and green opsins is largely due to differences at amino acid positions 277 and 285 in exon 5 (Neitz et al. 1989, 1991), which predicts that all crossovers upstream of exon 5 would be expected to result in hybrids with similar spectral characteristics.

A competing, though not mutually exclusive, hypothesis has been advanced by Pokorny and Smith (1987). They postulated that variation in either the amount of pigment synthesized or the number of the red versus green photoreceptors might account for the variation in color-discrimination ability within the protan and deutan series. In support of this hypothesis, Pokorny and Smith point to a number of studies (Nagy 1980; Breton and Cowan 1981; for review, see Pokorny and Smith 1982) which indicate that residual anomalous trichromacy is often demonstrated in nominal dichromats when Rayleigh matches are performed with large stimulus fields at high mean luminances. Similarly, Alpern and Wake (1977) showed that subjects nominally testing as dichromats by a Nagel anomaloscope were found to be anomalous trichromats by other methods. Thus, the failure to find a strict correspondence between genotype and phenotype may, in many cases, be due to the use of Nagel anomaloscopy. A close correspondence may be found by incorporating other psychophysical procedures into our protocol.

The most puzzling cases remain those subjects who have only a single red-pigment gene and yet are anomalous trichromats rather than dichromats (subjects 1897, 1933, 2185 in the deutan series). Because they have only a single pigment in the long-wavelength region of the spectrum, these subjects should be completely unable to discriminate the 541- and 644-nm lights in the anomaloscope from the 591-nm standard light (Cornsweet 1970; Wyszecki and Stiles 1982); yet they can do so. The use of color-match criterion is technically demanding. Any imperfections in the stimulus field that are due either to misalignment of the subject with the instrument or to other artifacts could cause the subject to refuse a match even though his refusal is not based on discrimination of the fields on the basis of color differences.

More generally, Nagel anomaloscopy may not provide a sufficiently precise and detailed description of the color-discrimination capacity of the red-green color-deficient subjects to allow precise quantitative correlations between genotype and phenotype. Whether more sophisticated psychophysical instrumentation or techniques would provide clarifying data and allow more perfect correlations between genotype and phenotype will need to be shown.

Finally, although subjects with red-green color-vision defects were almost always found to have abnormal gene arrays, our results clearly indicate that the converse is not true. We found four subjects who possessed a 5′ green-red 3′ hybrid gene, in addition to normal red- and green-pigment genes (fig. 6) but who gave normal anomaloscopic results. On the basis of molecular analysis alone, we would have predicted these subjects to be deutans. We have shown elsewhere that the frequency of red- and green-pigment hybrid genes is greater than would be expected from the frequency of color-vision defects in a Caucasian population (Drummond-Borg et al. 1989)—and that it is particularly greater than would be expected among African-Americans (Jorgensen et al. 1990). We hypothesized that some subjects with normal color vision possess abnormal gene arrays. Thus we have confirmed the suggestion that abnormal genes can be associated with normal anomaloscopic findings. In explaining these results, we hypothesize that the position of the fusion gene in an array influences the degree to which it is expressed. If the fusion gene happens to be 3′ downstream of the normal red- and green-pigment genes, it may not be expressed to an extent that will influence the subject's Rayleigh matches. This hypothesis predicts that all single red-green hybrid genes will manifest as protans and that all single green-red hybrid genes not associated with other green-pigment genes will manifest as deutans. An alternative, but less likely, explanation is that the hybrid genes in some individuals are not expressed because they carry a mutation either in the regulatory sequences or at splice junctions (the coding sequences of the hybrid genes in these individuals are normal). Further work on determining the actual position of hybrid genes in an array will be needed to resolve this question.

Acknowledgments

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