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Molecular Genetics of Inherited Variation in Human Color Vision

JEREMY NATHANS, THOMAS P. PIANTANIDA, ROGER L. EDDY, THOMAS B. SHOWS, DAVID S. HOGNESS

The hypothesis that red-green "color blindness" is caused by alterations in the genes encoding red and green visual pigments has been tested and shown to be correct. Genomic DNA's from 25 males with various red-green color vision deficiencies were analyzed by Southern blot hybridization with the cloned red and green pigment genes as probes. The observed genotypes appear to result from unequal recombination or gene conversion (or both). Together with chromosome mapping experiments, these data identify each of the cloned human visual pigment genes.

MOST HUMANS CAN MATCH ANY COLOR EITHER BY COMbining three suitably chosen primary colors or by combining two primaries and adding the third primary to the given color. For additive color mixture, such as when lights are mixed, the primaries are red, green, and blue. Thomas Young, 180 years ago, put forward the hypothesis that this phenomenon (trichromacy) is a consequence of humans having three independent light-sensitive mechanisms (*1*). We now know that Young's three mechanisms are embodied in three classes of cone photoreceptor cells in the human retina. Each class contains a different visual pigment, which determines the spectral sensitivity of all the cones of that class.

Most humans agree on the proportions of the three primaries required to match a given color. Among those who differ from color normals with respect to the proportions of the primaries, some require that the three be present in unusual proportions, and others require only two primaries. Individuals with the first type of variation are called anomalous trichromats and are presumed to have three classes of cones, one of which contains a photopigment with an anomalous absorption spectrum. Those with the second type of variation are called dichromats and are presumed to have only two of the three classes of cones. These types of variation can be further subdivided by psychophysical tests into classes whose color vision variation is attributable to alterations in either red, green, or blue cones. (As is discussed below, the blue cone defects are so rare that most of the data, especially with respect to subtypes of variation, comes from studies of the red and green cone sensitivities.)

The heritability of variations in color sensitivity has long been recognized (*2*). It is now clear that one locus is responsible for variations in red cone sensitivity and that a second locus is responsible for variations in green cone sensitivity (*3*). Both loci map to the distal part of the q arm of the X chromosome and are tightly linked to each other and to glucose-6-phosphate dehydrogenase (G-6-PD) (*4*). Variations in the blue cone sensitivity have recently been shown to segregate in an autosomal fashion (*5*). Among the red and green variants, available evidence points to allelism of those traits that affect a given cone type. However, a true complementation test (requiring expression of both alleles in the same cell) is not possible because each cell in a female expresses only one of her two X chromosomes (*6*). The evidence for allelism rests instead on the lack of recombination between two defects affecting a single cone type (*4, 5, 7*). It is possible to define a "dominance" hierarchy among alleles by observing the phenotypes of heterozygous women; in each case the allele that least diminishes color discrimination is "dominant" (*7*).

These data are consistent with a model in which the loci responsible for inherited variations in color vision correspond to the genes that encode the apoproteins of the three cone pigments. In support of this model, Rushton, as well as Alpern and Wake, have measured visual pigment absorption in the living human eye by reflection densitometry and found that dichromats lack one of the cone photopigments (*8*). More recently, this result has been confirmed and extended by microspectrophotometric analysis of single human cones from normal and dichromat retinas (*9*).

We have performed a direct test of the hypothesis that inherited variations in human color vision are caused by alterations in the genes that encode the red, green, and blue visual pigments. Our strategy has been to isolate these genes as recombinant DNA molecules and compare their structures among normal and mutant individuals. The X linkage of red and green defects simplifies this analysis. In males, only those genes resident on a single X chromosome are seen in Southern blots or in cloning experiments, and only the phenotype resulting from that X chromosome is observed in psychophysical experiments. This phenotype can be measured in a sample noninvasive test. Moreover, the incidence of red and green color vision variation is quite high—approximately 8 percent among Caucasian males.

We have described (*10*) the isolation and characterization of the three human cone pigment genes. We referred in (*10*), and do so here, to these genes by their true identities—that is, blue, green, and red pigment genes; however, this assignment requires the data presented below. We showed (*10*) that the red and green pigment genes were extraordinarily similar in DNA sequence (98 percent identity). This degree of homology and the high incidence of variation in green pigment gene number among color normal males, led us to propose a model in which red and green pigment genes reside in a head-to-tail tandem array.

Chromosomal locations of cloned visual pigment genes. As a

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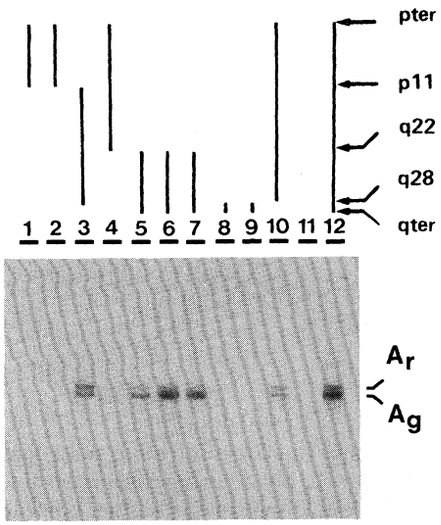


Fig. 1. Subchromosomal localization of green and red pigment genes. Shown is a Southern blot autoradiograph of Eco RI–digested DNA prepared from a panel of ten mouse-human hybrid cell lines (lanes 1 to 10), a mouse cell line (RAG, lane 11), and a human cell line (WI-38, lane 12). Each line has that fraction of the human X chromosome shown above the autoradiograph. The probe hybridizes to both green and red pigment genes. The gene mapping strategy and isolation of mouse-human hybrid cell lines have been described (*11*). Each of the mouse-human hybrids has a known X-autosome translocation as determined by karyotypic analysis, isozyme markers, and the HAT (hypoxanthine, aminopterine, thymidine) selection system (*11*). They are: lanes 1 and 2, a 15 to X translocation retaining Xpter-Xp11; lane 3, an X to 15 translocation retaining Xp11-Xqter; lane 4, an X to 11 translocation retaining Xpter-Xq22; lane 5, a 5 to X translocation retaining Xq22-Xqter; lanes 6 and 7, a 22 to X translocation retaining Xq22-Xqter; lanes 8 and 9, a 3 to X translocation retaining Xq28-Xqter, and lane 10, an X to 3 translocation retaining Xpter-Xq28. Hybridization methods are described in the legend to Fig. 3.

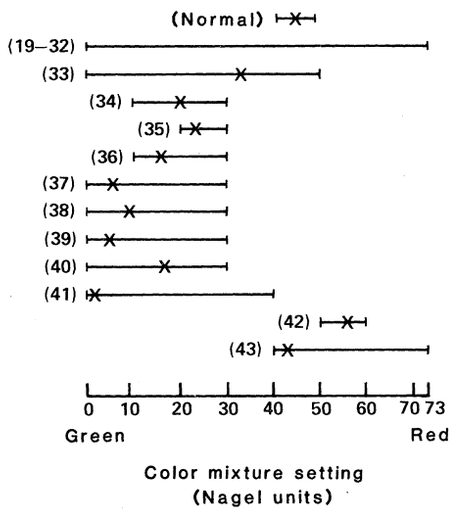


Fig. 2. Anomaloscope test data for 25 color variant males. Each subject is represented by a number next to the horizontal bar showing the range of ratios of red to green lights acceptable as a match of the standard yellow light. The midpoints of the free matching range are marked by crosses; that is, those red to green ratios chosen by the subject when he was free to adjust both the relative intensities of red and green lights as well as the absolute intensity of the standard yellow light. Subjects 19 to 32 accept any red to green ratio and are, therefore, dichromats. They were divided into G^-R^+ (19 to 26) or G^+R^- (27 to 32) classes on the basis of their relative sensitivities to the red primary. Subject 33 appears to show some residual discrimination; he is either $G''R^+$ or G^-R^+. Because he is missing all green pigment genes (Figs. 3 and 4) we classify him as G^-R^+. Subjects 34 to 36 are $G'R^+$; subjects 37 to 41 are $G''R^+$; subject 42 is G^+R'; and subject 43 is G^+R''.

first step in testing the hypothesis relating color blindness loci and visual pigment genes, we mapped the chromosomal locations of the three human cone pigment genes, as well as that of the gene encoding the rod pigment, rhodopsin. In one experiment a DNA probe derived from a green pigment gene [the rightmost 4 kilobases (kb) of clone gJHN9; see figure 5A in (*10*)] was hybridized to a Southern blot filter of Eco RI digested DNA from a panel of mouse-human hybrid cell lines that retain different human chromosomes. The probe is homologous to both red and green pigment genes and revealed the expected 9-kb size class of hybridization only in those lanes containing DNA from cell lines that retain the human X chromosome. In a second experiment we observed loss of that hybridization concomitant with loss of the human X chromosome from one of the cell lines. To map the subchromosomal locations of these sequences, we probed a Southern blot of Eco RI–digested DNA prepared from a panel of mouse-human hybrid cell lines that retain different parts of the human X chromosome. The blot was hybridized with a probe encompassing the second exon of a green pigment gene [contained in clone gJHN21; figure 5B in (*10*)]. This probe hybridizes with two Eco RI fragments: A_g from the green pigment genes and A_r from the red pigment gene (Fig. 3A). Because the ratio of green to red pigment genes varies among normal males (*10*) and the hybrids were generated by fusion of cells from different human donors (*11*), the ratio of A_g to A_r is expected to vary among the hybrids. Figure 1 shows that A_g and A_r segregate together in each case; they also segregate with human G-6-PD activity, and with the q22-q28 interval (*11*). This pattern matches that predicted for the loci responsible for variations in red and green color sensitivity and provides the first evidence for the identification of the red and green pigment genes. These experiments do not, however, allow us to determine which of the X-linked genes corresponds to the red locus and which to the green locus.

The gene encoding the human rod photopigment, rhodopsin (RHO) (*12*), was mapped to the third chromosome by Southern blotting of a panel of mouse-human hybrids that retain various fractions of the human karyotype (Table 1). This gene is present in a cell hybrid (XTR-22) that retains the q21-qter region of chromosome 3 as part of an X to 3 translocation. Therefore the human rhodopsin gene resides within the interval 3q21-3qter. The same panel was used to map the remaining visual pigment gene [shown in figure 1 in (*10*)] to chromosome 7. Given that this is the only visual pigment gene that maps to an autosome aside from rhodopsin, we presume that it encodes the blue pigment, consistent with the autosomal nature of inherited variation in blue sensitivity. The blue pigment gene (BCP; that is, blue cone pigment) is absent from a cell hybrid (JSR-17S) that retains the pter-q22 region of chromosome 7 as part of a 7 to 9 translocation. This gene therefore resides in the 7q22-7qter interval.

Identification of red and green pigment genes. To correlate genotype with phenotype, we examined 25 males with various forms of red or green color vision variation. Psychophysical test data obtained from these males are shown in Fig. 2. Briefly, the test (which measures only red and green cone sensitivities) consists of presenting a variable mixture of red and green lights on one half of a screen, and a variable intensity yellow light on the other half. The subject adjusts the ratio of the red and green lights and the intensity of the yellow light to produce a perfect match between the two halves of the screen. When the red-green ratio and the yellow intensity are adjusted to produce a match, the number of photons captured per second by red and green pigments is the same from both halves of the screen. The midpoints and ranges of this red-green ratio for 25 male subjects is shown in Fig. 2. All of the major groups of red and green color vision variation are represented.

Southern blots for three different restriction digests of genomic DNA from 15 dichromats and a color-normal control are shown in Fig. 3. The probes used for these blots hybridize only with the X-

linked loci under these conditions. It is immediately apparent that 14 out of 15 samples differ in the fragment pattern for at least one of the restriction digests from that in the color-normal controls [see figure 8 in (*10*)]. It is also apparent that more than one genotype can correspond to a given phenotype: the nine G^-R^+ (G^-, absent green sensitivity; R^+, normal red sensitivity) subjects fall into two groups, whereas the six G^+R^- (G^+, normal green sensitivity; R^-, absent red sensitivity) subjects are all different from each other (*12a*). These hybridization patterns suggest that gross changes in DNA rather than point mutation have produced at least 14 of these 15 mutant genotypes.

We consider first the assignment of gene identities based upon these data. We showed (*10*) that restriction fragments A_r, B_r, C_r, and D_r shown in Fig. 3A are all derived from one gene and that color-normal males all have the same copy number of this gene, which is probably one; by contrast restriction fragments A_g, B_g, C_g, and D_g derive from a different gene that varies in number among color-normal males. None of the nine G^-R^+ subjects have D_g and three of nine G^-R^+ subjects have A_g, B_g, and C_g (Fig. 3). However, all of the G^-R^+ subjects retain A_r, B_r, C_r, and D_r. Thus A_r, B_r, C_r, and D_r are associated with a functioning red mechanism, whereas A_g, B_g, C_g, and D_g are not. Indeed, absence of A_g, B_g, and C_g correlates partially, and absence of D_g correlates perfectly with absence of the green mechanism.

The situation among G^+R^- subjects is more complex. Four of six subjects have A_r, all six have B_r, four of six have C_r, and only one of six has D_r. However, every G^+R^- subject (6/6) has D_g, although A_g, B_g, and C_g are not always present (5/6, 4/6, and 5/6, respectively). We can summarize these data and those presented below from anomalous trichromats (as well as those from normals) by listing the following true statements: (i) G^+ is always associated with D_g, and usually, but not always, with A_g, B_g, and C_g; (ii) R^+ is always associated with A_r, B_r, C_r, and D_r; (iii) G^- is always associated with loss of D_g and sometimes with the combined loss of A_g, B_g, and C_g; (iv) R^- is usually associated with loss of D_r, sometimes with loss of A_r or C_r, and never with loss of B_r, and, in fact, (v) B_r is never lost. Taken together these associations imply that A_g, B_g, C_g, and D_g derive from the normal green pigment genes and that A_r, B_r, C_r, and D_r derive from the normal red pigment gene. Furthermore, the 5′ end of the red pigment gene, represented by fragment B_r (Fig. 3C),

Table 1. Chromosomal assignment of the rhodopsin gene and the blue pigment gene. Probes from each gene were hybridized to Southern blots of DNA from mouse-human hybrid cell lines. The 30 cell hybrids involve 14 unrelated human cell lines and four mouse cell lines (*11*). They were characterized by analysis of karyotype, mapped enzyme markers, and mapped cloned DNA probes (*11*). Presence (+) or absence (−) of a human chromosome is indicated. (+/+), (+/−), (−/+), (−/−): the first symbol within the parentheses indicates the presence (+) or absence (−) of the human gene; the second symbol within the parentheses indicates the presence (+) or absence (−) of the indicated chromosome. Percent discordancy indicates the percent discordant segregation for a probe and a chromosome. The chromosome which shows no (0 percent) discordancy with a human gene probe is the one from which that gene derives. Hybrid XTR-22 retains the 3q21-3qter region as part of an X to 3 translocation and, because the human rhodopsin gene is also retained, localizes that gene to this interval. Hybrid JSR-175 retains the 7pter-7q22 region and, because it does not retain the blue pigment gene, localizes that gene to the 7q22-7qter interval.

			Human Chromosomes																						
HYBRID	Rhodopsin	Blue Pigment	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Translocations
ATR-13	+	+	+	+	+	+	+	+	+	+	−	+	−	+	+	+	+	+	+	+	+	−	−	−	t	5/X
DUA-3BSAGA	−	+	−	+	−	−	−	−	+	+	−	−	−	−	+	+	−	−	+	−	−	−	−	−	−	
DUA-5BSAGA	+	−	−	−	+	−	+	−	−	−	−	−	+	−	−	+	−	−	+	+	−	−	+	−	−	
DUM-13	+	+	+	+	+	−	+	+	+	−	−	+	+	+	−	+	t	+	+	+	+	+	+	+	t	X/15 15/X
GAR-1	+	−	−	−	+	−	+	−	−	+	−	+	−	+	−	+	+	+	−	−	−	+	−	−	+	
ICL-15	−	−	−	−	−	−	−	−	−	+	−	−	−	+	−	−	−	−	+	−	−	+	+	−	−	
JSR-14	+	−	−	+	+	+	+	+	−	−	−	−	−	+	+	−	−	−	+	−	−	+	+	−	+	
JSR-175	+	−	+	+	+	−	+	−	t	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	−	7/9
JWR-26C	+	+	t	+	+	+	+	+	+	−	+	+	+	+	−	+	+	+	+	+	−	+	+	−	+	1/2
NSL-9	−	−	−	−	−	+	−	−	+	t	+	−	+	+	+	+	+	+	+	−	−	+	+	+	−	17/9
NSL-16	+	+	−	−	+	+	+	−	+	+	t	+	−	+	−	+	+	+	+	+	−	+	+	−	−	17/9
REW-7	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	−	+	+	+	+	+	+	+	
REW-11	−	+	−	−	−	+	−	−	+	−	−	−	+	+	+	−	−	+	−	−	−	+	+	+	+	
REX-11BSAgB	+	−	−	−	+	−	−	−	−	−	−	+	−	−	−	+	+	−	−	+	−	−	−	−	−	
REX-11BSHF	+	−	−	−	+	−	−	−	−	−	−	+	−	−	−	+	−	−	−	+	−	−	−	t	t	22/X
REX-26	+	+	+	+	+	−	−	+	+	+	+	+	+	−	+	+	+	+	+	+	−	+	t	t	t	22/X
SIR-8	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+	+	+	
TSL-1	+	−	−	−	+	−	−	−	−	+	+	+	−	+	+	−	+	+	+	−	+	+	−	−	−	
VTL-6	−	+	−	+	−	−	−	+	+	+	−	+	+	−	−	−	+	−	+	−	+	+	+	+	−	
VTL-8	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	−	+	−	−	+	+	+	−	
VTL-17	−	+	−	−	−	+	−	+	−	−	−	+	+	−	+	+	−	−	+	−	−	+	+	−	−	
WIL-2	−	−	−	−	−	−	−	−	−	+	−	−	−	+	−	−	+	−	+	−	−	−	+	−	+	
WIL-6	−	+	−	+	−	+	+	+	+	+	−	+	+	−	−	+	−	−	+	−	+	+	+	−	+	
WIL-7	+	−	−	+	+	−	+	+	−	+	−	+	+	−	+	+	−	−	+	+	−	−	+	−	+	
WIL-8	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
WIL-8X	+	+	−	−	+	+	+	−	+	+	−	+	+	+	−	+	−	−	+	+	+	+	+	−	+	
WIL-14	+	+	+	−	+	−	+	+	−	+	−	+	−	+	−	+	+	−	+	−	−	−	−	−	+	
WIL-15	+	+	−	+	+	+	−	+	+	−	−	+	+	+	+	+	+	−	+	+	−	+	+	−	+	
XER-11	+	+	+	−	+	+	−	+	+	+	−	+	t	+	+	−	+	+	+	+	+	+	+	+	t	11/X X/11
XTR-22	+	−	−	+	t	+	+	+	−	+	−	+	+	−	−	−	+	−	−	+	+	+	+	+	+	X/3

Rhodopsin		Chromosome	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X
	(+/+)		9	12	20	13	15	10	12	13	5	19	13	15	10	18	14	11	17	18	8	13	16	7	11
	(+/−)		11	9	0	8	6	11	8	8	15	2	7	6	11	3	6	10	4	3	13	8	5	12	5
	(−/+)		0	3	0	2	3	2	5	6	0	4	4	4	5	4	4	2	8	0	2	7	0	4	3
	(−/−)		9	6	9	7	6	7	4	3	8	5	5	5	4	5	5	7	1	9	7	2	1	5	6
		% Discordancy	38	40	0	33	30	43	45	47	54	20	38	33	53	23	34	40	40	10	50	50	43	57	32

Blue Pigment		Chromosome	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X
	(+/+)		8	11	12	12	11	9	17	12	3	15	12	13	9	14	11	9	16	11	9	12	14	7	9
	(+/−)		8	6	5	5	6	8	0	5	13	2	4	4	8	3	5	8	1	6	8	5	3	9	4
	(−/+)		1	4	8	3	7	3	0	7	2	8	5	6	6	8	7	4	9	7	1	8	10	4	5
	(−/−)		12	9	4	10	6	10	12	6	10	5	8	7	7	5	6	9	4	6	12	5	3	8	7
		% Discordancy	31	33	45	27	43	37	0	48	54	33	31	33	47	37	41	48	33	43	30	43	43	46	36

NOTE: "t" in the table indicates a translocation, no intact chromosome.

appears to occupy a privileged position—it is neither duplicated nor deleted. We show below that the complexity of these genotypes, especially G^+R^-, can be explained by proposing that they arose via intragenic recombination. Thus, green function and red function correlate best with just a fraction of each gene, D_g and D_r, respectively. We infer that the region corresponding to the D_g compared to D_r difference (in the fifth exon) is tightly linked to sequence differences that determine the spectral absorbance of the cone photopigments.

Molecular models for color variant genotypes. The analysis presented above relied only on scoring each restriction digest for the presence or absence of hybridizing fragments. Another result of these experiments is that none of the blots have hybridization bands at positions other than those seen in normal DNA. Moreover, the Eco RI digest shown in Fig. 3B was also probed with the 3′ proximal two-thirds of a red pigment complementary DNA (cDNA) clone [a Bam HI–Eco RI fragment from hs7; figure 5C in (*10*)]. This probe encompasses the 3′ half of exon 2 and all of exons 3 to 6; it therefore hybridizes strongly to a 9-kb Eco RI fragment and weakly to 9.2-kb (A_g) and 11.1-kb (A_r) Eco RI fragments (Fig. 3A). No fragments other than these are seen following hybridization of this probe to the blots shown in Fig. 3B. An analogous result was obtained following hybridization to Eco RI–digested anomalous trichromat DNA. The above data suggest that the observed DNA rearrangements involve homologous crossing-over or gene conversion (or both) because nonhomologous rearrangements produce restriction fragments that usually differ in size from those of unrearranged DNA. Generation of these homologous rearrangements is not surprising in light of the high degree of sequence homology (98 percent) throughout almost the entire length of the

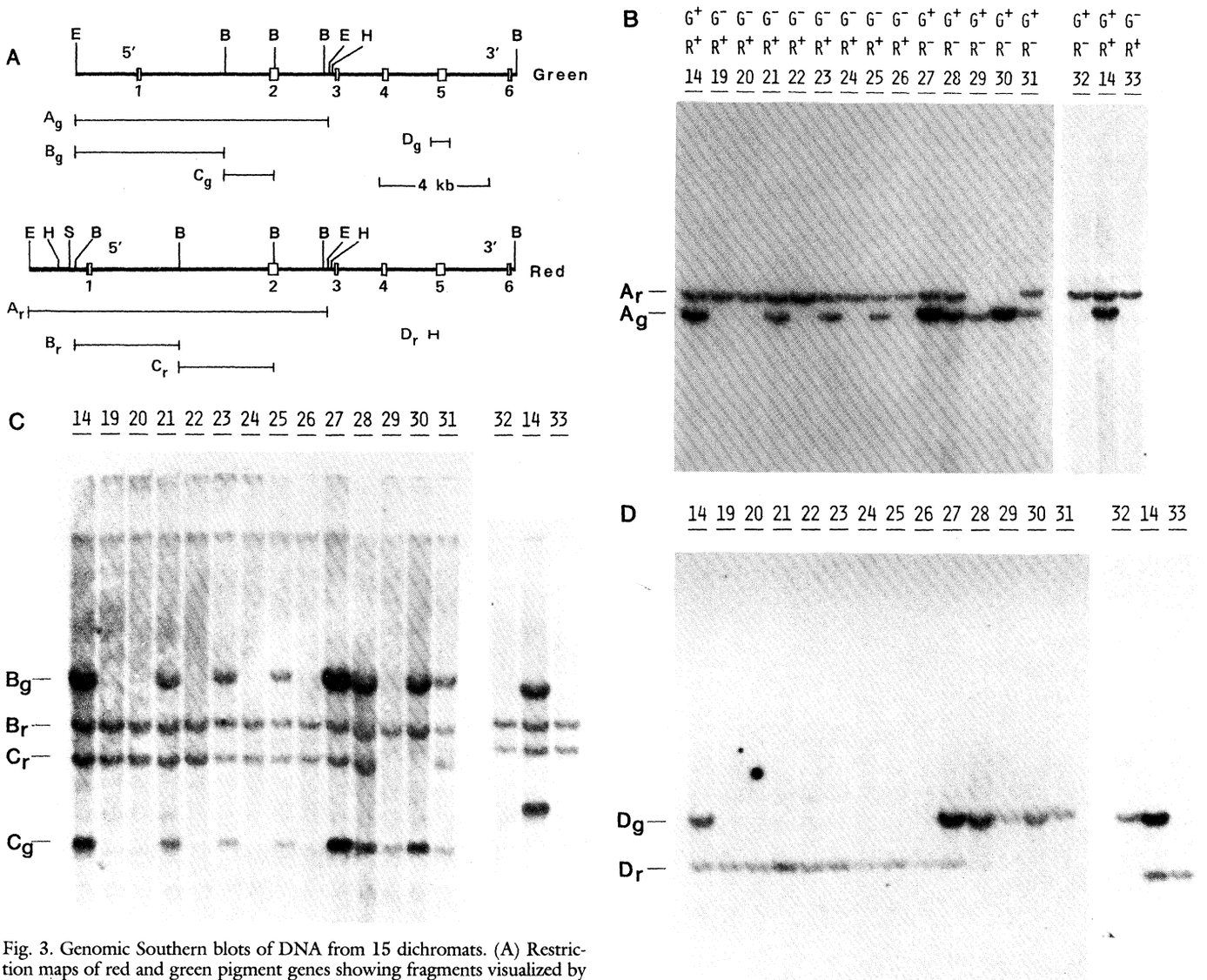


Fig. 3. Genomic Southern blots of DNA from 15 dichromats. (A) Restriction maps of red and green pigment genes showing fragments visualized by Southern blotting. The probes used to visualize these fragments are more than 98 percent identical to both genes. A_g and A_r are two Eco RI fragments derived from green and red pigment genes, respectively, and are visualized with a probe from exon 2. B_g and B_r, and C_g and C_r are fragments resulting from Bam HI and Eco RI double digestion and are visualized with a probe encompassing exon 1 and the 5′ half of exon 2. D_g and D_r are fragments resulting from Rsa I digestion which share a common left border but differ on their right borders because the green pigment gene lacks but the red pigment gene has an Rsa I site in the fifth exon. They are visualized with a probe from the 3′ end of the fourth intron. (B to D) Three pairs of genomic Southern blots of DNA from 15 dichromats and one color normal. Each number and phenotype refers to the individual whose DNA is in that lane and whose anomaloscope test data are shown in Fig. 2. (B) Eco RI, (C) Eco RI and Bam HI, (D) Rsa I. Approximately 10 μg of DNA, digested with the indicated enzymes, was placed on each lane. Filters were hybridized and washed under standard conditions (*18*) and exposed to preflashed x-ray film at −70°C with an intensifying screen.

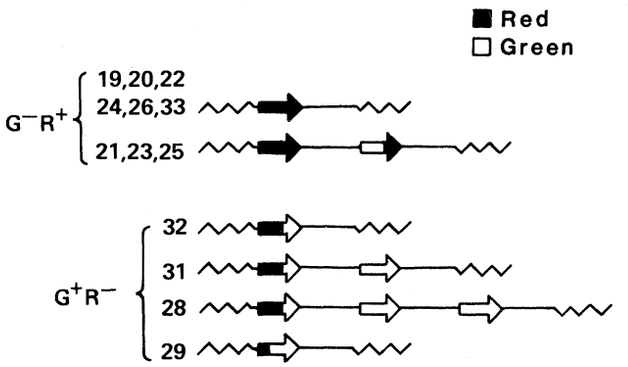


Fig. 4. Proposed arrangement of green and red pigment genes in dichromats. Each arrow represents a single gene: the base corresponds to the 5′ end and the tip to the 3′ end. Zigzag lines represent single-copy flanking DNA and thin lines represent homologous intergenic sequences. The genotypes and subject numbers represented by each diagram are at the left. Presented here, as an example, are the data and methods used to deduce the genotypes of subjects 21, 23, and 25. Peak areas were measured by scanning the autoradiographs shown in Fig. 3, B, C, and D, and ratios of these areas were calculated (Table 2). Subjects 21, 23, and 25 had A_g:A_r ratios of 1.10, 1.21, and 1.22, respectively; B_g:B_r ratios of 1.10, 1.69, and 1.35, respectively; C_g:C_r ratios of 0.69, 0.92, and 0.72, respectively; and D_g:D_r ratios of 0 for all three. For comparison, the peak area ratios corresponding to a 1:1 gene ratio have been calculated from the Southern blots of 18 color-normal males [shown in (*10*) figures 8 and 9]. These calculations show that the 18 color-normal males have a total of 18 red pigment genes and 37 green pigment genes and that the peak area ratios (mean ± SD) corresponding to a 1:1 gene ratio are as follows A_g:A_r, 0.97 ± 0.06; B_g:B_r, 2.00 ± 0.49; C_g:C_r, 0.88 ± 0.18; D_g:D_r, 1.24 ± 0.30. The A_g:A_r ratios cluster at nearly integral values because these two fragments appear to bind to the nitrocellulose and hybridize with nearly equal efficiencies. Fragments B_g and D_g appear to bind or hybridize better, respectively, than do the smaller B_r and D_r fragments. Therefore, the ratios B_g:B_r and D_g:D_r cluster at values greater than 1. Since subjects 21, 23, and 25 have no D_g with which to compare D_r, the copy number of D_r was estimated by normalizing each D_r peak area to the background smear present in that track and comparing this value to that of the color-normal standard. For subjects 21, 23, and 25 these values of D_r peak areas (arbitrary units) are: 3.43, 2.04, and 1.88, respectively. The areas of the smears (in different arbitrary units) are: 4.05, 2.50, and 1.75, respectively. Therefore, the normalized values of D_r are 0.85, 0.82, and 1.07, respectively. On the same blot the color normal control (subject 14), who has single copy of D_r, has a D_r peak area of 2.08 and a smear area of 3.85, giving a normalized D_r area of 0.54. As discussed in the text, the gene rearrangements appear to involve only homologous events. Hence, only complete genes are assumed to be present and the sum of the members of each fragment class—A, B, C, and D—are equal. Given these constraints, these data predict that subjects 21, 23, and 25 have one copy each of A_g, A_r, B_g, B_r, C_g, and C_r; two copies each of D_r; and no copies of D_g.

red and green pigment genes and the already documented propensity of the green pigment genes to vary in number among normal individuals (*10*).

By including in our analysis the relative intensities of the autoradiographic bands, we can calculate (using values from normal DNA as a standard) the stoichiometries of the various bands for each subject. From these stoichiometries and from the conclusion that only homologous events have occurred (see above), we can construct plausible models of the arrangements of these genes. Therefore, we measured peak areas and calculated ratios of peak areas for bands within a single track (A_g:A_r, B_g:B_r, C_g:C_r, and D_g:D_r) (Table 2); these data should be insensitive to variations in amount of DNA loaded per track and are the basis for our models. As a check on these data, we also estimated the amount of DNA loaded per track by scanning across the smear generated by reprobing the blots with labeled total human genomic DNA. [See (*10*); in some autoradiographs the background smear of hybridization produced by the pigment gene probe was sufficient for this purpose and reprobing was unnecessary.] These estimates of DNA loaded per track were used to normalize the measured peak areas (see legend to Fig. 4 for an example of these calculations). These models are not in all cases the only arrangements consistent with the data. In particular, the distance between genes and the relative order of the genes along the chromosome cannot be determined from these data. Ultimately, molecular cloning and DNA sequencing will be required to elucidate the precise structures of these variant genotypes.

Models for dichromacy. We consider first the dichromat genotypes. Quantitation of the hybridization patterns for the DNA from subjects 19, 20, 22, 24, 26, and 33 (all G^-R^+) indicates that they are identical within experimental error. All six subjects have a single red pigment gene and no green pigment gene (Fig. 4). We assume that these genotypes arose from a homologous but unequal exchange [such as the one in figure 10B in (*10*)]. In the absence of a green pigment gene those cells that were destined to become green cones may express the remaining red pigment gene by default. This general idea has been put forward in the past to account for the fact that dichromats have normal visual acuity (*13*) and an increase in the sensitivity of the unaffected mechanism [for example, increased green sensitivity in G^+R^- dichromats; (*14*)]. Subjects 21, 23, and 25, are also G^-R^+ and identical to one another within experimental error. These three subjects have one copy each of A_g and A_r, B_g and B_r, and C_g and C_r, two copies of D_r, and no D_g. Therefore, we propose that they have a single normal red pigment gene and a single hybrid gene in which the 5′ part of a green pigment gene (A_g, B_g, C_g) has been joined to the 3′ part of a red pigment gene (D_r) (Fig. 4). We further propose that the hybrid gene includes sufficient material derived from the red pigment gene that its spectral absorbance is identical or nearly identical to that of the red pigment.

Table 2. Ratios of Southern blot peak areas. For comparison the ratios that correspond to a 1:1 gene ratio are A_g:A_r, 0.97 ± 0.06; B_g:B_r, 2.00 ± 0.49; C_g:C_r, 0.88 ± 0.18; D_g:D_r, 1.24 ± 0.30 (see legend to Fig. 4). Symbols: inf, infinite, that is, the denominator is zero; *, probably too small due to Southern blot transfer artifact; **, probably too large due to Southern blot transfer artifact.

Subject genotype	Numbers	A_g:A_r	B_g:B_r	C_g:C_r	D_g:D_r
G^-R^+	19	0	0	0	0
G^-R^+	20	0	0	0	0
G^-R^+	21	1.10	1.10	0.69	0
G^-R^+	22	0	0	0	0
G^-R^+	23	1.21	1.69	0.92	0
G^-R^+	24	0	0	0	0
G^-R^+	25	1.22	1.35	0.76	0
G^-R^+	26	0	0	0	0
G^+R^-	27	6.08	5.40	3.77	5.87
G^+R^-	28	1.95	3.02	1.08*	inf
G^+R^-	29	inf	0	inf	inf
G^+R^-	30	inf	2.21	inf	inf
G^+R^-	31	0.93	1.50	1.07	inf
G^+R^-	32	0	0	0	inf
G^-R^+	33	0	0	0	0
$G'R^+$	34	3.97	6.76	1.50	0.33
$G'R^+$	35	2.22	4.07	1.83	0
$G'R^+$	36	4.46	6.96	4.66	0.71
$G''R^+$	37	4.42	8.87	3.91	1.99
$G''R^+$	38	3.54	5.20	1.82	1.04
$G''R^+$	39	2.00	3.71	2.86**	0.40
$G''R^+$	40	2.31	5.36	1.42	0.56
$G''R^+$	41	3.00	7.63	2.95	1.64
G^+R'	42	1.70	2.94	1.63	inf
G^+R''	43	4.97	7.89	3.39	inf

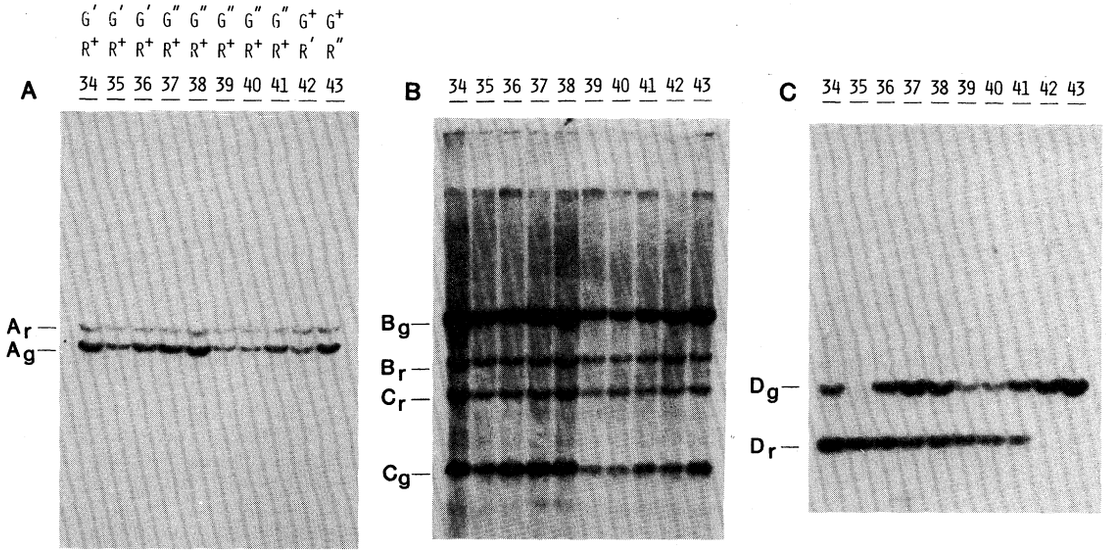


Fig. 5. Genomic Southern blots of DNA from anomalous trichromats. Each number and genotype refers to the individual whose DNA is loaded in that lane. (A) Eco RI digestion; (B) Bam HI and Eco RI double digestion; (C) Rsa I digestion. Fragment identities and experimental methods are described in the legend of Fig. 3A.

If pigment gene expression is controlled by 5′ proximal sequences, then the hybrid gene should be expressed in cells that were destined to become green cones. As a result the spectral sensitivity of these cells would be the same as that of the red cones.

As already mentioned the six G^+R^- genotypes are all different. Consider subjects 28, 31, and 32. Each has a single copy of A_r, B_r, and C_r; all lack D_r. They differ in their content of material derived from the green pigment gene: subject 32 has one copy of D_g and nothing else, subject 31 has two copies of D_g and one copy of A_g, B_g, and C_g, and subject 28 has three copies of D_g and two copies of A_g, B_g, and C_g. Models that account for these various stoichiometries are shown in Fig. 4. These three genotypes resemble one another in having one hybrid gene (5′ red–3′ green) and either zero, one, or two intact green pigment genes. We postulate that the hybrid gene produces a greenlike pigment in cells that would have become red cones.

Subject 29 (G^+R^-) has only one copy of fragments A_g, B_r, C_g, and D_g, and no other bands. Because the difference in size between A_r and A_g arises from a difference in the lengths of the first introns of the red and green pigment genes, these fragments do not serve as good markers for recombination events upstream of that intron. Instead B_r and B_g represent the most 5′ proximal landmarks which distinguish red from green pigment genes. Thus, subject 29 has a single hybrid gene in which only the part of the red pigment gene that is furthest toward the 5′ end has been retained (Fig. 4). Subject 30 (G^+R^-) is similar except that either one or two intact green pigment genes are also present. (The measured band intensities do not allow an unambigious assignment of stoichiometries for this subject.)

Subject 27 is the only G^+R^- subject who has all of the fragments corresponding to an intact red pigment gene (A_r, B_r, C_r, and D_r). At the same time he has more green pigment genes (either four or five) than we have seen in any normal subject (*10*). We are, at present, uncertain of the exact number and arrangement of his genes.

In summary, dichromasy caused by defects in the red or green mechanisms appears to be produced by various unequal exchanges or gene conversions. Different combinations of hybrid and normal genes can produce the same phenotype. We suggest that this heterogeneity may explain the observation that under very rigorous test conditions dichromats of a given type frequently do not accept each other's spectral matches (*15*).

Models for anomalous trichromacy. Ten anomalous trichromats were tested (Fig. 2), and their DNA was analyzed by Southern blotting (Fig. 5). In each case only those fragment sizes predicted from the normal restriction maps are seen. (This is also seen when the Eco RI digest is reprobed with a cDNA fragment encompassing exons 2 to 6.) Therefore we will consider only events involving homologous exchange. We consider first subjects 42 and 43, the former classified as G^+R' (R′, anomalous red sensitivity) and the latter G^+R'' (R″, extremely anomalous red sensitivity). Both subjects have one copy of A_r, B_r, and C_r, but lack D_r. They differ in that subject 42 has two copies of A_g, B_g, and C_g, and three copies of D_g, whereas subject 43 has approximately four copies of A_g, B_g, and C_g, and approximately five copies of D_g. Both subjects, therefore, have one copy of a hybrid gene (5′ red–3′ green) as well as a number of normal green pigment genes (Fig. 6). We predict that the red cones would in each case express the hybrid gene. The two different phenotypes could be caused by differences in the exact point of crossover, which might well determine the light-absorbing properties of the hybrid pigment.

In psychophysical studies of G^+R' and G^+R'' subjects (*16*), the anomalous red sensitivity curve was found in the interval between the normal red and green sensitivity curves. The decrease in color discrimination is attributed to the resultant decrease in the difference between red and green cone outputs. The shift of red cone sensitivity toward shorter wavelengths also alters the red-green ratio required to match a standard yellow. In extreme anomalous trichromacy the interval between normal green sensitivity and the short-wavelength-shifted red sensitivity is very small. This gives rise to large errors in color matching. The observation of hybrid genes,

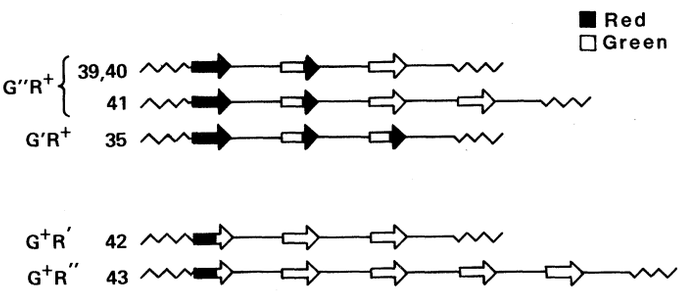


Fig. 6. Proposed arrangement of green and red pigment genes in anomalous trichromats. See legend to Fig. 4 for a description of the symbols.

which might plausibly encode pigments with spectral properties part way between those of the normal red and green pigments, fits well with these psychophysical data. The finding of approximately four green pigment genes in subject 43 is curious, but may not be relevant to the phenotype.

Four of eight $G'R^+$ (G', anomalous green sensitivity) and $G''R^+$ (G", extreme anomalous green sensitivity) (numbers 35, 39, 40, and 41) have a sufficiently small total number of genes that we could assign them unambiguous fragment stoichiometries. Subjects 39 and 40 have one copy of A_r, B_r, and C_r, two copies of D_r, two copies of A_g, B_g, and C_g, but only one copy of D_g. Subject 41 is similar, except one additional copy each of A_g, B_g, C_g, and D_g is superimposed. Subject 35 is unusual in having one copy of A_r, B_r, and C_r, three copies of D_r, two copies of A_g, B_g, and C_g, but none of D_g. The remaining four subjects (numbers 34, 36, 37, and 38) have a total of either four or five genes, including one or more hybrid genes, but quantitation of their hybridization band intensities do not permit an unambiguous assignment of fragment stoichiometries.

Our interpretation of these data for subjects 35, 39, 40, and 41 is shown in Fig. 6. Each has a single intact red pigment gene and some combination of intact or hybrid (or both) green pigment genes. In each case the hybrids are 5' green–3' red. If we suppose that green cones express all genes with 5' sequences derived from a green pigment gene, then we would predict the production in those cones of a mixture of normal green or hybrid pigments (or both). The action spectrum of such a cell would then be shifted to the mean of the absorption spectra of its pigments. Psychophysical experiments indicate that G' and G" defects result, respectively, from smaller or greater shifts of green sensitivity toward the red sensitivity curve (*16*). If the shifted green sensitivity curve results from averaging the sensitivity curves of two or more pigments, then we might expect $G'R^+$ genotypes to have more normal green pigment genes than $G''R^+$ genotypes. In our sampling of three $G'R^+$ and five $G''R^+$ subjects we do not see this pattern; in fact, we see a small bias toward the reverse. It is therefore likely that the exact nature of the hybrid pigment is important and varies from subject to subject.

Although we could not deduce unambiguous stoichiometries for all eight green anomalous subjects, together they define a consistent pattern. All eight have one intact red pigment gene, as evidenced by the presence of single copies of A_r, B_r, and C_r, but all eight have at least two copies of D_r. Moreover, as a class they have on average more total genes than do normals (Fig. 7). We showed in (*10*) that normals have on average two copies of A_g, B_g, C_g, and D_g for each copy of A_r, B_r, C_r, and D_r, and therefore an average total of three genes. Green anomalous subjects have on average three copies of A_g, B_g, and C_g for each copy of A_r, B_r, and C_r giving an average total of four genes. In contrast, the ratio of D_g to D_r in green anomalous subjects is significantly less than that of normals. Therefore green anomalous subjects must have hybrid genes with the structure 5' green–3' red.

How might the observed green anomalous genotypes be created? The required event must produce both an increase in gene number and create a 5' green–3' red hybrid gene. An intragenic recombination between a red and a green pigment gene (Fig. 8) is such an event. Since the 3' end of the red pigment gene is always accompanied by those green pigment genes distal to it, the total number of genes on the green anomalous chromosome will increase. In contrast, a gene conversion would not alter the total number of genes. The other product of this event should have on average fewer total genes and confer on a male a G^+R^-, G^+R', or G^+R'' phenotype.

Frequencies of color variant types. The frequencies of different anomalies in the red and green mechanisms have been accurately measured among Caucasians. We have calculated means and standard deviations of these values obtained from eight large population studies (*17*). The frequency of all types of red and green variant alleles is 8.08 ± 0.37 percent. Among the variant alleles 15.5 ± 5.2 percent are G^-R^+, 15.6 ± 5.2 percent are G^+R^-, 56.2 ± 4.6 percent are $G'R^+$ or $G''R^+$, and 12.5 ± 4.0 percent are G^+R' or G^+R''. (Anomalous and extreme anomalous trichromats are counted together for this analysis.) The most striking feature of this distribution is the asymmetry in anomalous trichromat frequencies.

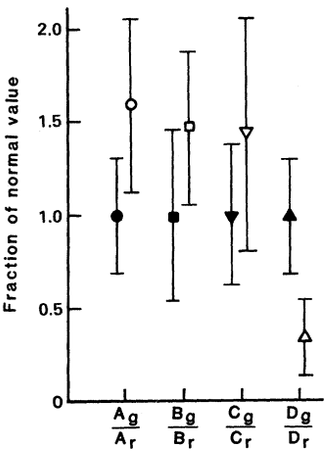


Fig. 7. Ratios of Southern blot band intensities: comparison of $G'R^+$ and $G''R^+$ to G^+R^+. Each point indicates the mean and standard deviation of the indicated ratio of fragments derived from the green pigment gene to those derived from the red pigment gene: A_g:A_r, B_g:B_r; C_g:C_r, and D_g:D_r (Fig. 3A). These ratios are plotted as the fraction of the corresponding ratios for the 18 color normals described in (*10*). For the color-normal data (closed symbols) the values, therefore, all center at 1.0. Data for the eight $G'R^+$ and $G''R^+$ subjects are shown by open symbols. The large standard deviations reflect the inhomogeneity of each population.

The models of unequal recombination just presented can account qualitatively for this asymmetry. The unequal intragenic exchanges (Fig. 8) illustrate a general feature of all such events. The recombination product carrying a 5' green–3' red hybrid gene also carries one normal red pigment gene and at least one normal green pigment gene. We propose that this hybrid gene would be expressed in the green cones together with the normal green pigment gene or genes. This gene arrangement therefore confers a $G'R^+$ or $G''R^+$ phenotype. In contrast, the other recombination product carries either a single 5' red–3' green hybrid gene (Fig. 8B) or that hybrid plus one

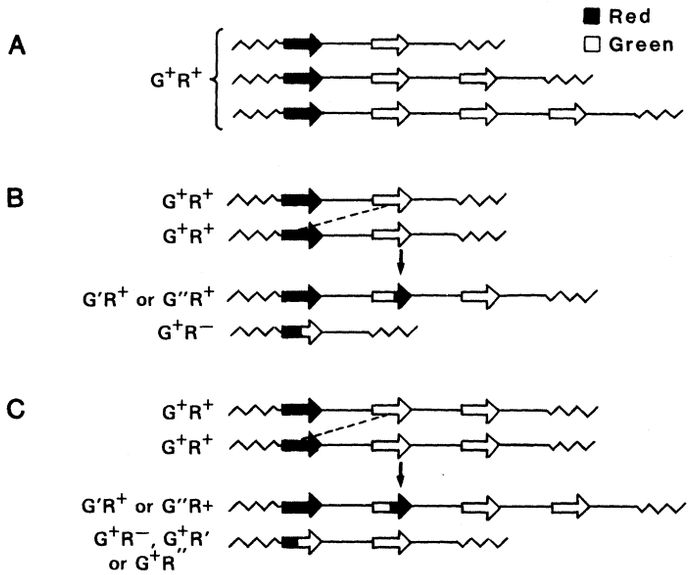


Fig. 8. Proposed products of unequal intragenic recombination between red and green pigment genes. At the left are the corresponding phenotypes. See legend to Fig. 4 for further explanation of symbols. (A) Proposed genotypes for a sampling of 18 color-normal males. (B) Recombination event in which one product retains only a 5' red–3' green hybrid gene. (C) Recombination event in which both products retain intact green pigment genes.

or more normal green pigment genes (Fig. 8C). This hybrid is presumed to be expressed in the red cones. In the former case it confers a G^+R^- phenotype, in the latter case it confers either G^+R^-, G^+R', or G^+R'' phenotypes, depending presumably on the exact site of intragenic recombination.

Herein lies the asymmetry. This mechanism produces $G'R^+$ and $G''R^+$ in excess over G^+R' and G^+R'', the difference being the number of G^+R^-. Moreover, if the fitness of dichromats is lower than that of anomalous trichromats, then $G'R^+$ and $G''R^+$ genotypes will accumulate to levels greater than the sum of G^+R', G^+R'', and G^+R^- genotypes.

Finally, G^-R^+ genotypes are produced by at least two different mechanisms. Unequal intergenic recombination, resulting in a change in the number of green pigment genes, gives rise to either two G^+R^+ products or to one G^+R^+ product and one G^-R^+ product, the latter produced by the complete loss of all green pigment genes [for example, subjects 19, 20, 22, 24, 26, and 33 shown in Fig. 4; see also figure 10B in (*10*)]. In contrast, those G^-R^+ genotypes containing 5′ green–3′ red hybrid genes (subjects 21, 23, and 25) (Fig. 4) cannot be produced from the normal genotypes (Fig. 8A) by a single recombination event. After such an event, the 3′ part of each 5′ green–3′ red hybrid gene remains linked to one or more downstream green pigment genes. These three subjects lack an intact green pigment gene, and therefore their genotypes are derived from either a gene conversion event or a sequence of two or more unequal recombinations.

These experiments verify the long-standing hypothesis that the loci responsible for inherited red-green color blindness are the genes encoding the red and green visual pigments. A test of the analogous hypothesis regarding blue color blindness and the blue pigment gene is now also possible. The arrangement of green and red pigment genes observed in color variant subjects reveals a unifying theme: in at least 24 out of 25 cases, either unequal homologous recombination or gene conversion has produced an arrangement of pigment genes different from those arrangements observed in color normals. This propensity for homologous events is probably a consequence of the proximity and high degree of sequence homology between red and green pigment genes. It is probably responsible, at least in part, for the high frequency (8 percent among Caucasian males) of red-green color blindness. Other genetic events, such as point mutation and nonhomologous rearrangement, probably occur at a far lower frequency. The finding of hybrid genes in anomalous trichromats fits well with the observation that anomalous green and red sensitivities lie in the interval between normal green and red sensitivities. In contrast, point mutation would not be expected to preferentially produce shifts toward either long or short wavelengths.

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12a. Abbreviations: G^+R^+, normal color vision; $G'R^+$, anomalous green sensitivity (deuteranomaly); $G''R^+$, extreme anomalous green sensitivity (extreme deuteranomaly); G^-R^+, absent green sensitivity (deuteranopia); G^+R', anomalous red sensitivity (protanomaly); G^+R'', extreme anomalous red sensitivity (extreme protanomaly); and G^+R^-, absent red sensitivity (protanopia).

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