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Genetic Heterogeneity among Blue-Cone Monochromats

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Summary

Thirty-three unrelated subjects with blue-cone monochromacy or closely related variants of blue-cone monochromacy were examined for rearrangements in the tandem array of genes encoding the red- and green-cone pigments. In 24 subjects, eight genotypes were found that would be predicted to eliminate the function of all of the genes within the array. As observed in an earlier study, the rearrangements involve either deletion of a locus control region adjacent to the gene array or loss of function via homologous recombination and point mutation. One inactivating mutation, Cys^{203}-to-Arg, was found in 15 probands who carry single genes and in both visual pigment genes in one subject whose array has two genes. This mutation was also found in at least one of the visual pigment genes in 1 subject whose array has multiple genes and in 2 of 321 control subjects, suggesting that preexisting Cys^{203}-to-Arg mutations constitute a reservoir of chromosomes that are predisposed to generate blue-cone–monochromat genotypes by unequal homologous recombination and/or gene conversion. Two other point mutations were identified: (*a*) Arg^{247}-to-Ter in one subject with a single red-pigment gene and (*b*) Pro^{307}-to-Leu in one subject with a single 5′ red–3′ green hybrid gene. The observed heterogeneity of genotypes points to the existence of multiple one- and two-step mutational pathways to blue-cone monochromacy.

Introduction

Normal human color vision involves a comparison of the relative extents of excitation of three classes of cone photoreceptors. The most common forms of inherited color-vision deficiency involve an alteration in one of the three cone systems (Boynton 1979). These alterations cause a loss of cone function (dichromacy) or a shift in the spectral sensitivity of one of the cone visual pigments (anomalous trichromacy). Dichromacy and anomalous trichromacy affecting the red and green cones arise from rearrangements in the genes encoding the red- and green-sensitive cone pigments, located on the long arm of the X chromosome (Nathans et al. 1986*a*; Deeb et al. 1992). These highly homologous genes reside in a head-to-tail tandem array, an arrangement that predisposes them to unequal intra- and intergenic homologous recombination (Nathans et al. 1986*b*; Vollrath et al. 1988; Feil et al. 1990). Greater than 95% of color-vision variation in the red- and green-cone systems arises from gene rearrangements of this type.

Blue-cone monochromacy is a rare X-linked condi-

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tion characterized by an absence of red- and green-cone function (Blackwell and Blackwell 1961; Alpern 1974; Pokorny et al. 1979). Under photopic conditions, vision is mediated exclusively by blue cones (Hess et al. 1989). Under these conditions, the blue-cone monochromat experiences a colorless world: without a comparison between different photoreceptor classes the visual system cannot resolve the independent variables of wavelength composition and light intensity. It is interesting that, at intermediate light levels, blue-cone monochromats show a weak interaction between rod and blue-cone signals which permits crude hue discrimination (Reitner et al. 1991). Blue-cone monochromacy is typically associated with low acuity, in the range 0.3–0.1, which is exacerbated in some individuals by a progressive degeneration of the central retina, the region most enriched in cone photoreceptors (Fleischman and O'Donnell 1981; Nathans et al. 1989).

In earlier work we identified eight different rearrangements in the red- and green-pigment gene array in blue-cone monochromats from 12 families (Nathans et al. 1989). The rearrangements were found to be of two types. In one type, unequal homologous recombination had reduced to one the number of visual pigment genes in the array. In three families with this type of rearrangement, a point mutation was observed which disrupted a conserved disulfide bond by replacing Cys^{203} with Arg (C203R; amino acid substitutions are referred to by the identity of the wild-type residue, abbreviated by using the single-letter amino acid designation, followed by the codon number, followed by the introduced residue). In the second type of rearrangement, six different deletions ranging in size from 0.6 kb to 55 kb were found in, or adjacent to, otherwise typical gene arrays. All of these deletions encompass a common region between 3.1 kb and 3.7 kb 5′ of the array. Recent experiments in which sequences 5′ of the red- and green-pigment gene array direct expression of a beta-galactosidase reporter gene in transgenic mice indicate that the region between 3.1 kb and 3.7 kb 5′ of the array functions as an essential activator of cone-specific gene expression (Wang et al. 1992).

The present paper describes a molecular genetic analysis of 33 new families with blue-cone monochromacy or closely related variants of blue-cone monochromacy. One of the goals of this study is to determine the range and relative frequencies of genetic events responsible for this disorder. The molecular heterogeneity reported here suggests that some of the clinical heterogeneity among blue-cone monochromats may have a genetic basis.

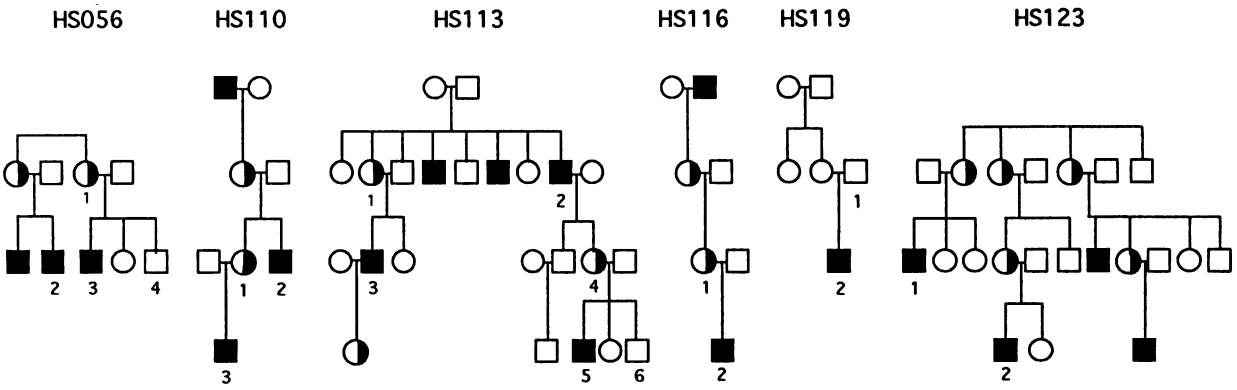
Subjects, Material, and Methods

Human Subjects

The diagnosis of blue-cone monochromacy was based on either a history of severe congenital color-blindness and a pedigree consistent with an X-linked mode of inheritance (fig. 1) or, in the absence of a conclusive pattern of inheritance, psychophysical data indicating the presence of blue-cone function and the absence of red- and green-cone function. Most subjects also have reduced acuity, some degree of photophobia, and a history of nystagmus in childhood. In those subjects who have undergone more extensive psychophysical testing, photopic threshold measurements indicate an absence of red- and green-cone function and the presence of blue-cone function. For some subjects, clinical and psychophysical data have been published elsewhere, as follows: in the family of HS123, subjects 1 and 2 correspond to III-1 and IV-1, respectively, in Pagon et al. (1987); in the family of HS129, subjects 1, 2, 3, and 4 correspond to III-7, either III-8 or III-9, III-22, and IV-18, respectively, in Fleischman and O'Donnell (1981); HS1075 and HS1188 correspond to BTW and HH, respectively, in Hansen (1979); HS1066, HS1067, HS1068, HS1077, and HS1078 correspond to KS, MP, PS, SB, and FB, respectively, in Hess et al. (1989) and Reitner et al. (1991); and the families of HS1580, HS1581, and HS1582 correspond to families 41, 47, and 79, respectively, in Andreasson and Tornquist (1991). Control samples were obtained from male participants in a study unrelated to vision and from cadets at the United States Air Force Academy. Proband HS129 is African-American; all other probands are of European descent. The control subjects were sampled from the U.S. population and are predominantly Caucasian.

DNA Collection and Analysis

Venous blood samples (5–20 cc) were obtained from each subject and were processed as described elsewhere (Sung et al. 1991*a*). Southern blot analysis of the red- and green-pigment gene array was performed with the following combinations of restriction digests and probes: to visualize fragment Z, *Hin*dIII digests were probed with an 800-bp *Bam*HI-*Hin*dIII partial-digest fragment located between 8.0 kb and 8.8 kb 5′ of the red-pigment gene transcription start site (Nathans et al. 1989); to visualize fragments B_g, B_r, C_g, and C_r (subscripts "g" and "r" refer to fragments derived from the green- and red-pigment genes, respectively), *Eco*RI-*Bam*HI double digests first were probed with a 300-bp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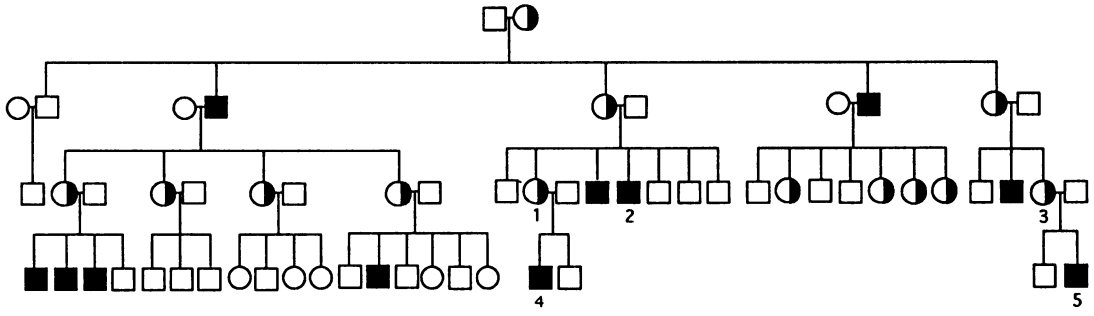
HS113
HS116
HS119
HS123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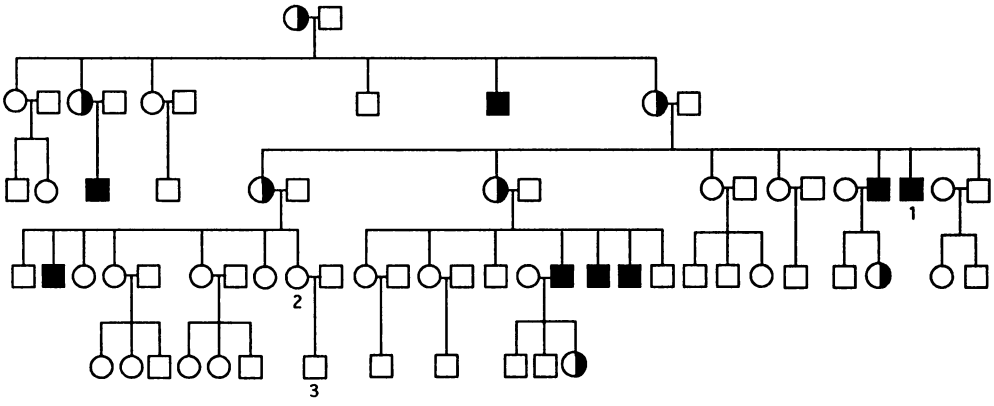
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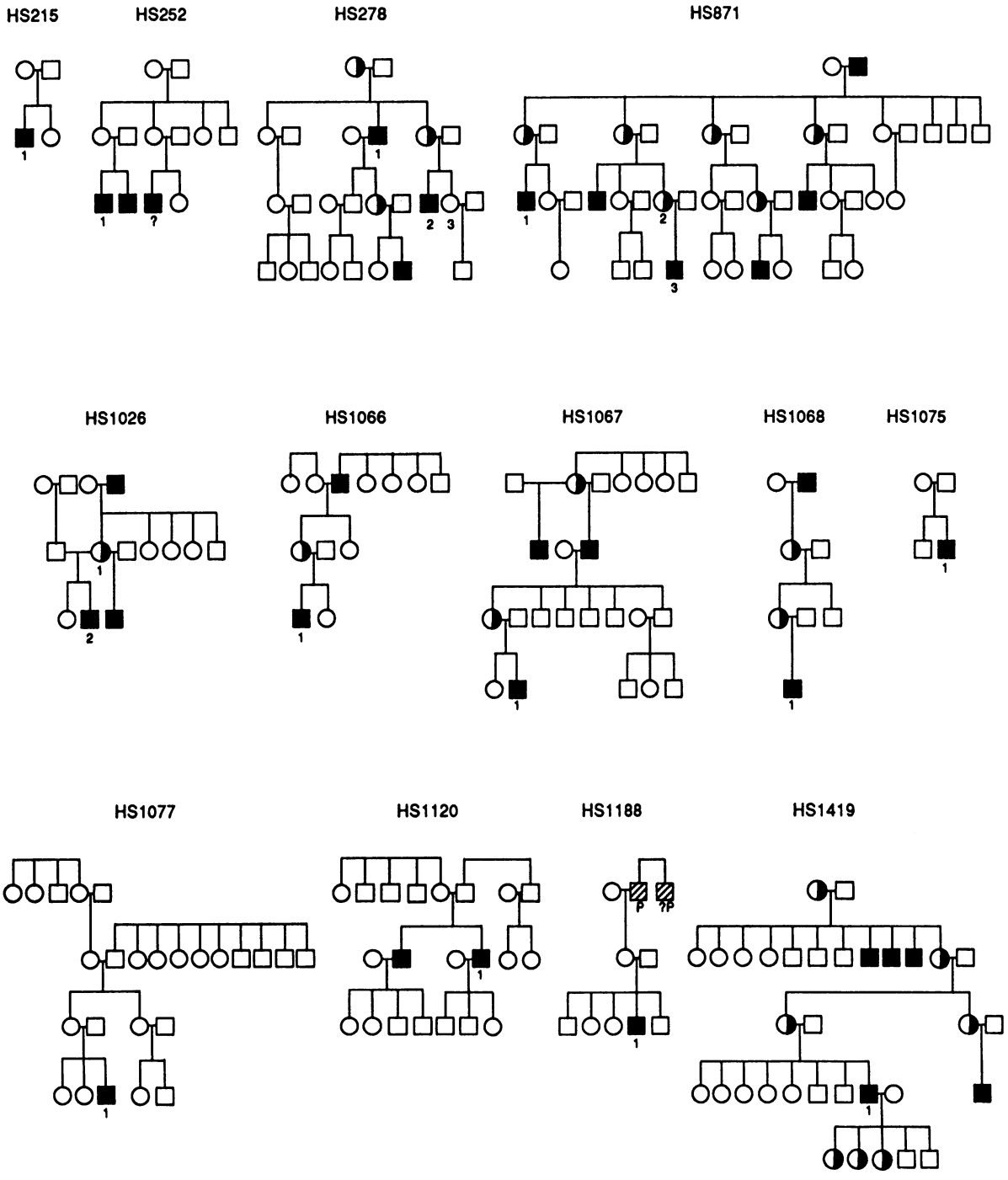
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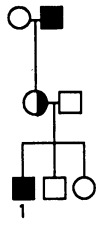
HS1556



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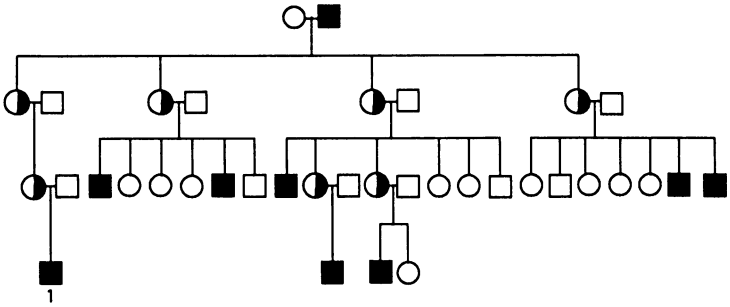

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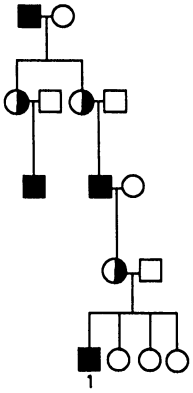

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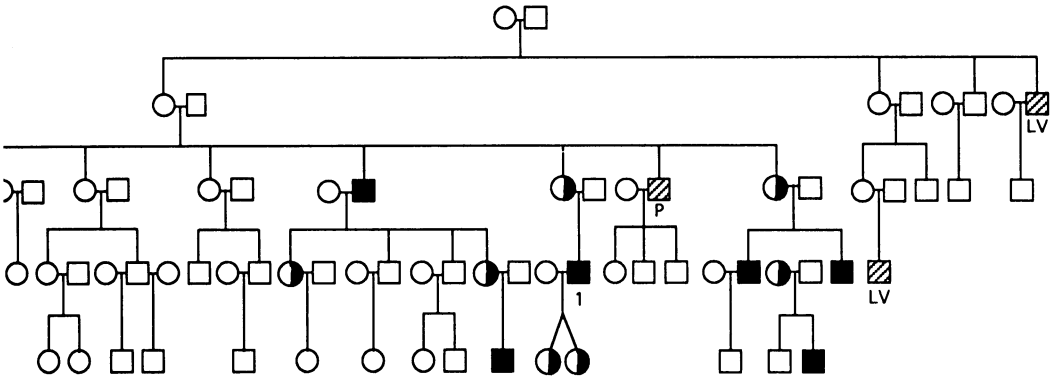

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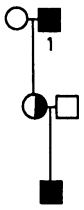
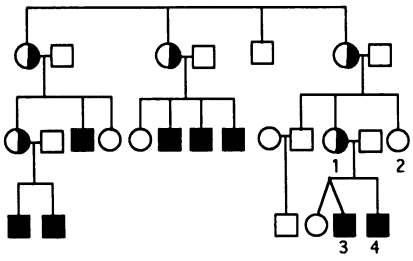


HS1770



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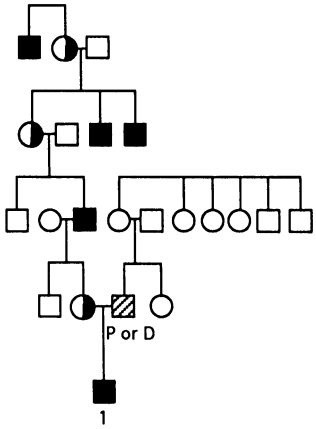

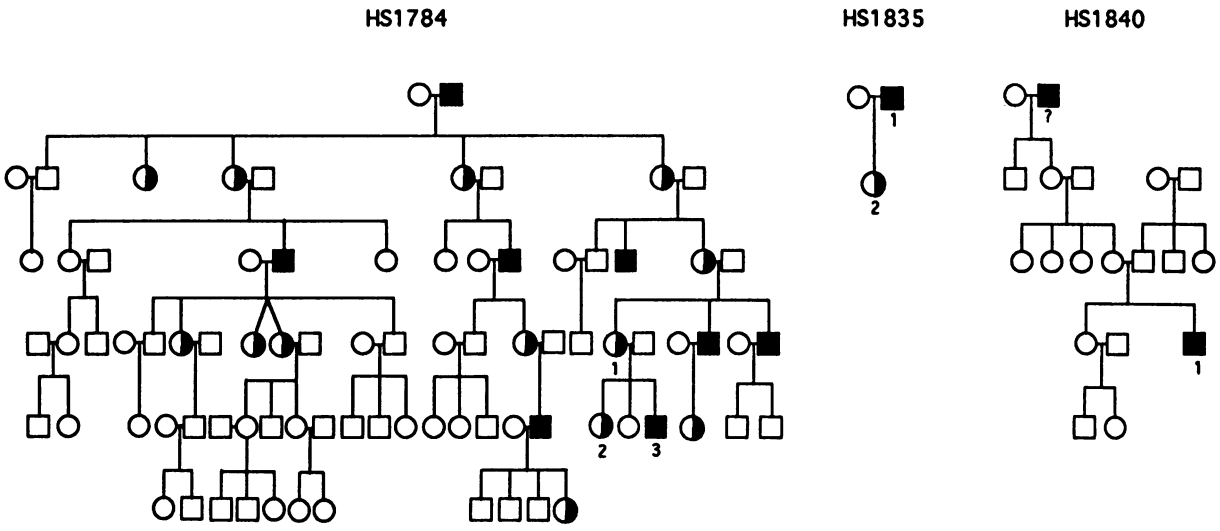

Figure 1 Pedigrees of blue-cone monochromat families. Blackened symbols denote affected subjects; half-blackened symbols denote obligate carriers; hatched symbols denote variant color vision; "P" denotes "protan"; "D" denotes "deutan"; "LV" denotes "low visual acuity"; and a question mark (?) denotes uncertain diagnosis. In some sibships, the birth order is not known. The "HS" number above each pedigree refers to the proband in that pedigree. Subjects analyzed in this study are marked by a number on the pedigree diagram; the proband's number in the pedigree is indicated in parentheses in table 2.

*Eco*RI-*Bam*HI fragment from red-pigment cDNA clone hs7 encompassing both exon 1 and the 5′ half of exon 2 (Nathans et al. 1986*b*) and then, to visualize fragments E_g, E_r, F_g, and F_r, were reprobed with a 900-bp *Eco*RI-*Bam*HI fragment from red-pigment cDNA clone hs7 encompassing both the 3′ half of exon 2 and exons 3–6; and to visualize fragments D_g and D_r, *Rsa*I digests were probed with a 450-bp fragment (created by deletion mutagenesis) located in intron 4 between 300 bp and 750 bp 5′ of exon 5.

The red- and green-pigment gene promoter region and six exons were amplified by PCR using primers located immediately 5′ and 3′ of the transcription unit and within each intron approximately 50 bp from the intron-exon junction (table 1 and fig. 2). Two primer pairs were used to amplify the region between 3.1 kb and 3.7 kb 5′ of the red- and green-pigment gene array in two partially overlapping segments of 320 bp and 340 bp (table 1; Nathans et al. 1989). The products of these PCR reactions were analyzed for small rearrangements by electrophoresis on a 4% neutral acrylamide gel. PCR products derived from exon 4 were tested for the presence of the C203R mutation by cleavage with *Bst*UI, followed by electrophoresis on either a 4% neutral acrylamide gel or a 2% agarose gel. This mutation converts the sequence 5′ TGCG to 5′ CGCG and thereby introduces a single *Bst*UI cleavage site into the PCR product (Nathans et al. 1989). For some subjects the presence or absence of an *Rsa*I site in exon 5 was determined by cleavage of PCR products rather than by Southern blot as described above. For sequencing of PCR products, the amplified DNA segments were first subcloned into pUC118 or pUC119. To control for errors in the PCR, sequences were obtained by using templates prepared from either pools of several hundred recombinants or two or more independent recombinants.

For allele-specific oligonucleotide hybridization, an aliquot of each PCR reaction mixture (approximately 100 ng of DNA) was denatured and deposited onto a GeneScreen Plus filter (Du Pont) under gentle vacuum. For each mutant, a ^{32}P-labeled 13-mer probe, the sequence of which is identical to, and centered about, the mutant sequence, was annealed in 4 × SSC, 100 μg salmon sperm DNA/ml, 5 × Denhardt's solution, 0.05% SDS for 5 h at 23°C. The filters were washed in 0.4 × SSC, 0.1% SDS at temperatures between 25°C and 40°C to optimize the difference between hybridization to wild-type and mutant sequences.

To clone the HS1068 deletion breakpoint, 50 μg of genomic DNA was cleaved with *Hin*dIII, and fragments between 3.5 kb and 4.5 kb in length were purified by preparative agarose gel electrophoresis and ligated to *Hin*dIII-digested lambda 590 vector DNA (Murray et al. 1977). Recombinant phage were identified by hybridization with the 800-bp *Bam*HI-*Hin*dIII

Table 1

Primers Used for Amplification of Red- and Green-Pigment Gene Sequences

Name	Sequence
JN-278	5′ ACGTAGAATTCGGATGGCTGGCCAGCCTTGT 3′
JN-279	5′ GCATGGAATTCACAAACCCCACCCGAGTTAG 3′
JN-280	5′ GCATGAAGCTTAGGAGTCTCAGTGGACTCAT 3′
JN-283	5′ ACGTAGAATTCAATGACCCAGCTCAGTGCAA 3′
JN-284	5′ GCATGAAGCTTGTGGGGGCTGGCACACGTGT 3′
JN-285	5′ GCATGAAGCTTCCGCCACTGCTCCCGCTCCT 3′
JN-286	5′ ACGTGAAGCTTCAGGTGGGGCCATCACTGCA 3′
JN-287	5′ ACGTGGAATTCCCTCTCCTCCTCCCCACAAC 3′
JN-289	5′ GTCTAAGCAGGACAGTGGGAAGCTTTGCTT 3′
JN-290	5′ ACGTGAAGCTTGTGAATGAGTGGTTTCCGCC 3′
JN-291	5′ ACGTGGAATTCGGTATAGACAGGCGGTGCTG 3′
JN-294	5′ ACGTGAAGCTTGATAAATTACATTTATTTTACAGGG 3′
JN-295	5′ ACGTGGAATTCAGGGAAGGCTCGGGCACGTA 3′
JN-379	5′ ACGTAAAAGCTTTAAGGTCACAGAGTCTGACC 3′
JN-422	5′ ACGTCGAATTCGGTGGGAGGAGGAGGTCTAA 3′
JN-423	5′ ACGTCAAGCTTGGTGGCCCCCAGTGCAGCC 3′
JN-454	5′ GCATGGAATTCCAACCCCCGACTCACTAT 3′
JN-455	5′ GCATGAAGCTTGAGTGGGATCTGCTGATG 3′

NOTE.—For amplifying the six exons, primers were chosen which encompass regions of 100% identity between red- and green-pigment genes. For primer locations, see fig. 2.

partial-digest fragment used to visualize fragment Z (see above). The endpoints of the deletion were mapped to a resolution of 100–200 bp by the following two methods: (*a*) ^{32}P labeling of the corresponding ends of wild-type and HS1068 breakpoint restriction fragments followed by partial digestion with restriction enzymes that recognize 4-bp sites (Smith and Bernstiel 1976) and (*b*) Southern blot hybridization to restriction digests of cloned wild-type genomic segments by using the HS1068 breakpoint clone as a probe (Nathans et al. 1986*b*).

Results

Thirty-three unrelated males with blue-cone monochromacy or close variants of blue-cone monochromacy were recruited from the United States, Canada, Germany, Denmark, Sweden, and Norway. Thirty-one of the subjects met the diagnostic criteria described under Subjects, Material, and Methods. We also included in this study two simplex subjects (HS119 and HS215) with less certain diagnoses, under the assumption that these individuals might be blue-cone monochromats. In these two cases the mutant allele could have been either inherited through the female lineage for several generations or generated recently through new mutation. Pedigrees were available for 31 families (fig. 1). In all of the pedigrees the pattern of inheritance is consistent with X-linkage; however, in only 21 of the pedigrees is that mode of inheritance unequivocally established by the pattern of affected relatives.

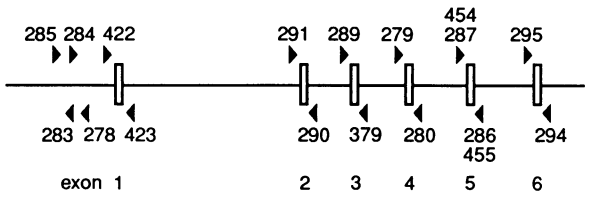

Figure 2 Locations of primers used for PCR amplification of red- and green-pigment gene sequences. Primer designations are as in table 1.

Large-Scale Rearrangements within the Red- and Green-Pigment Gene Array

As a first step in analyzing the red- and green-pigment genes for rearrangements associated with blue-cone monochromacy, we assessed the structure of this locus by Southern blotting using hybridization probes to visualize restriction fragments B_g, B_r, C_g, C_r, D_g, D_r, E_g, E_r, F_g, F_r, and Z (table 2 and fig. 3). The wild-type arrangement consists of a head-to-tail tandem array of two or more repeat units of 39 kb that are 98% identical at the DNA level (Nathans et al. 1986*b*; Vollrath et al. 1988;

Table 2

Summary of Southern Blot and PCR Analysis for Each Proband

Family[a]	Restriction Fragment[b] (Size of Wild-Type Fragment [kb])									Codon 203[c]
	B_g (5.4)	B_r (4.1)	C_g (1.8)	C_r (3.3)	D_g (1.36)	D_r (0.84)	E_g, E_r (1.8)	F_g, F_r (6.5)	Z (7.2)	
1 HS056 (2)	−	+	−	+	−	+	+	+	+	Arg
2 HS110 (3)	+	+	+	+	+	+	+	+	+	Cys
3 HS113 (5)	+	+	+	+	+	+	+	+	+	Cys
4 HS116 (2)	+	+	+	+	−	+	+	+	+	Cys
5 HS119 (2)	+	+	+	+	+	+	+	+	+	Cys
6 HS123 (1)	+	+	+	+	+	−	+	+	+	Cys + Arg
7 HS129 (4)	+	−	+	+	+	+	+	+	11[d]	
8 HS130 (1)	+	−	+	+	+	+	+	+	11[d]	
9 HS215	−	+	−	+	+	−	+	+	+	Arg
10 HS252	+	+	+	+	+	+	+	+	+	Cys
11 HS278 (2)	+	+	+	+	+	+	+	+	4.5[d]	
12 HS871 (3)	−	+	−	+	+	−	+	+	+	Cys
13 HS1026 (2)	−	+	−	+	+	−	+	+	+	Arg
14 HS1066	−	+	−	+	+	−	+	+	+	Arg
15 HS1067	−	+	−	+	−	+	+	+	+	Cys
16 HS1068	+	−	+	+	+	+	+	+	4[d]	
17 HS1075	−	+	−	+	+	−	+	+	+	Arg
18 HS1077	−	+	−	+	+	−	+	+	+	Arg
19 HS1078	−	+	−	+	+	−	+	+	+	Arg
20 HS1120	+	+	+	+	+	+	+	+	+	Cys
21 HS1188	−	+	−	+	+	−	+	+	+	Cys
22 HS1419	−	+	−	+	+	−	+	+	+	Arg
23 HS1556	−	+	+	−	+	−	+	+	+	Cys
24 HS1580	−	+	−	+	+	−	+	+	+	Arg
25 HS1581	−	+	−	+	+	−	+	+	+	Arg
26 HS1582	−	+	−	+	+	−	+	+	+	Arg
27 HS1751	−	+	−	+	+	−	+	+	+	Arg
28 HS1752	−	+	−	+	+	−	+	+	+	Arg
29 HS1770 (3)	+	+	+	+	+	+	+	+	+	Arg
30 HS1773	+	−	+	+	+	+	+	+	11[d]	
31 HS1784 (3)	+	−	+	+	+	+	+	+	11[d]	
32 HS1835 (1)	−	+	−	+	+	−	+	+	+	Arg
33 HS1840 (1)	−	+	−	+	+	−	+	+	+	Arg

[a] No. in parentheses indicates the position of the proband in the pedigree shown in fig. 1, for those families with more than one participant.

[b] For map positions, see fig. 3. For each restriction fragment, a plus sign (+) denotes presence of a hybridizing fragment of wild-type mobility, and a minus sign (−) denotes absence of a hybridizing fragment of wild-type mobility.

[c] Presence at position 203 of either cysteine or arginine (or both, in those subjects with two or more genes), for subjects with a fragment Z of normal size.

[d] Size of fragment (in kb).

Feil et al. 1990). Each 39-kb repeat unit contains a 15-kb transcription unit, the 5′ end of which abuts one edge of the unit. The red-pigment gene lies at the 5′ end of the entire array and is therefore adjacent to unique flanking DNA. In this study, all Southern blot probes and PCR primers that derive from within the repeat units hybridize efficiently to both red- and green-pigment genes.

Among the 33 probands, nine different Southern blot patterns were observed (table 2 and fig. 4). One group of subjects (HS129, HS130, HS278, HS1068, HS1773, and HS1784) carries DNA rearrangements that delete sequences 5′ of the red- and green-pigment genes. In four of these DNA samples (HS129, HS130, HS1773, and HS1784), the novel junction fragment generated by *Hin*dIII cleavage is indistinguishable in its

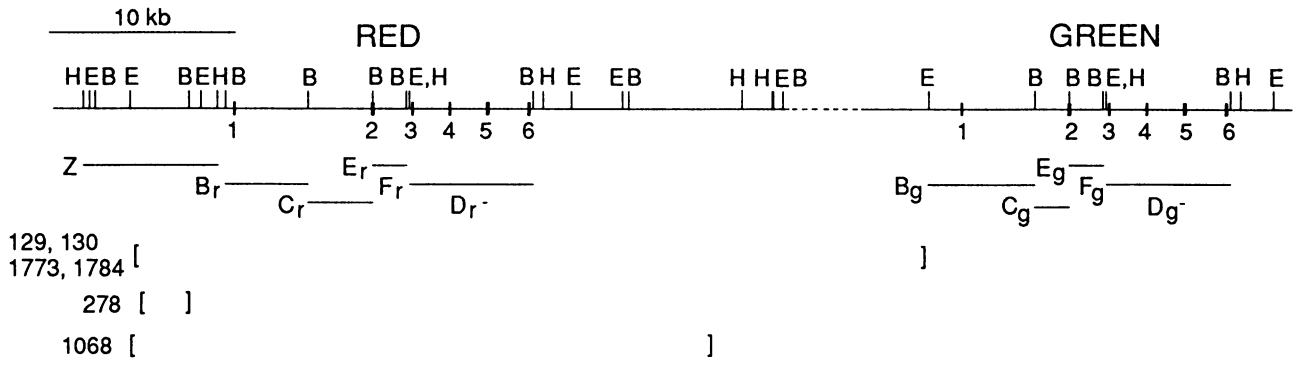

Figure 3 Restriction map of the human red- and green-pigment gene array. *Top,* Wild-type array showing the locations of red- and green-pigment gene exons 1–6. "B" = *Bam*HI; "E" = *Eco*RI; and "H" = *Hin*dIII. The number of green-pigment genes varies among individuals with normal color vision. *Middle,* Restriction fragments analyzed by Southern blot hybridization. Subscripts "r" or "g" refer to the gene—red or green pigment, respectively—from which the fragment originates. Fragment Z derives from unique 5′ flanking DNA. For fragment lengths, see table 2; fragments E_g and E_r are the same length, and fragments F_g and F_r are the same length. *Bottom,* Nonhomologous deletions responsible for blue-cone monochromacy. Numbers indicate the probands who carry each deletion.

mobility from that of a previously characterized blue-cone monochromat with a 41-kb deletion (HS107; Nathans et al. 1989) as determined by Southern blotting of *Hin*dIII-cut samples loaded on adjacent gel lanes. Identical patterns of restriction fragments B_g, B_r, C_g, C_r, D_g, and D_r were also observed. An analogous identity was observed between the Southern blot patterns of HS278 and that of a second previously characterized blue-cone monochromat with a 2.5-kb deletion (HS106; Nathans et al. 1989). Southern blotting of DNA from subject HS1068 revealed a novel deletion that removes sequences both within and upstream of the red-pigment gene. The 4-kb deletion breakpoint fragment generated from this subject's DNA by digestion with *Hin*dIII was cloned, restriction mapped, and used as a hybridization probe to restriction digests of genomic clones from the red- and green-pigment gene array. Alignment of the restriction map of the deletion breakpoint clone with that of a wild-type array shows that HS1068 carries a 36-kb deletion that removes sequences between 6 kb 5′ and 30 kb 3′ of the red-pigment gene transcription start site. At least one intact green-pigment gene and one 5′ green–3′ red hybrid gene remain in the HS1068 visual pigment gene array (table 2 and figs. 3 and 4).

A second group of subjects carries only a single visual pigment gene in the red- and green-pigment gene array, presumably the product of unequal homologous recombination. As seen in table 2, for each of the restriction fragments that distinguish the red- and green-pigment genes (B, C, and D), these subjects show only one of the two fragment types. Two subjects (HS056 and HS1067) carry a single red-pigment gene, the product of unequal intergenic recombination; others (e.g., HS215) carry a single 5′ red–3′ green hybrid gene, the product of unequal intragenic recombination.

In a third group of subjects, Southern blotting reveals that the red- and green-pigment gene array consists of two or more intact visual pigment genes and an intact 5′ flanking region. Southern blots of DNA from HS110, HS113, HS119, HS252, HS1120, and HS1770 show a wild-type pattern; Southern blots of DNA from HS123 reveal a 5′ red–3′ green hybrid gene in place of the normal red-pigment gene, a genotype that would be predicted to produce either red$^-$ dichromacy (protanopia) or red anomalous trichromacy (protanomaly; Nathans et al. 1986*a;* Deeb et al. 1992); and Southern blots of DNA from HS116 reveal a 5′ green–3′ red hybrid gene in place of the normal green-pigment gene, a genotype that would be predicted to produce either green$^-$ dichromacy (deuteranopia) or green anomalous trichromacy (deuteranomaly).

Coding-Region Point Mutations

In an earlier study, three blue-cone monochromat families were identified in which the red- and green-pigment gene array had been reduced by unequal homologous recombination to a single 5′ red–3′ green hybrid gene carrying a substitution of Cys^{203} by Arg (C203R; Nathans et al. 1989). This mutation creates a novel *Bst*UI site in exon 4. All of the blue-cone monochromat samples that did not contain upstream deletions were screened for this mutation by PCR amplification of exon 4 followed by *Bst*UI cleavage (fig. 5).

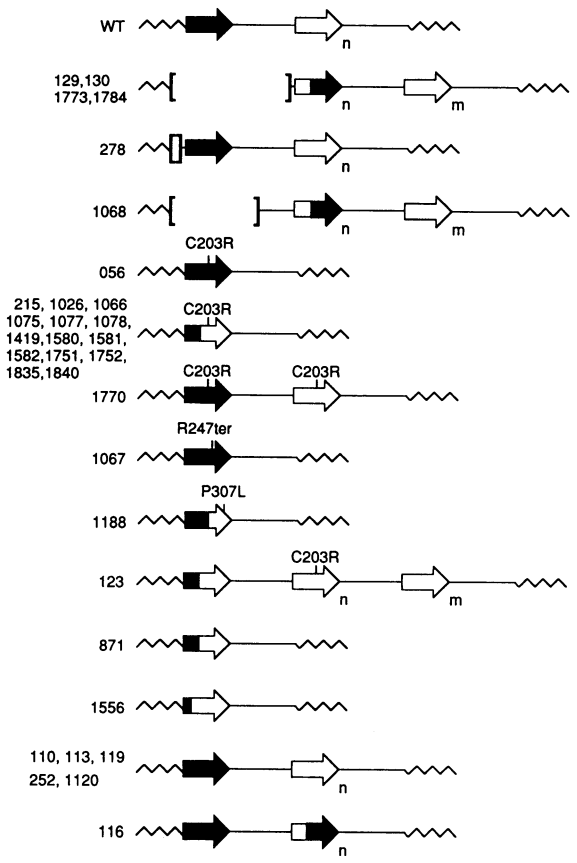

Figure 4 Schematic representations of the red- and green-pigment gene arrays found in blue-cone monochromats. "WT" = wild-type array showing a single red-pigment gene 5′ of a variable number of green-pigment genes. Each rightward-pointing arrow represents a visual pigment gene: the base and tip of the arrow correspond, respectively, to the 5′ and 3′ ends of the transcription unit. A blackened rightward-pointing arrow denotes sequences derived from the red-pigment gene; and an unblackened rightward-pointing arrow denotes sequences derived from the green-pigment gene. Straight lines represent homologous intergenic DNA; and zigzag lines represent unique flanking DNA. Brackets demarcate deleted sequences. Subscripts "m" and "n" indicate one or more green-pigment genes. In those arrays with more than one green-pigment gene, the order of the genes in the array is not known. Numbers indicate the probands who carry each deletion. In subject HS123 it is not known which gene carries the C203R mutation.

Fourteen subjects were found to carry a single 5′ red–3′ green hybrid gene with the C203R mutation. One subject (HS056) carries the C203R mutation in a red-pigment gene. Proband HS1770 and his affected brother carry one red- and one green-pigment gene, both of which contain the C203R mutation (fig. 5). One subject, HS123, who has a 5′ red–3′ green hybrid gene and at least one green-pigment gene, carries one or more genes with the C203R mutation and one or more genes with a cysteine at position 203 (fig. 4).

To assess the frequency of the C203R mutation in the normal gene pool, we analyzed DNA by PCR amplification and *Bst*UI digestion of exon 4 from 321 control subjects. The C203R mutation was found in 0/107 young adults known to have good vision and in 2/214 adult males who were recruited for an unrelated study and whose visual status is not known, suggesting a frequency in the gene pool of approximately .5%.

Four subjects (HS871, HS1067, HS1188, and HS1556) whose red- and green-pigment gene arrays consist of only a single visual pigment gene do not carry the C203R mutation. To determine whether these genes contain other point mutations, the six exons, together with 114 bp of 5′ flanking DNA and approximately 50 bp of flanking intron sequence adjacent to each exon, were amplified by PCR, cloned, and sequenced (table 1). Two of the genes contain sequence changes that would be predicted to have functional consequences. As shown in figure 6, HS1067 carries the substitution cytidine^{1233} to thymidine in exon 4 which leads to the replacement of Arg^{247} by a termination codon (R247ter), and HS1188 carries the substitution cytidine^{1414} to thymidine in exon 5, which leads to the replacement of Pro^{307} by Leu (P307L; for numbering system, see Nathans et al. 1986*b*) . Both mutations involve a cytidine-to-thymidine transition in a 5′ CpG sequence. As shown in figure 7, Pro^{307} is located within the same transmembrane alpha helix as Lys^{312}, the site of covalent attachment of 11-*cis* retinal.

Among those subjects who do not carry upstream deletions or the C203R mutation, only HS1067 and HS1188 carry the R247ter or P307L mutations, respectively, as determined by hybridization of an allele-specific oligonucleotide probe to slot blots of PCR-amplified DNA (data not shown). To determine whether small sequence changes in the essential region between 3.1 kb and 3.7 kb 5′ of the red-pigment gene could explain the color-vision defect observed in the remaining subjects, a region between 3,078 bp and 3,759 bp 5′ of the red-pigment gene—encompassing the smallest 5′ flanking deletion found to date in a blue-cone monochromat—was amplified as two overlapping PCR fragments and analyzed on a 4% native polyacrylamide gel (table 1; Nathans et al. 1989; Wang et al. 1992). In each case the electrophoretic mobility of the PCR product matched that of the corresponding wild-type product, thereby excluding DNA rearrangements larger than several base pairs.

In addition to the 33 probands described above,

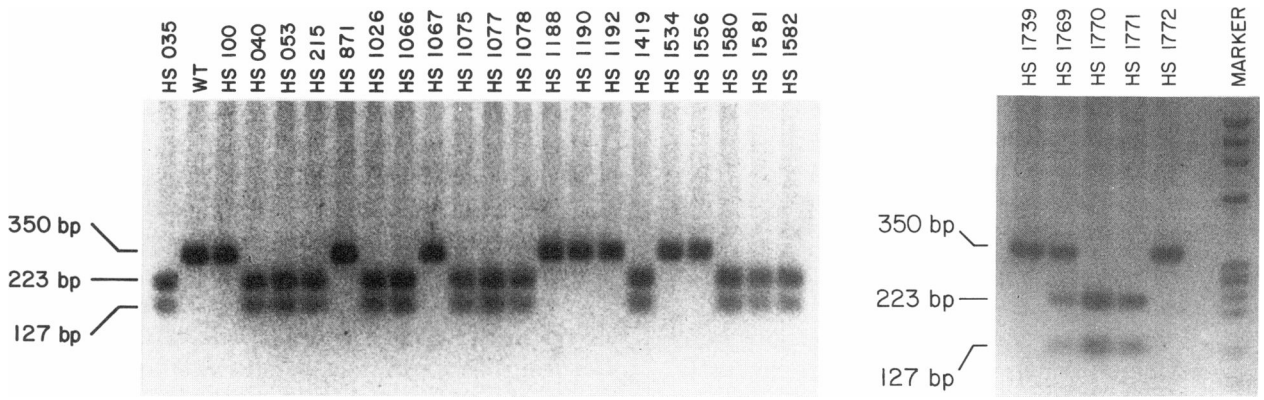

Figure 5 Identification of C203R mutations by PCR and *Bst*UI cleavage. Exon 4 was amplified using primers that hybridize to intron regions that are identical in the red- and green-pigment genes. The PCR products were digested with *Bst*UI and resolved on 1.5% agarose gels. Negative images of the ethidium bromide–stained gels are shown. The wild-type PCR product of 350 bp lacks a *Bst*UI site; substitution of cytidine for thymidine at position 1101 produces the C203R mutation and creates a *Bst*UI site which divides the PCR product into fragments of 127 bp and 223 bp (Nathans et al. 1989; for numbering system, see Nathans et al. 1986*b*). *Left*, *Bst*UI cleavage of exon 4 PCR products from probands who carry a single red or red-green hybrid gene. Controls HS035 and HS040 are known to carry the C203R mutation, and HS100, HS1190, HS1192, and HS1534 are known to carry cysteine at position 203, as determined by DNA sequencing (Nathans et al. 1989; J. Nathans, unpublished data). HS053 is the affected first cousin of HS056 (fig. 1). *Right*, *Bst*UI cleavage of exon 4 PCR products from members of family HS1770. Affected subjects HS1770 and HS1771 (subjects 3 and 4, respectively, in the pedigree in fig. 1) carry one red- and one green-pigment gene, both of which carry the C203R mutation; HS1769 (subject 1 in the pedigree in fig. 1) is an obligate carrier female, and HS1772 (subject 2 in the pedigree in fig. 1) is her sister. HS1739 is a control who does not carry the C203R mutation.

DNA analyses were performed on 30 relatives in 14 families (fig. 1). In each case in which a genotypic anomaly had been identified in the proband, this anomaly was observed to cosegregate with blue-cone monochromacy.

Discussion

Among 33 subjects with blue-cone monochromacy or closely related variants of blue-cone monochromacy, eight different sequence arrangements that would be predicted to eliminate the function of all of the genes within the array were identified in the red- and green-pigment gene array. Three of these genotypes have been found in an earlier study (genotypes E, F, and G in fig. 6 of Nathans et al. 1989). Of the 12 blue-cone monochromat genotypes reported to date that carry inactivating mutations, 8 have been found in only one family each. One genotype, consisting of a single 5′ red–3′ green hybrid gene that carries a C203R mutation, has been found in 17 families, accounting for approximately one-half of all blue-cone monochromat genotypes. The large number of rare blue-cone monochromat genotypes suggests that many remain to be discovered.

Deletions in the Locus Control Region (LCR)

Blue-cone monochromat genotypes fall into two broad classes. One class consists of nonhomologous deletions that encompass a region between 3.1 kb and 3.7 kb 5′ of the red-pigment gene transcription start site. The present study brings to seven the total number of alleles in this class. In each case one or more of the visual pigment gene transcription units remains intact. An analogous set of deletions 5′ of the epsilon-gamma-delta-beta–globin gene array causes thalassemia because of loss of expression of the linked globin genes (Van der Ploeg et al. 1980; Curtin et al. 1985; Taramelli et al. 1986). The deleted region adjacent to the globin

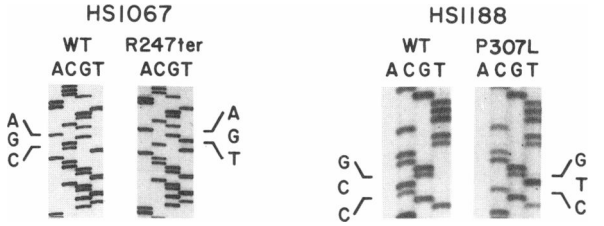

Figure 6 Variant nucleotide sequences from exon 4 of the single red-pigment gene of subject HS1067 and from exon 5 of the single 5′ red–3′ green hybrid gene of subject HS1188. Variant and normal DNA sequences were determined from cloned PCR products. The mutations have been independently confirmed by restriction endonuclease cleavage of genomic PCR products (R247ter creates a *Dde*I site, and P307L eliminates an *Nae*I site) and by hybridization with an allele-specific oligonucleotide.

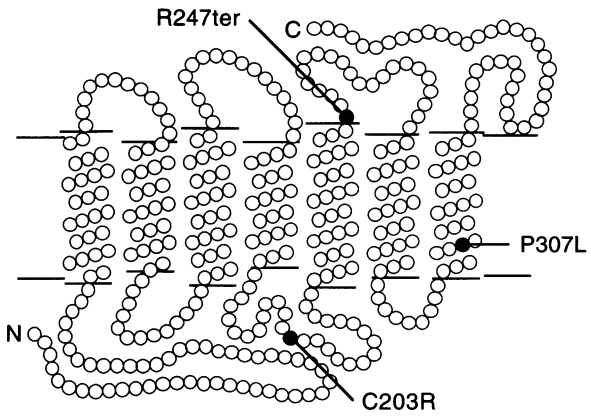

Figure 7 Model of the red and 5′ red–3′ green hybrid pigments in the photoreceptor membrane showing the locations of point mutations identified in blue-cone monochromats. Each circle represents an amino acid. "N" = amino-terminus; and "C" = carboxy-terminus. The amino-terminus faces the extracellular space.

genes is referred to as the "LCR," and, by analogy, the essential region 5′ of the red- and green-pigment genes is also referred to as an "LCR."

The role of the red- and green-pigment gene LCR in transcriptional regulation has recently been analyzed in transgenic mice (Wang et al. 1992). A 6.5-kb segment beginning immediately 5′ of the red-pigment gene transcription initiation site was found to direct expression of a beta-galactosidase reporter gene exclusively to cone photoreceptors, whereas an otherwise identical construct that lacked sequences between 3.1 kb and 3.7 kb 5′ of the transcription initiation site showed no beta-galactosidase expression. In the human retina, these sequences could interact via bound proteins with promoter proximal sequences to activate transcription of either the red- or green-pigment genes in the appropriate cone type. The observation that all seven different blue-cone monochromat deletions encompass a common region suggests that this may be the only sequence within or near the visual pigment gene array that is required for activation of both red- and green-pigment genes.

Coding-Region Point Mutations

The second class of genotypes found among blue-cone monochromats contains amino acid changes that are predicted to inactivate the encoded visual pigment(s) (fig. 7). This class is most commonly associated with red- and green-pigment gene arrays that have been reduced by unequal homologous recombination to a single repeat unit. Two of 20 arrays of this type carry uncommon point mutations: a stop-codon mutation in exon 4 and a missense mutation in exon 5. In the remaining 18 arrays (3 examples in Nathans et al. 1989 and 15 examples in the present study) the mutation is C203R in exon 4. The C203R mutation was found 17 times in 5′ red–3′ green hybrid genes and once in a red-pigment gene (HS056). In the latter case it may have arisen as the product of either a gene conversion or double homologous recombination event with a green pigment gene that carried the C203R mutation. This mutation was also found in both visual pigment genes in a family with two genes in the array (HS1770), as well as in at least one gene in a family with multiple genes (HS123). The latter family may carry additional mutations in those genes that are not inactivated by the C203R mutation.

Recent experiments by Winderickx et al. (1992*a*) show that, among males with two or more distinguishable types of green-pigment genes, only one type—and most likely only one gene—is expressed. Winderickx et al. suggest that the expressed green pigment gene may be the one that is closest to the LCR, presumably reflecting a proximity effect in LCR-promoter interactions. This line of reasoning suggests that, among blue-cone monochromats with multiple genes in the red- and green-pigment gene array, only the two genes closest to the LCR need to acquire inactivating point mutations to eliminate red- and green-cone function.

In nine of the subjects reported here, the structure of the red- and green-pigment gene array does not reveal the genetic mechanism of the severe color-vision defect. In two subjects (HS871 and HS1556) who carry a single 5′ red–3′ green hybrid gene in their array, we have been unable to find a mutation in the exons or immediately flanking intron and promoter sequences by sequencing of cloned DNA. In the absence of inactivating mutations, these genotypes would be expected to produce dichromacy rather than monochromacy. Five subjects (HS110, HS113, HS119, HS252, and HS1120) carry gene arrays that resemble the wild type by Southern blotting, and two subjects (HS116 and HS123) carry gene arrays that would be predicted to alter only one of the two pigments. As the red- and green-pigment genes from these latter seven subjects have not been sequenced, they could contain novel point mutations.

Multiple Pathways to Blue-Cone Monochromacy

The present work extends the general conclusion of Nathans et al. (1989) that there are different mutational pathways to blue-cone monochromacy (fig. 8). Among the 45 families examined in the two studies, 35 were

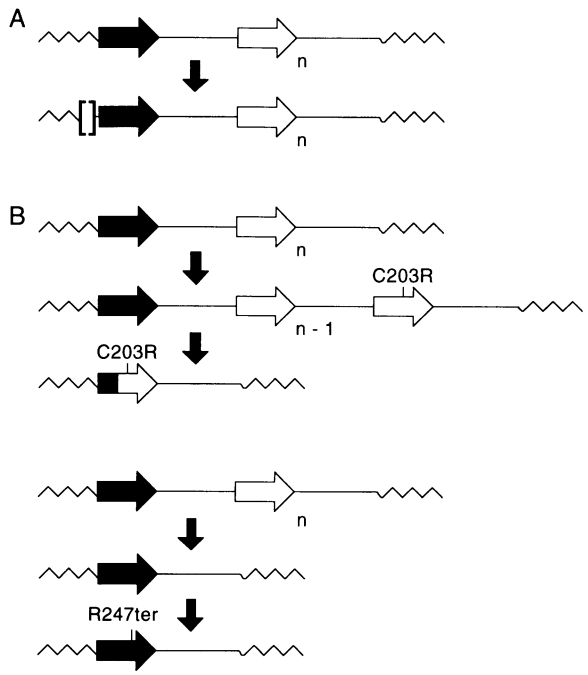

Figure 8 Multiple pathways for the generation of blue-cone monochromacy. *A*, One-step deletion of the red- and green-pigment gene LCR, located between 3.1 kb and 3.7 kb 5′ of the red-pigment gene. *B*, Two examples of two-step pathways involving both point mutation and either homologous recombination or gene conversion. In the example involving R247ter, the temporal order of the two steps is unknown. Symbols are as defined in the legend of fig. 4.

found to carry 1 of 12 different genotypes that inactivate both red- and green-pigment genes. A one-step mutational pathway, consisting of nonhomologous deletion of the LCR, produces 40% (14/35) of blue-cone monochromat genotypes in which the mutational event has been defined. A heterogeneous group of multistep pathways, involving a combination of point mutation and homologous unequal recombination and/or gene conversion, produce the remaining 60% (21/35). Many of these latter pathways produce visual pigment genes that carry the C203R mutation.

The presence of the C203R mutation in a number of different blue-cone monochromat gene arrays indicates that this mutation predated the unequal homologous recombination events that generated the final blue-cone monochromat genotype. Winderickx et al. (1992*b*) have arrived at the same conclusion based on their finding of this mutation in (*a*) all of the green-pigment genes in a dichromat who is missing green-cone function and (*b*) one of several green-pigment genes in 1/65 subjects with normal color vision and in 1/63 subjects with anomalous color vision. Our survey of 107 subjects with normal vision and of 214 males whose vision status is unknown indicates that this mutation is likely to be present in the gene pool at a frequency of approximately .5%. This mutation eliminates a conserved disulfide bond (Karnik et al. 1988; Karnik and Khorana 1990) and, in this respect, appears to resemble those destabilizing rhodopsin mutations responsible for autosomal dominant retinitis pigmentosa (Sung et al. 1991*b*; fig. 7). It will be of interest to determine whether those individuals with normal color vision who carry the C203R mutation in one of their green-pigment genes show any evidence of retinal disease later in life.

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