Molecular Genetics of Human Blue Cone Monochromacy

JEREMY NATHANS,* CAROL M. DAVENPORT, IRENE H. MAUMENEE, RICHARD ALAN LEWIS, J. FIELDING HEJTMANCIK, MICHAEL LITT, EVERETT LOVRIEN, RICHARD WELEBER, BRIAN BACHYNSKI, FRED ZWAS, ROGER KLINGAMAN, GERALD FISHMAN

Blue cone monochromacy is a rare X-linked disorder of color vision characterized by the absence of both red and green cone sensitivities. In 12 of 12 families carrying this trait, alterations are observed in the red and green visual pigment gene cluster. The alterations fall into two classes. One class arose from the wild type by a two-step pathway consisting of unequal homologous recombination and point mutation. The second class arose by nonhomologous deletion of genomic DNA adjacent to the red and green pigment gene cluster. These deletions define a 579–base pair region that is located 4 kilobases upstream of the red pigment gene and 43 kilobases upstream of the nearest green pigment gene; this 579–base pair region is essential for the activity of both pigment genes.

NORMAL HUMAN COLOR VISION IS MEDIATED BY THREE types of photoreceptors called cones. One type is maximally sensitive at 565 nanometers (nm) (the red cones), a second is maximally sensitive at 535 nm (the green cones), and the third is maximally sensitive at 440 nm (the blue cones). A fourth type of photoreceptor, rods, are maximally sensitive to light of 500 nm, mediate vision in dim light, and contribute little to color sense. To match the appearance of a given colored light, normal observers require a combination of at most three independently adjustable lights. Their color vision is trichromatic. In contrast, the several percent of men who have X-linked, recessively inherited, color vision deficiency as a consequence of loss of either red or green cone pigments require a combination of at most only two independently adjustable lights to match a given light. Their color vision is dichromatic.

Achromatopsia, the true absence of color discrimination, is rare, affecting approximately one person in one hundred thousand. Achromats can match any two colored lights by suitably adjusting their relative intensities (*1*). Their color vision is monochromatic. The first detailed description of achromatopsia in the Western scientific literature appeared in 1777 (*2*). The subject of that report "could never do more than guess the name of any color; yet he could distinguish white from black, or black from any light or bright color. . . . He had two brothers in the same circumstances as to sight; and two brothers and sisters who, as well as his parents, had nothing of this defect."

In principle, achromatopsia arising at the photoreceptor level could result from the absence or malfunction of any two of the three cone types or of all three cone types. In the former case, the single remaining cone type would mediate color matches at high luminance and the rod system would, as in normals, mediate matches at low luminance. In the latter case, all color matches would be rod mediated. Achromatopsia could also arise from deficiencies in higher order neural processing. Indeed, acquired achromatopsia is occasionally seen after cortical damage (*3*).

Categories of achromats. Studies of congenital achromatopsia over the past several decades have made it possible to define the following three categories. (i) Typical rod monochromats have normal levels of rhodopsin and normal rod function but lack all sensitivity mediated by cone pigments (*4, 5*). In some individuals the central cone rich region of the retina (the fovea) mediates a light response with the directional sensitivity and dark adaptation properties of cones but with the action spectrum of rhodopsin. This has led to the suggestion that in these individuals rhodopsin may be functioning in both rods and cones (*5, 6*). (ii) Atypical rod monochromats behave as if they have only rod vision. However, reflection densitometry shows that their retinas contain normal quantities of cone pigments (*1*), suggesting that the defect occurs distal to the point of light absorption. (iii) Blue cone monochromats have rods and blue cones (*1, 4, 7, 8*).

Clinically, individuals in all three categories share a number of features, especially at a young age. Regardless of category, achromats classically have very poor or no color discrimination and on a Farnsworth D-15 or Farnsworth-Munsell 100 Hue Test show no clear axis, that is, no preference for confusing any subset of hues; they also have low acuity, typically 20/80 to 20/200; a pendular nystagmus that decreases with age; photophobia; and retinas that appear normal or nearly so (*5, 9*). Blue cone monochromats can be distinguished from both types of rod monochromats by psycho-

J. Nathans and C. M. Davenport are in the Howard Hughes Medical Institute at the Department of Molecular Biology and Genetics and the Department of Neuroscience, and I. H. Maumenee is in the Wilmer Ophthalomologic Institute, The Johns Hopkins University School of Medicine, Baltimore, MD 21205. R. A. Lewis is at the Cullen Eye Institute, the Department of Ophthalmology, and the Institute for Molecular Genetics, and J. F. Hejtmancik is in the Howard Hughes Medical Institute and the Institute for Molecular Genetics, Baylor College of Medicine, Houston, TX 77030. M. Litt is in the Department of Biochemistry, E. Lovrien is in the Department of Medical Genetics, and R. Weleber is in the Department of Medical Genetics and the Department of Ophthalmology, Oregon Health Sciences University, Portland, OR 97201. B. Bachynski is in the Department of Ophthalmology, Henry Ford Hospital, Detroit, MI 48202. F. Zwas is in the Kresge Eye Institute, Wayne State University School of Medicine, Detroit, MI 48201. R. Klingaman is in the Department of Biomedical Sciences, Southern College of Optometry, Memphis, TN 38104. G. Fishman is in the Department of Ophthalmology, University of Illinois College of Medicine, Chicago, IL 60612.

*To whom correspondence should be directed.

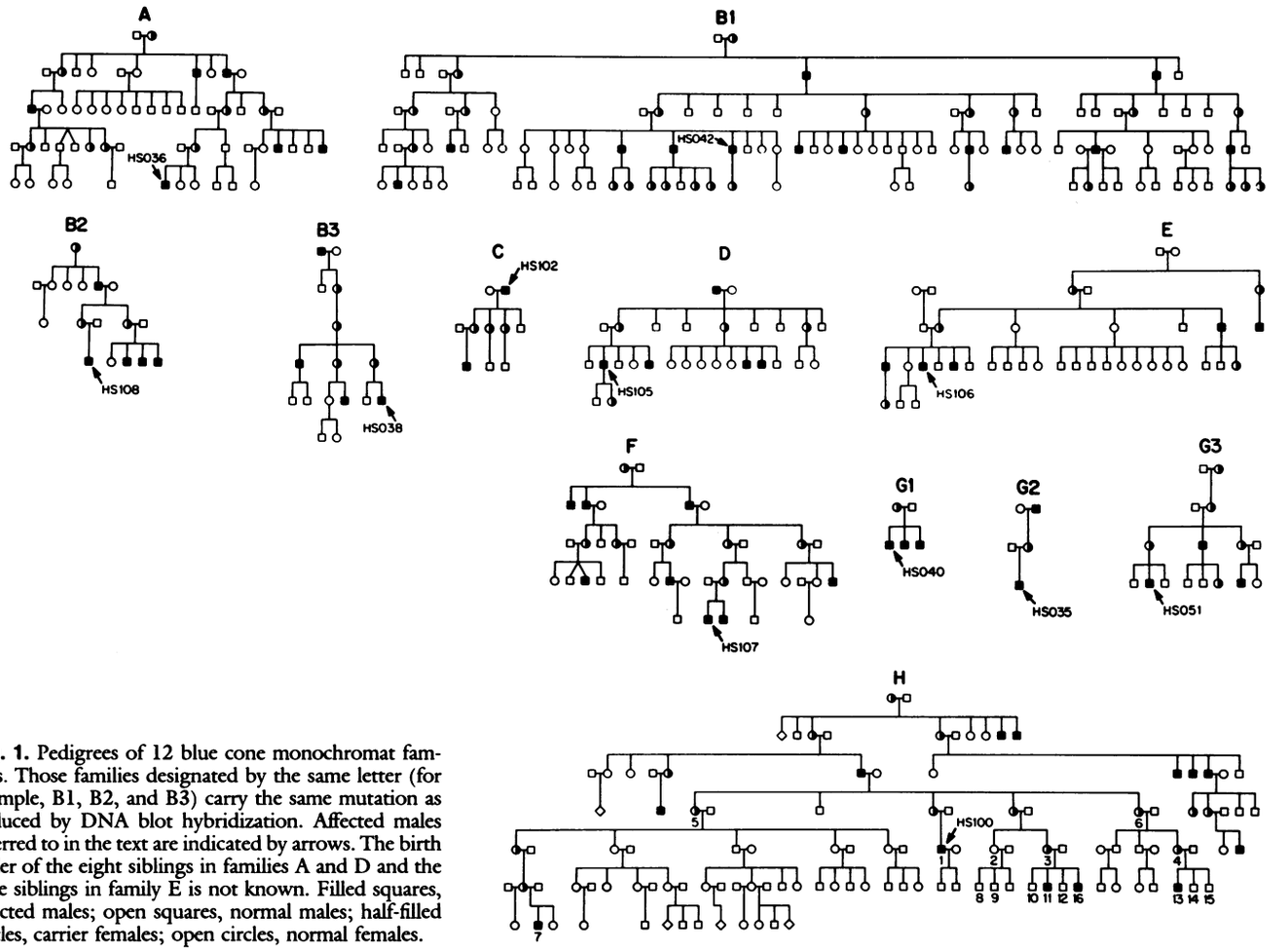


Fig. 1. Pedigrees of 12 blue cone monochromat families. Those families designated by the same letter (for example, B1, B2, and B3) carry the same mutation as deduced by DNA blot hybridization. Affected males referred to in the text are indicated by arrows. The birth order of the eight siblings in families A and D and the nine siblings in family E is not known. Filled squares, affected males; open squares, normal males; half-filled circles, carrier females; open circles, normal females.

physical threshold testing or electroretinographic studies at different wavelengths. These tests measure which, if any, cone mechanisms are active. Classification also relies on family history because blue cone monochromacy is inherited as an X-linked trait, whereas both types of rod monochromacy are autosomal recessive (*9, 10*). Blue cone monochromacy is also referred to as π_1 monochromacy or X-linked incomplete achromatopsia (*9*).

In this article we report our analysis of the molecular basis of blue cone monochromacy in 12 families. We were guided by the hypothesis that this trait may represent an inherited loss of both red and green pigments. This hypothesis was strengthened by recent mapping studies based on restriction fragment length polymorphisms that placed blue cone monochromacy in the same chromosomal region as the red and green pigment genes (*11*).

Red and green visual pigment gene structure. The common X-linked dichromacies result from alterations in the red and green visual pigment genes (*12*). These genes are arranged in a head-to-tail tandem array near the tip of the long arm of the X chromosome. The red pigment gene lies at the edge of the array with its 5′ end abutting single-copy flanking DNA; one or more green pigment genes are found at intervals of 39 kilobases (kb) downstream of the red pigment gene. Red and green pigment genes are highly homologous and appear to have arisen from a common ancestral gene following a recent gene duplication. Almost all inherited variations in red-green color discrimination arise from unequal homologous recombination within this tandem array, resulting in the deletion of genes or the generation of red-green or green-red hybrid genes.

Such unequal homologous recombination can reduce to one the

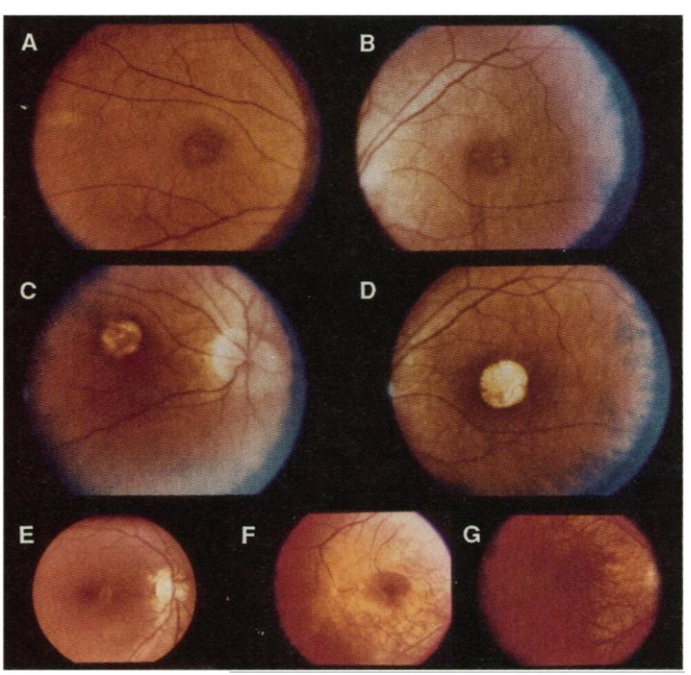


Fig. 2. Fundus photographs of blue cone monochromat males. Right eye (**A**) and left eye (**B**) of HS106 at age 27. Right eye (**C**) and left eye (**D**) of HS106 at age 37. Right eyes of HS035 (**E**), HS107 (**F**), and HS108 (**G**). HS106 shows a progressive bilateral central retinal degeneration; HS035, HS107, and HS108 have retinas of normal appearance bilaterally.

number of X-linked visual pigment genes but cannot eliminate the last remaining gene. Therefore, a Red$^-$Green$^-$ (R^-G^-) phenotype cannot be produced by unequal homologous recombination starting from R^-G^+, R^+G^- or R^+G^+, nor can it arise from homologous crossing over between R^-G^+ and R^+G^- genotypes. If alterations in these genes do result in blue cone monochromacy, those alterations must involve some other mechanism or mechanisms.

Clinical characteristics and DNA blot hybridization patterns. Affected individuals in the 12 families studied (Fig. 1) display little or no color discrimination at either high or low light levels (Table 1). At intermediate levels, where both rod and cone systems are active, many patients can make crude dichromatic discriminations, presumably by convergence of rod and cone signals. Nystagmus, photophobia, and low acuity may or may not be present, even within a pedigree. Most of the individuals have retinas that appear normal, but, in some, a progressive central retinal dystrophy was observed as they grew older (Fig. 2). The dystrophic region corresponds to the fovea, the cone rich area responsible for high acuity vision, and the immediately surrounding retina. The pedigrees of all 12 families show only males affected and an absence of male to male transmission, a pattern of inheritance consistent with X-linkage. In most families, other modes of inheritance can be ruled

Table 1. Clinical characteristics of affected males. For families C and H, two affected males are described (Fig. 1): HS98 is the affected grandson of HS102; HS99 is individual 13 in pedigree H. Farnsworth D-15 and Farnsworth-Munsell 100 hue tests consist, respectively, of a series of 15 and 85 caps of graded hue. Individuals with normal color vision can arrange the caps in a circular order that minimizes the change in hue between adjacent caps. Dichromats make errors along particular "confusion axes"; monochromats make many errors with no preferred axis. Ishihara plates test red-green color discrimination and Ichikawa plates test for the presence of a blue cone mechanism. Sloan achromatopsia plates match different hues to varying shades of gray: The ability to make a match across a range of hues indicates achromatopsia, and the gray levels of each match define the active photoreceptor type. The increment threshold test matches a standard light to a test light of variable intensity and variable wavelength: The matches define the active photoreceptor type. The electroretinogram (ERG) measures light-dependent electrical signals from photoreceptors and higher order retinal cells. Photopic (bright) light is used to measure cone function; scotopic (dim) light is used to measure rod function. [These tests are described in (*9*).] Clinical data for families B2, C, E, and F were provided by I.H.M.; for A, B1, B3, D, and G3 by R.A.L. and J.F.H.; for G1 by B.B. and F.Z.; for G2 by G.F. and R.K.; and for H by R.W., E.L., and M.L.; sc, scotopic; ph, photopic; b, blue; g, green; r, red; N.D., not determined.

Family (individual)	Color Vision Testing	Electroretinogram	Acuity	Photophobia	Nystagmus	Ophthalmoscopy	Age
A (HS036)	D-15, F-M 100: many errors, no axis	ph: greatly decreased	20/80	present	present	normal	12
B1 (HS042)	ph increment threshold: no r or g sensitivity, normal b sensitivity	N.D.	20/400	N.D.	mild	normal	21
B2 (HS108)	Ishihara plates: 5/26 correct Sloan Achromatopsia plates: rod matches	N.D.	20/100 - 20/200	present	present	normal	18
B3 (HS038)	color plates: absent r-g discrimination, weak b-y discrimination	ph: absent sc: normal	20/60	present	mild	normal	13
C (HS102)	no color discrimination by history	N.D.	20/160	N.D.	present	macular lesion	46
(HS98)	Ishihara plates: fail	N.D.	3/30 - 3/50	present	decreased since birth	normal	3.5
D (HS105)	F-M 100: many errors, no axis weak b-y discrimination	N.D.	20/300 - 20/400	N.D.	present	normal	27
E (HS106)	D-15: many errors, no axis Ishihara plates: fail	ph: absent sc: normal	20/80 - 20/200	absent	in early childhood	progressive degeneration	37
F (HS107)	Ishihara plates: fail Ichikawa plates: pass	ph: absent sc: normal	20/100 - 20/200 (corrected)	absent	in early childhood	normal	7
G1 (HS040)	D-15, F-M 100: many errors, no axis ph increment threshold: no r or g sensitivity, normal b sensitivity Ishihara: fail	ph: greatly decreased sc: normal	20/200	absent	mild	normal	15
G2 (HS035)	D-15: many errors, no axis ph color matching: b cone matches only ph increment threshold: no r or g sensitivity, normal b sensitivity	ph: greatly decreased sc: normal ph flicker: no r, normal b response	20/200 - 20/100	present	present	normal	19
G3 (HS051)	absent r-g discrimination	N.D.	20/200	N.D.	present	distorted macular reflex	13
H (HS100)	poor or absent color discrimination by history	long wave ph: greatly decreased; short wave ph: present	20/200	N.D.	mild	atrophy secondary to severe myopia	50
(HS99)	D-15, F-M 100: many errors, no axis	long wave ph: greatly decreased shortwave ph: present sc: normal	20/200	N.D.	mild	normal	7

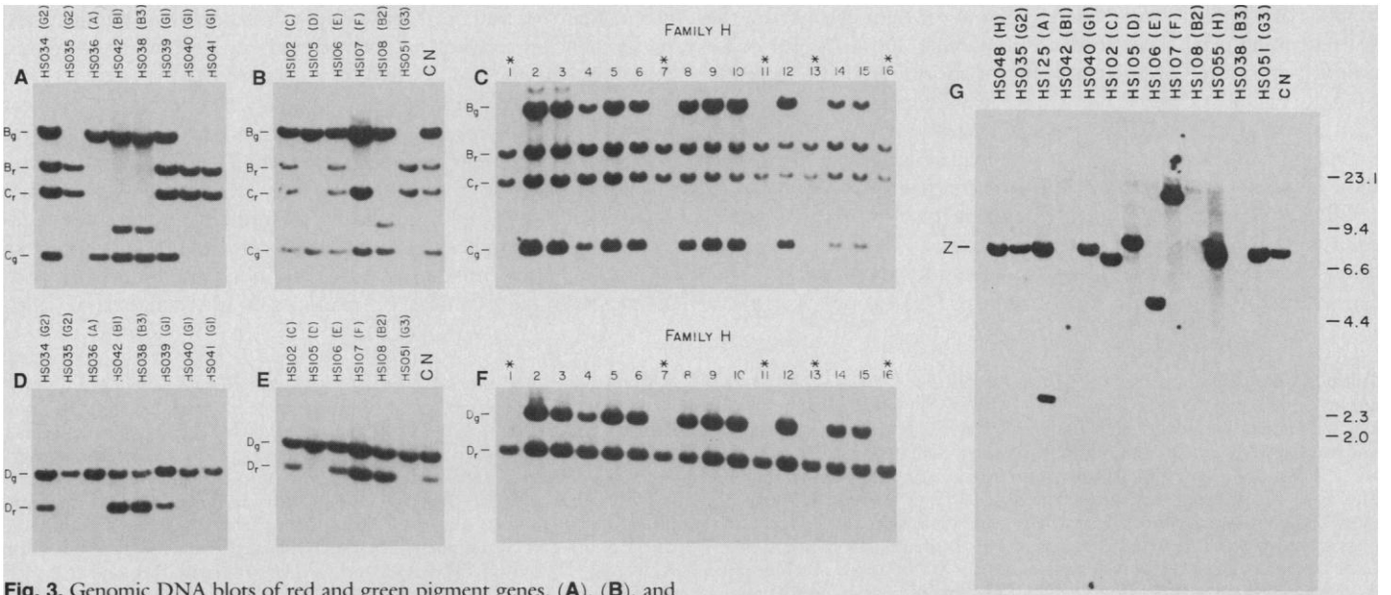


Fig. 3. Genomic DNA blots of red and green pigment genes. (**A**), (**B**), and (**C**): Bam HI–Eco RI double digests probed with the 5′ proximal 350 bp of red cDNA clone hs7 (*12*), encompassing exon 1 and the 5′ one-half of exon 2. (**D**), (**E**), and (**F**): Rsa I digests probed with a 400-bp segment from the 3′ edge of green intron 4. (**G**): Hind III digests probed with an 800-bp Bam HI–Hind III partial digest fragment located between 8.2 and 9.0 kb 5′ of the red pigment gene. Fragments B_r (4.1 kb), C_r (3.3 kb), and D_r (841 bp) are derived from the red pigment gene; B_g (5.4 kb), C_g (1.8 kb), and D_g (1360 bp) are derived from the green pigment gene; fragment Z (7.2 kb) is upstream of the red pigment gene. (See Fig. 4 for restriction map.) Lanes are labeled in (A), (B), (D), and (E) according to individual and family designations in Fig. 1. CN, color-normal. (A) and (D), HS034 is the mother of HS035; HS039 and HS041 are, respectively, the mother and affected brother of HS040. Lane numbers in (C) and (F) refer to numbered individuals in family H in Fig. 1; HS100 is number 1, asterisks mark affected males. In (G), HS125 is an obligate carrier female from family A; HS036, an affected male from family A, has the same 2.5-kb Hind III fragment seen in HS125 but lacks the 7.2-kb fragment Z. Preparation of genomic DNA and DNA blot hybridization were as described (*12*).

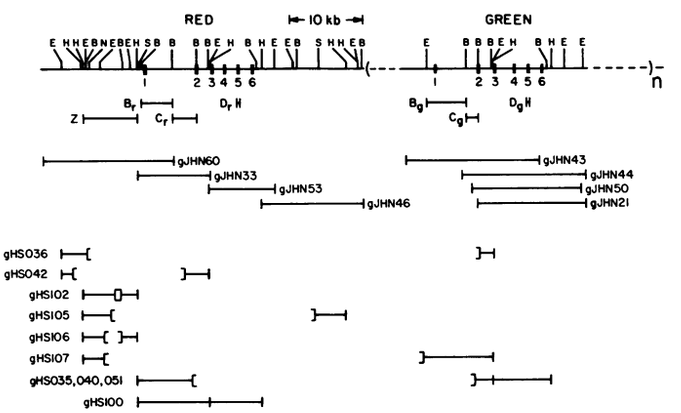


Fig. 4. Restriction maps of cloned color-normal and mutant red and green pigment genes. (Top) Restriction map of normal genomic DNA. B, Bam HI; E, Eco RI; H, Hind III; N, Not I; S, Sal I. Numbers 1 to 6 mark red and green pigment gene exons. The number of green pigment genes varies among color-normals as indicated by subscript n. Restriction fragments shown below the genes refer to DNA blot hybridization bands in Fig. 3. Center, genomic phage clones from color-normal DNA are labeled gJHN. (Bottom) Genomic phage clones from X-linked incomplete achromats. Deleted sequences are indicated by brackets. The gHS035, gHS040, gHS051, and gHS100 genes were cloned as two adjacent Hind III fragments of 10 and 7 kb. In HS035, HS040, and HS051 a 5′ red, 3′ green hybrid gene was generated by unequal homologous recombination. Genomic clones were isolated in lambda 607 (gHS036 and gHS042), lambda 590 (gHS035, gHS040, gHS051, gHS100, gHS102, gHS105, and gHS106), or lambda 762 (gHS107) as described (*12*). Two or more independent recombinants were isolated and analyzed for each mutant. Deletion breakpoints were defined by restriction mapping to a resolution of several hundred base pairs.

out. We refer to each family by letter or by a combination of letter and number (for example, G1, G2, G3); different letters designate different mutations.

Blot hybridization of DNA from affected individuals using probes from within and around the red and green pigment genes demonstrates a clear difference from color normals in all 12 families. The alterations fall into two classes: (i) nonhomologous deletions in or adjacent to the red pigment gene, and (ii) loss by unequal homologous recombination of all but one of the genes from the tandem array.

Nonhomologous deletion mutants. The DNA blot hybridization patterns of affected individuals in families A through F reveal nonhomologous rearrangements. (In Fig. 3 the color normal DNA hybridization patterns are shown for comparison.) In families A through F one (or more) of the normal fragments is missing, and a new fragment is seen at a different mobility. This was first observed in Bam HI–Eco RI double digests of DNA from affected individuals HS036 and HS042 (families A and B1, respectively) probed with a 350-bp fragment of red pigment complementary DNA (cDNA) encompassing exon 1 and the entire 5′ half of exon 2. The DNA's of the red and green pigment genes are 98 percent identical, and therefore probes derived from coding regions or introns from either one hybridize to both (*12*). The color normal pattern with this probe consists of four fragments: B_g and C_g derived from the green pigment gene and B_r and C_r derived from the red pigment gene (Figs. 3 and 4). In HS036 and HS042, B_r and C_r are missing, but B_g and C_g are retained. In HS042 there was a new hybridizing fragment at a mobility different from that of the normal fragments. When a probe from the 3′ one-half of exon 2 was used a different (and novel) fragment was seen in the DNA of HS036. To isolate the rearranged segments, we analyzed Eco RI digests of HS036 and HS042 DNA's for the corresponding novel fragments which were then cloned from libraries of Eco RI digested and size-selected DNA.

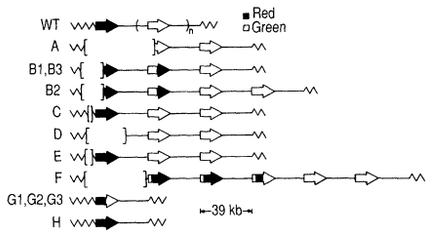


Fig. 5. Schematic representation of blue cone monochromat genotypes. Each arrow represents a visual pigment gene: the base corresponds to the 5′ end, the tip corresponds to the 3′ end, straight lines represent homologous intergenic DNA, and zig-zag lines represent unique flanking DNA. Brackets demarcate the missing sequences.

Restriction maps of these cloned DNA's together with the DNA blot data in Fig. 3 show that HS042 has lost the first exon, the first intron, and upstream flanking sequences of the red pigment gene and that HS036 has lost the first exon and first intron of a green pigment gene, as well as the entire red pigment gene and its upstream flanking sequences (Fig. 4). To define the extent of the deleted upstream flanking sequences, we prepared a Sau 3A partial digest genomic library from the DNA of a male (J.N.) with normal color vision and whose visual pigment genes served as the reference in previous studies (*12*). The library was screened with a segment derived from the upstream region of the gHS042 deletion breakpoint clone. Clone gJHN60 was isolated and shown to contain the upstream end points of both the HS036 and HS042 deletions as well as part of the red pigment gene (Fig. 4). The gJHN60 restriction map shows the HS036 and HS042 deletions to be 54 and 16 kb, respectively.

Using segments of gJHN60 as DNA blot probes revealed a loss or rearrangement of sequences upstream of the red pigment gene in affected individuals from families A through F (Fig. 3G). By contrast, in 14 of 14 color-normal males examined by DNA blotting the Hind III restriction map of the 8.6-kb region upstream of the red pigment is unaltered. Families B1, B2, and B3 (subjects HS042, HS038, and HS108) share the same novel bands in Eco RI, Eco RI–Bam HI, or Hind III DNA blots, and therefore appear to carry the same deletion. DNA encompassing each different alteration was cloned from either Eco RI or Hind III preparative digests as outlined above. Restriction mapping of the cloned DNA's shows that HS102, HS105, HS106, and HS107 have deletions of 0.5 kb, 29 kb, 2.5 kb, and 41 kb, respectively.

All of the nonhomologous deletion chromosomes retain one or more green pigment genes or green-red hybrid genes as determined by DNA blotting. Two of six deletions lie entirely upstream of the red pigment gene transcription unit. The HS102 and HS106 deletions remove sequences no closer than 3.8 kb upstream of the red pigment gene transcription start site. The presence or absence of red or green coding sequences can be seen in Fig. 3. Fragments B_g, C_g, and D_g (derived from the first, second, and fifth exons, respectively, of the green pigment gene) are present in all of these deletion chromosomes. B_r and C_r (derived from the first and second exons, respectively, of the red pigment gene) are present in DNA from HS102 and HS106 and absent from HS036, HS042, and HS105; in HS107, C_r is present and B_r is missing. D_r (derived from the fifth exon of the red pigment gene) is present in HS042, HS102, HS106, and HS107 and absent in HS036 and HS105.

Scanning densitometry was used to measure the intensities of the DNA blot hybridization bands (Table 2). Ratios of peak areas were calculated both within a sample (for example, B_g:B_r, C_g:C_r, and D_g:D_r) and between each monochromat sample and the normal DNA after correcting for variation in the amount of DNA loaded in each lane. From these ratios, the numbers and types of remaining visual pigment genes were deduced (Fig. 5). Two of the six deletions coexist with green-red hybrid genes, not a statistically significant difference from the 6 percent of green-red hybrid containing X chromosomes seen in the general population (*12, 13*).

```
AAACCTGTCT CTACTAAAAA TACAAAGAAA AAATTAGCCG GGCATGGTGG TGCATGCCTG TAGTCCCAGC TACTCAGGAC ACTGAGGCTG AGGAGAATTG

                                        ► Deletion HS107
CTTGAACCCG GGAGGCAGAG GTTGTAGTGA GCAAAGATCG CGCCACTGCA CTCCAGCCTG GGCAACAGAG TGAGACTCCA CGTCAAAAAA AAAAAAAAAA

AAGACATGTC AGAAACAAAG AAAGAAAGAC ATGACAGGCT CAGAATCAAT GTCAACCTGG GCAAAGGTCA GAAACGGAAC AGGGACCCTT TCTTTGTGGG

CAGGTTTGAC ATGAAAGGTT CAGCGACTTC AACTCTTCTG ATTCTTTCTT CTTGAGCCCA TTTTGGTAAG TTGTGTTTTC CTAAAAGTTT GTCCATTCCA

                    ► Deletion HS106
TCCAAATGTT CACATTAGTC AGGATCTGTG AAGGATGTCA ATGGCTGTGA GGCGAGGGCC CTTGCCAGTC CCATGTTCGT TATTTATGGA TGCCTGATGC

  ... one and one-half kb ...    GTCCCTCTGG CCTTTCCCCA GGGGCCCTCT TTCCTTGGGG CTTTCTTGGG CCGCCACTGC TCCCGCTCCT

                                 ► Deletion HS102
CTCCCCCCAT CCCACCCCCT CACCCCCTCG TTCTTCATAT CCTTCTCTAG TGCTCCCTCC ACTTTCATCC ACCCTTCTGC AAGAGTGTGG GACCACAAAT

GAGTTTTCAC CTGGCCTGGG ACACACGTGC CCCCACAGGT GCTGAGTGAC TTTCTAGGAC AGTAATCTGC TTTAGGCTAA AATGGGACTT GATCTTCTGT

TAGCCCTAAT CATCAATTAG CAGAGCCGGT GAAGGTGCAG AACCTACCGC CTTTCCAGGC CTCCTCCCAC CTCTGCCACC TCCACTCTCC TTCCTGGGAT

GTGGGGGCTG GCACACGTGT GGCCCAGGGC ATTGGTGGGA TTGCACTGAG CTGGGTCATT AGCGTAATCC TGGACAAGGG CAGACAGGGC GAGCGGAGGG

CCAGCTCCGG GGCTCAGGCA AGGCTGGGGG CTTCCCCCAG ACACCCCACT CCTCCTCTGC TGGACCCCCA CTTCATAGGG CACTTCGTGT TCTCAAAGGG

CTTCCAAATA GCATGGTGGC CTTGGATGCC CAGGGAAGCC TCAGAGTTGC TTATCTCCCT CTAGACAGAA GGGGAATCTC GGTCAAGAGG GAGAGGTCGC

 CCTGTTCAAG GCCACCCAGC CAGCTCATGG CGGTAATGGG ACAAGGCTGG CCAGCCATCC CACCCTCAGA AGGGACCCGG TGGGGCAGGT GATCTCAGAG
Deletion HS102 ◄
    Deletion HS106 ◄

 GAGGCTCACT TCTGGGTCTC ACATTCTTGG ATCC
```

Fig. 6. Nucleotide sequence of wild-type DNA upstream of the red pigment gene showing the HS102, HS106, and HS107 deletion breakpoints. The HS102 deletion is 579 bp in length; the HS106 deletion is 2.5 kb; the HS107 deletion is 41 kb in length. Wild-type sequences were determined on both strands with modified T7 polymerase (Sequenase, United States Biochemicals) and the dideoxy method. In the region sequenced, HS102 and HS106 differ from normal only by the deletion event and HS107 only by the deletion event and one nucleotide substitution.

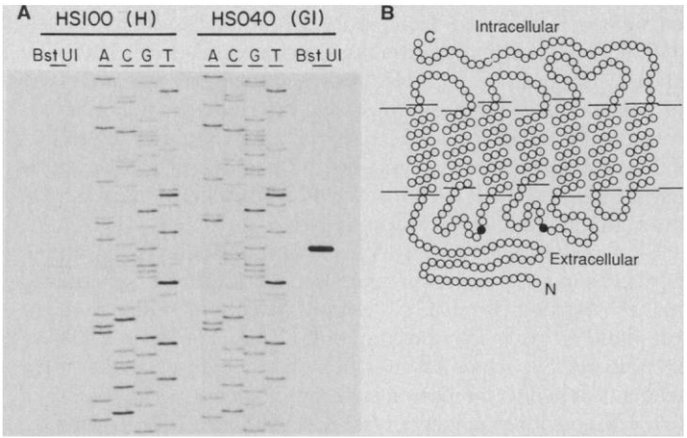


Fig. 7. (**A**) The T to C mutation in the coding strand of HS040 produces a cysteine-to-arginine substitution and creates a Bst U1 site. The central eight tracks are dideoxy sequences of the noncoding strand of exon 4 cloned from HS100 and HS040. Flanking the sequencing tracks are pooled samples of the four sequencing reactions cut with Bst U1, which cleaves at $^{5'}$CGCG$^{3'}$. HS100, like wild type, lacks a Bst U1 site in this region. (**B**) Conserved cysteines at positions 126 and 203 (corresponding to 110 and 187 in bovine rhodopsin) are shown in the visual pigment secondary structure model of Hargrave (*14*).

HS108 carries the same deletion as HS038 and HS042, but differs from them by the presence of an additional green pigment gene. Presumably an unequal homologous recombination event removed or added a green pigment gene some time after the nonhomologous deletion event.

The five larger deletions encompass the 579-bp region missing from the smallest deletion (HS102). The nucleotide sequences across the HS102 and HS106 breakpoints and from the corresponding region of wild-type DNA reveals simple breakage and fusion events (Fig. 6). Interestingly, the 3′ breakpoints of HS102 and HS106 are only four nucleotides apart. Nucleotides 501 to 1334 of Fig. 6, which encompass the 579-bp deletion, show no statistically significant homology on either strand to any vertebrate sequences in the NBRF (National Biomedical Research Foundation) DNA database. Likewise, the conceptual translation products of all six reading frames of this segment show no statistically significant homology to any vertebrate sequences in the NBRF protein database.

Homologous deletion and point mutation. The DNA blot hybridization patterns of affected individuals in families G1, G2, G3, and H reveal loss by homologous recombination of all but one of the visual pigment genes from the tandem array. They show no evidence of nonhomologous deletion events when tested with the red and green pigment gene probes described above. In families G1, G2, and G3 the presence of fragments B_r, C_r, and D_g and the absence of fragments of B_g, C_g, and D_r, indicates the presence of a single 5′ red, 3′ green hybrid gene—presumably the product of an intragenic unequal homologous recombination event. In family H the presence of fragments B_r, C_r, and D_r, and the absence of fragments B_g, C_g, and D_g, indicates the presence of a single red pigment gene, presumably the product of an intergenic unequal homologous recombination event. In each of these four families, DNA blots show the expected color-normal Hind III restriction fragments between 8.6 kb upstream and 3 kb downstream of the remaining gene.

These two types of gene arrangements had been seen in studies of inherited dichromacy (*12*). Males who carry only a single red pigment gene and who are missing all green pigment genes are phenotypically G^-R^+; those carrying only a single 5′ red, 3′ green hybrid are G^+R^-. We guessed, therefore, that the G^-R^- phenotype in families G1, G2, G3, and H was caused by a superimposed point mutation not detectable by DNA blotting. To test this hypothesis genomic clones containing the red or red-green hybrid genes were isolated from the DNA of affected males in each of the four families.

Nucleotide sequencing of both strands of the six exons and immediately flanking intron regions from HS040 (family G1) revealed a red-green hybrid gene in which exons 2 through 6 were derived from a green pigment gene. The sequence also shows a single change from thymine to cytosine in exon 4 at nucleotide 1101 [see (*12*) for the numbering system], producing a change from cysteine to arginine at amino acid 203. This nucleotide sequence change creates a Bst U1 site (Fig. 7A), whose presence in the red-green hybrid genes from HS035, HS040, and HS051 (families G2, G1, and G3) indicates that they carry the same cysteine to arginine mutation. This cysteine (corresponding to position 187 in the bovine rhodopsin numbering system) is conserved among all visual pigments sequenced to date (*12, 14*) and is predicted to lie in the second extracellular loop. A second conserved cysteine, at position 110 in the bovine rhodopsin numbering system, is located in the first extracellular loop (Fig. 7B). All but two of the G protein–activating receptors sequenced—including visual pigment (*14*), muscarinic (*15*), adrenergic (*16*), serotonin (*17*), dopamine (*18*), substance K (*19*), α-factor (*20*), and cyclic adenosine monophosphate (cyclic AMP) receptors (*21*)—have cysteines in both of these loops. In the two exceptions, the yeast a-factor receptor and the angiotensin receptor, cysteine is absent from both loops (*20, 22*). Moreover, Karnik *et al.* have shown by in vitro mutagenesis that of the ten cysteines in bovine rhodopsin, only the two at positions 110 and 187 are required for visual pigment formation (*23*). A similar experiment with the beta-adrenergic receptor shows that the ho-

Table 2. DNA blot band intensities and deduced fragment copy numbers. DNA blots (Fig. 3) were scanned and the hybridization band areas measured. Variation in the relative amounts of DNA in each track was measured by then probing blots A, B, D, and E with ^{32}P-labeled human genomic DNA. The ratios of peak areas in the resulting hybridization to dispersed repetitive sequences were used to normalize each of the band intensities to those of the color-normal sample(s) run on the same gel. (In Fig. 3, the color-normal tracks are shown only for blots B and E. In each instance the normal sample is known to contain one red and two green pigment genes.) The normalized values, in arbitrary units, are listed. To determine fragment copy number (in parentheses) we also calculated the ratios of fragment peak areas within a track. These ratios are highly reproducible and are not affected by track-to-track variation in the amount of DNA loaded.

	B_g	B_r	C_g	C_r	D_g	D_r
HS035	0 (0)	14.7 (1)	0 (0)	13.9 (1)	34.9 (1)	0 (0)
HS036	21.9 (1)	0 (0)	10.9 (1)	0 (0)	61.4 (2)	0 (0)
HS042	34.8* (2)	0 (0)	15.2* (2)	0 (0)	40.9 (1)	68.6 (2)
HS038	26.8* (2)	0 (0)	13.8* (2)	0 (0)	32.1 (1)	62.9 (2)
HS040	0 (0)	15.0 (1)	0 (0)	20.1 (1)	37.0 (1)	0 (0)
HS041	0 (0)	18.0 (1)	0 (0)	16.8 (1)	35.0 (1)	0 (0)
C N	45.5 (2)	15.1 (1)	21.3 (2)	18.1 (1)	66.1 (2)	33.6 (1)
HS102	64.0 (2)	15.9 (1)	11.7 (2)	15.8 (1)	88.7 (2)	35.6 (1)
HS105	59.1 (2)	0 (0)	14.1 (2)	0 (0)	64.9 (2)	0 (0)
HS106	69.8 (2)	16.4 (1)	11.3 (2)	13.2 (1)	79.4 (2)	37.7 (1)
HS107	108.7† (5)	0 (0)	18.7 (2)	44.9 (3)	85.4 (3)	61.4 (2)
HS108	69.6 (3)	0 (0)	16.8 (3)	0 (0)	53.8 (2)	47.2 (2)
HS051	0 (0)	19.3 (1)	0 (0)	18.1 (1)	39.5 (1)	0 (0)
C N	55.9 (2)	14.1 (1)	12.1 (2)	13.3 (1)	72.0 (2)	23.0‡ (1)
HS100	0 (0)	34.1 (1)	0 (0)	28.8 (1)	0 (0)	40.1 (1)
C N	69.2 (2)	44.2 (1)	41.1 (2)	30.8 (1)	70.3 (2)	40.8 (1)

*Low because of DNA degradation. †Low because of film saturation. ‡Low because of a blot transfer artifact. C N, color-normal.

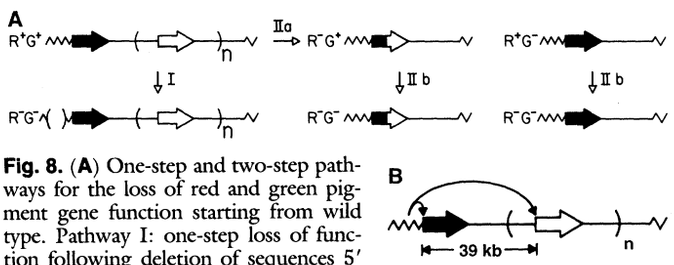


Fig. 8. (**A**) One-step and two-step pathways for the loss of red and green pigment gene function starting from wild type. Pathway I: one-step loss of function following deletion of sequences 5′ of the gene cluster. Pathway II: two-step loss of function following inter- or intragenic unequal homologous recombination (IIa) and point mutation (IIb). (**B**) Working model to explain G^-R^- phenotype of upstream deletions. Controlling sequences between 3.8 and 4.4 kb 5′ of the red pigment gene are proposed to activate red and green pigment gene transcription in cones.

mologous pair of cysteines are essential for the activity of that receptor (*24*). Most likely, this pair of cysteines forms a conserved disulfide bond that is important for protein stability.

The nucleotide sequence of both strands of the six exons, immediately flanking intron region, and the promoter regions of the red pigment gene from HS100 (pedigree H) reveals three nucleotide and two corresponding amino acid changes in exon 3: guanine 1005 to adenine, guanine 1007 to thymine, and adenine 1026 to guanine, producing a change from valine to isoleucine at amino acid 171 and a change from isoleucine to valine change at amino acid 178. Both of these positions are in putative transmembrane segment 4. The conservative nature of these two substitutions suggests that they may not be responsible for the mutant phenotype. If the relevant mutation lies beyond the region sequenced, it is likely to be near the red pigment gene; in family H, the red pigment gene cosegregates with blue cone monochromacy in 11 out of 11 meioses (Fig. 3F).

Two mutational pathways to blue cone monochromacy. The molecular basis of human blue cone monochromacy can now be defined. Examination of 12 families has revealed in each case a rearrangement in, or adjacent to, the red and green pigment gene cluster. Within each family the rearrangement cosegregates with the mutant phenotype (20 affected males and 11 unaffected males were examined).

Starting from a color-normal tandem array of red and green pigment genes, two mutational pathways to blue cone monochromacy were observed (Fig. 8). One pathway consists of a two-step loss of function. In the first step homologous unequal recombination reduces to one the number of genes in the tandem array. Approximately 1 percent of X chromosomes in the Caucasian population have this arrangement, and males who inherit them are dichromats (*12, 13, 25*). In the second step a mutation inactivates the remaining gene; the two examples presented here carry point mutations.

A second pathway consists of a one-step loss of both red and green pigment gene function by deletion of sequences between 3.8 and 4.3 kb upstream of the red pigment gene transcription start site. These sequences are 43 kb upstream from the nearest green pigment gene transcription start site (*12*). Additional green pigment genes, which are present in some mutant chromosomes (as they are in wild type), are even further removed from the deleted sequences by multiples of 39 kb per gene. These upstream sequences could be part of an adjacent gene whose action is required for red and green cone function. Alternatively, this region may contain one or more elements required in *cis* for the correct transcription of both red and green pigment genes. The common 579-bp sequence missing from all six deletion mutants could interact via bound proteins with the promoter regions of the red and green pigment genes or could act indirectly by controlling superhelicity or intranuclear localization of adjacent sequences.

Comparison with (ϵγδβ)° thalassemias. The upstream deletions that we report are reminiscent of those seen in two (ϵγδβ)° thalassemias in which absence of distant upstream sequences results in loss (in *cis*) of β-globin gene expression (*26*). One deletion of 99 kb leaves the β-globin gene and 2.5 kb of upstream flanking DNA intact. A second deletion of more than 100 kb begins 25 kb upstream of the β-globin gene and leaves the $^A\gamma$, δ, and β genes intact. Within the common deleted region as well as downstream of the globin genes are clusters of erythroid-specific deoxyribonuclease I (DNase I) "hypersensitivity" sites (*27, 28*). A 20-kb fragment encompassing the upstream DNase I sites together with a 15-kb fragment encompassing the downstream DNase I sites confer on a linked human β-globin gene uniformly high, tissue-specific expression independent of chromosomal position in transgenic mouse fetuses (*28*). These observations support a model in which distant sequences act to coordinate tissue-specific gene expression. The upstream sequences missing in blue cone monochromats may be playing that role in the retina.

Blue cone monochromacy, like (ϵγδβ)° thalassemia, is a trait that involves loss of function in more than one gene. As such, it represents a favorable situation for identifying alterations in long range cis-acting controlling sequences. Apart from deletion of the entire gene cluster, deletions of controlling sequences are likely to be the only one-step mutational events that can give the double mutant phenotype. In contrast, single gene loss-of-function mutations most commonly arise from mutations in protein coding regions (*29*). In this study, two of eight alleles arose by point mutation starting from dichromat genotypes that are found on only 1 percent of X chromosomes. The other six alleles arose by deletion in a pool of the remaining 99 percent of X chromosomes. Thus, had the pool size been equal for both classes of mutation—as it would be for single gene targets—we would expect approximately 200 point mutations per six controlling region deletions.

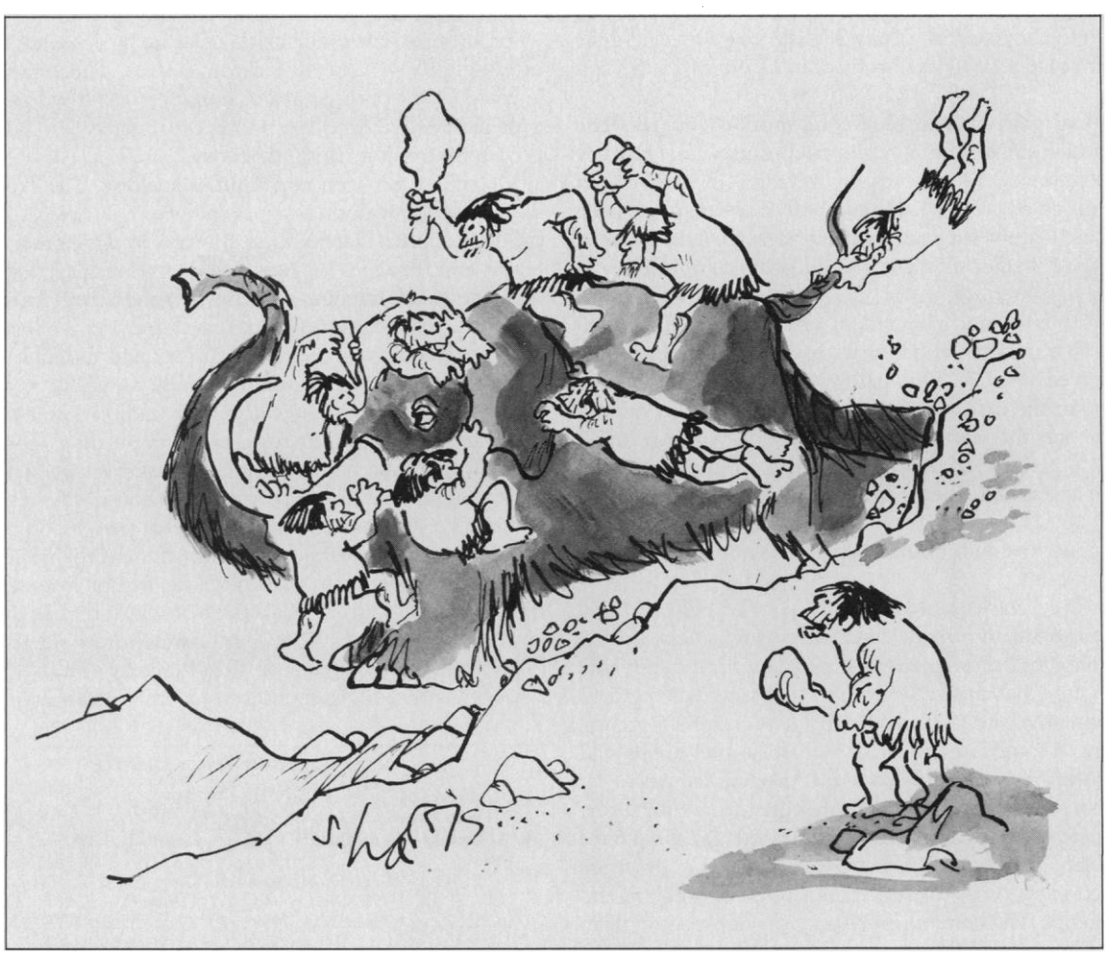
Molecular genetics and ophthalmology. The experiments presented here draw upon several experimentally favorable attributes of the human visual system. First, humans recognize and can accurately report abnormalities in their vision. As a result, many people with heritable visual impairments come to the attention of an ophthalmologist. Second, human visual psychophysics is a highly developed science; it offers accurate, sensitive, and noninvasive tests for defining phenotypes. Third, inherited variations in visual system function rarely affect longevity or fecundity. These mutations are, therefore, expected to increase in the population. For example, the incidence of inherited variation in red-green color vision among Caucasian males is now 8 percent (*25*). Fourth, the retina is the only part of the central nervous system that can be observed directly. Monitoring its appearance through the ophthalmoscope makes it possible to follow at the tissue level the natural history of a disorder. In our study, a slow progressive central retinal dystrophy was observed in patient HS106. This natural history suggests by analogy that some peripheral retinal dystrophies may be caused by mutations in the genes encoding rhodopsin or other rod proteins.

REFERENCES AND NOTES

1. M. Alpern, *Invest. Ophthalmol.* **13**, 648 (1974).
2. J. Huddart, *Philos. Trans. R. Soc.* **67**, 260 (1777).
3. A. Damasio, T. Yamada, H. Damasio, J. Corbett, J. McKee, *Neurology* **30**, 1064 (1980).
4. H. R. Blackwell and O. M. Blackwell, *Vision Res.* **1**, 62 (1961).
5. L. L. Sloan, *J. Opt. Soc. Am.* **44**, 117 (1954).
6. ———, *Am. J. Ophthalmol.* **46**, 81 (1958); M. Alpern, H. F. Falls, G. B. Lee, *ibid.* **50**, 996 (1960); H. F. Falls, J. R. Wolter, M. Alpern, *Arch. Ophthalmol.* **74**, 610 (1965).
7. M. Alpern, G. B. Lee, B. E. Spivey, *Arch. Ophthalmol.* **74**, 334 (1965); J. Pokorny,

V. C. Smith, R. Swartley, *Invest. Ophthalmol.* **9**, 807 (1970); M. Alpern, G. B. Lee, F. Maaseidvaag, S. S. Miller, *J. Physiol.* **212**, 211 (1971); N. W. Daw and J. M. Enoch, *Vision Res.* **13**, 1669 (1973).
8. V. C. Smith, J. Pokorny, F. W. Newell, *Ophtalmologica* **177**, 197 (1978); *Am. J. Ophthalmol.* **87**, 393 (1979).
9. J. Pokorny, V. C. Smith, G. Verriest, A. J. L. G. Pinckers, in *Congenital and Acquired Color Vision Defects* (Grune & Stratton, New York, 1979), p. 183; R. A. Lewis, R. G. Weleber, A. Eisner, in *Retinal Dystrophies and Degenerations*, D. A. Newsome, Ed. (Raven Press, New York, 1988), pp. 21, 115, and 233.
10. B. E. Spivey, *Arch. Ophthalmol.* **74**, 327 (1965); J. A. Fleischman and F. E. O'Donnell, *ibid.* **99**, 468 (1981).
11. R. A. Lewis *et al.*, *ibid.* **105**, 1055 (1987); E. S. Jones, I. H. Maumenee, D. Zhee, L. Hyvarinen, P. C. Huang, in preparation.
12. J. Nathans, D. Thomas, D. S. Hogness, *Science* **232**, 193 (1986); J. Nathans, T. P. Piantanida, R. L. Eddy, T. B. Shows, D. S. Hogness, *ibid.*, p. 203; D. Vollrath, J. Nathans, R. W. Davis, *ibid.* **240**, 1669 (1988).
13. M. Drummond-Borg, S. S. Deeb, A. G. Motulsky, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 983 (1989).
14. Yu.A. Ovchinnikov *et al.*, *Bioorg. Khim.* **9**, 1331 (1983); P. A. Hargrave *et al.*, *Biophys. Struct. Mech.* **9**, 235 (1983); J. Nathans and D. S. Hogness, *Cell* **34**, 807 (1983); *Proc. Natl. Acad. Sci. U.S.A.* **81**, 4851 (1984); J. E. O'Tousa *et al.*, *Cell* **40**, 839 (1985); C. S. Zuker, A. F. Cowman, G. M. Rubin, *ibid.*, p. 851; A. C. Cowman, C. S. Zuker, G. M. Rubin, *ibid.* **44**, 705 (1986); C. Montell, K. Jones, C. Zuker, G. M. Rubin, *J. Neurosci.* **7**, 1558 (1987); C. S. Zuker, A. F. Cowman, G. M. Rubin, *ibid.*, p. 1550; M. Takai, A. Yasui, F. Tokunago, *Vision Res.* **28**, 471 (1988); W. Baehr, J. D. Falk, K. Bugra, J. T. Triafantafyllos, J. F. McGinnis, *FEBS Lett.* **238**, 253 (1988).
15. T. Kubo *et al.*, *Nature* **323**, 411 (1986); T. Kubo *et al.*, *FEBS Lett.* **209**, 367 (1986); E. G. Peralta *et al.*, *Science* **236**, 600 (1987); T. I. Bonner, N. J. Buckley, A. C. Young, M. R. Brann, *ibid.* **237**, 527 (1987); E. G. Peralta *et al.*, *EMBO J.* **6**, 3923 (1987).
16. R. A. F. Dixon *et al.*, *Nature* **321**, 75 (1986); Y. Yarden *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 6795 (1986); B. K. Kobilka *et al.*, *Science* **238**, 650 (1987).
17. D. Julius, A. B. MacDermott, R. Axel, T. M. Jessell, *Science* **241**, 558 (1988); B. K. Kobilka *et al.*, *Nature* **329**, 75 (1987); A. Fargin *et al.*, *ibid.* **335**, 358 (1988); D. B. Pritchett *et al.*, *EMBO J.* **7**, 4135 (1988).
18. J. R. Bunzow *et al.*, *Nature* **336**, 783 (1988).
19. Y. Masu *et al.*, *ibid.* **329**, 836 (1987).
20. N. Nakayama, A. Miyajima, K. Arai, *EMBO J.* **4**, 2643 (1985).
21. P. S. Klein *et al.*, *Science* **241**, 1467 (1988).
22. D. Young, G. Waitches, C. Birchmeier, G. Fasano, M. Wigler, *Cell* **45**, 711 (1986); T. R. Jackson, L. A. C. Blair, J. Marshall, M. Goeder, M. R. Hanley, *Nature* **335**, 437 (1988).
23. S. S. Karnik, T. P. Sakmar, H.-B. Chen, H. G. Khorana, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 8459 (1988).
24. R. A. F. Dixon *et al.*, *EMBO J.* **6**, 3269 (1987).
25. J. Francois, in *Heredity in Ophthalmology* Eds. (Mosby, St. Louis, 1961), p. 430; R. W. Pickford, *Br. J. Physiol. Optics* **14**, 2 (1957).
26. L. H. T. Van der Ploeg *et al.*, *Nature* **283**, 637 (1980); R. Taramelli *et al.*, *Nucleic Acids Res.* **14**, 7017 (1986); P. Curtin *et al.*, *J. Clin. Invest.* **76**, 1554 (1985).
27. D. Tuan, W. Solomon, L. S. Qiliang, M. L. Irving, *Proc. Natl. Acad. Sci. U.S.A.* **32**, 6384 (1985).
28. F. Grosveld, G. B. Van Assendelft, D. R. Greaves, G. Kollias, *Cell* **51**, 975 (1987).
29. J. T. Stout and C. T. Caskey, *Annu. Rev. Genet.* **19**, 127 (1985).
30. We thank all of the blue cone monochromats and their families for their participation; S. Desiderio, S. Merbs, D. Nathans, R. Reed, D. Valle, C. Weitz, and D. Zack for comments on the manuscript; and T. Chase for assistance in preparing the manuscript. Supported by the Howard Hughes Medical Institute and by the National Eye Institute, NIH, The L. Kriebl and Walter Edel funds (I.H.M.), The National Retinitis Pigmentosa Foundation Fighting Blindness (R.A.L. and R.W.), and Research to Prevent Blindness (R.A.L.).

29 March 1989; accepted 11 July 1989



" Just what we need—Mass Transit."