Color Vision

From Genes to Perception

Edited by

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Contents

1

Opsin genes, cone photopigments, color vision, and color blindness

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In this chapter, we introduce the molecular structure of the genes encoding the human cone photopigments and their expression in photoreceptor cells. We also consider the consequences that alterations in those genes have on the spectral sensitivity of the photopigments, the cone photoreceptor mosaic, and the perceptual worlds of the color normal and color blind individuals who possess them. Throughout, we highlight areas in which our knowledge is still incomplete.

Trichromacy. Human color vision is trichromatic; this has three consequences. First, as was recognized in the eighteenth century (e.g., Le Blon, 1722; see Birren, 1963, 1980), but only formally postulated (Grassman, 1853) and verified (Maxwell, 1855, 1860) in the nineteenth century, the number of independent variables in color vision is three. That is, all colors can be matched by just three parameters: either by the three primaries of additive light mixture – typically, violet, green, and red – or by the three primaries of subtractive pigment mixture – typically, cyan, yellow, and magenta.

Second, as intimated by Palmer (1777, 1786; see also Voigt, 1781; Walls, 1956; Mollon, 1997), definitively stated by Young (1802, 1807), and revived by Helmholtz (1852), trichromacy is not a physical property of light but a physiological limitation of the eye: All color perceptions are determined by just three physiological response systems.

Third, as pointed out by Maxwell (1855) and applied by König and Dieterici (1886), a linear transform must exist between the tristimulus color matching properties of the eye, as established by the three primaries of additive light mixture, and the spectral sensitivities of the three physiological systems mediating the matches (see Chapter 2).

The three physiological response systems are universally acknowledged to be the three types of retinal photoreceptor cell, each containing a different photopigment: the short (S)-, middle (M)-, and long (L)-wave sensitive cones.[1] These have distinct, spectral sensitivities (Fig. 1.1A) or absorption spectra (Fig. 1.1B), which define the probability of photon capture as a function of wavelength. The absorbance spectra of the S-, M-, and L-cone photopigments overlap considerably, but have their wavelengths of maximum absorbance (λ_{max}) in different parts of the visible spectrum: ca. 420, 530, and 558 nm, respectively. When estimated in vivo, the λ_{max}'s are shifted to longer wavelengths (ca. 440, 545, and 565 nm, respectively) by the transmission properties of the intervening ocular media: the yellowish crystalline lens and the macular pigment of the eye (see Chapter 2).

The individual cone photopigments are blind to the wavelength of capture; they signal only the rate at

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[1]The fourth type of photoreceptor cell, the rods, contain rhodopsin as their photopigment. They are by far the most prevalent in the human retina, constituting more than 95% of all photoreceptor cells. However, they do not contribute to color vision, except under limited, twilight conditions (see section on rod monochromacy). Under most daylight conditions, where we enjoy color vision, the rod photoreceptor response is saturated by excessive light stimulation.
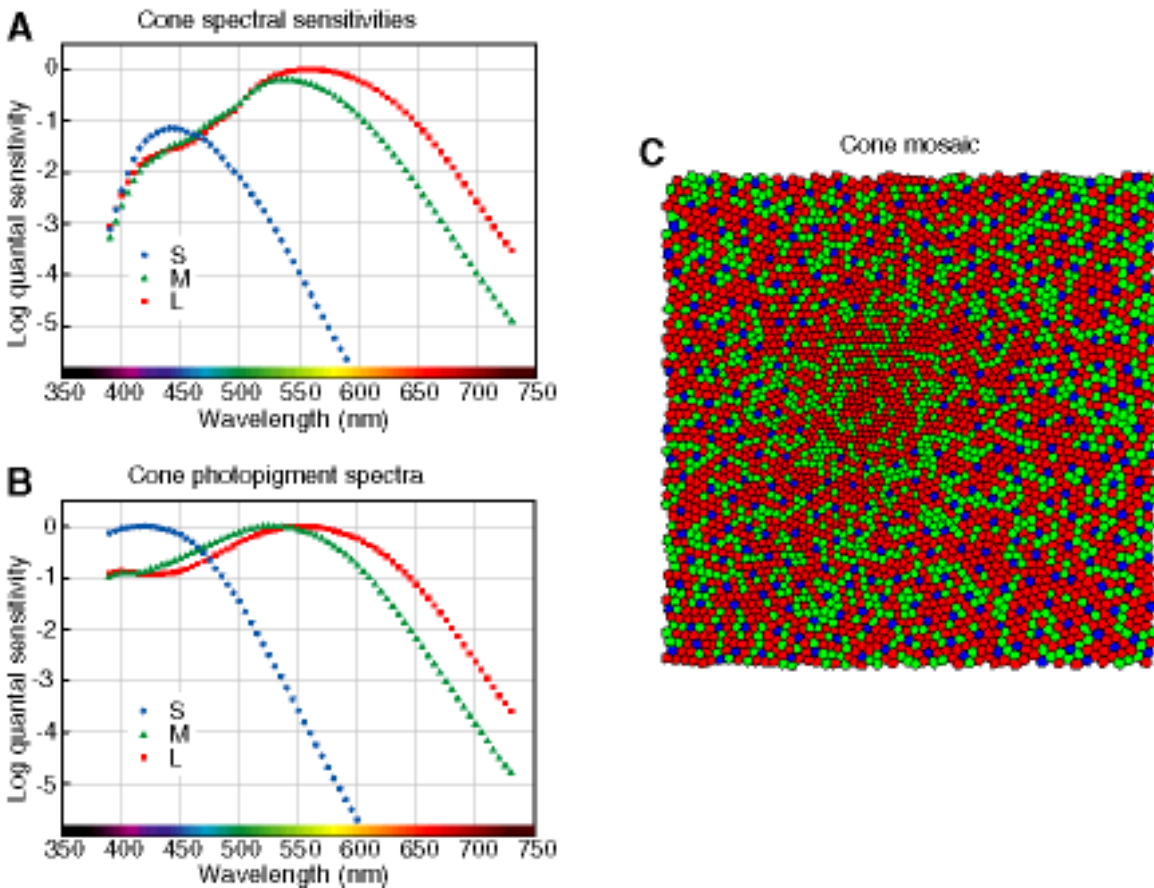
Figure 1.1: Cone spectral sensitivities and their representations in the photoreceptor mosaic. (A) Estimates of the light absorbing properties of the L- M-, and S-cones, measured at the cornea, as a function of wavelength (see Chapter 2, Table 2.1, for values). The heights of the curves have been adjusted according to the assumption that the relative cone sensitivities depend on the relative numbers of the different cone types; namely, that 7% of all cones contain the S-cone pigment and that, of the remaining 93%, those containing the L-cone pigment are 1.5 times more frequent than those containing the M-cone pigment (see Chapter 2). (B) The cone pigment absorption spectra. These were determined from the cone spectral sensitivity functions, by correcting the latter for the filtering of the ocular media and the macular pigment and for the self-screening of the pigment in the outer segment (see Chapter 2, Table 2.1, for values). (C) The cone mosaic of the rod-free inner fovea of an adult human retina at the level of the inner segment (tangential section). Superior is at the top and nasal to the left. The region is ca. 1 deg of visual angle in diameter (ca. 300 μm). The center coordinates of the cone cross sections shown were obtained from the retina of a 35-year-old male (Curcio & Sloan, 1992). The outer dimensions of the cone cross sections have been defined mathematically by Voronoi regions and computer-colored according to the following assumptions: (1) only three cone opsin genes, those encoding the S-, M-, and L-cone pigments are expressed; (2) the inner roughly circular area (ca. 100 μm or 0.34 deg in diameter), displaced slightly to the upper left quadrant of the mosaic, is free of S-cones (Curcio et al., 1991); (3) S-cone numbers in the rest of the retina do not exceed 7% and are semiregularly distributed (Curcio et al., 1991); and (4) there are approximately 1.5 times as many L- as M-cones in this region of the retina and they are randomly distributed (see Chapter 5). The diameters of the cross sections in the center are slightly smaller than those at the outer edge to allow for close packing.

which photons are caught (cf. Rushton, 1972). Lights of different spectral distributions, therefore, will appear identical, if they produce the same absorptions in the three cone photopigments, and different, if they do not (see Chapter 2). Thus color vision – the ability to discriminate on the basis of wavelength – requires comparisons of photon absorptions in different photopigments. And, accordingly, trichromatic color vision requires three such independent comparisons. Merely summing the absorptions in the three cone photopigments at some later neural stage will permit brightness or contrast discrimination, but not color vision.

The loss of one of the cone photopigments, as occurs in certain congenital disorders, reduces (photopic) color vision to two dimensions or dichromacy. The loss of two further reduces it to one dimension or monochromacy. And, the loss of all three completely extinguishes it. Vision, then, is purely scotopic and limited to the rods.

Cone pigments and visual pathways. In man and the higher primates, the primary visual or retino-geniculostriate pathway has evolved into three postreceptoral neuronal systems for transmitting the cone signals that arise from the photopigment absorptions (see Chapter 11). These have been characterized as: (i) a luminance subsystem, which mainly carries information about luminance contrast by summing the relative rates of quantum catch in the M- and L-cones (and is sensitive to high spatial and temporal frequencies); (ii) a yellow-blue color subsystem, which mainly carries information about color contrast by comparing the relative rate of quantum catch in the S-cones with those in the M- and L-cones; and (iii) a red-green color subsystem, which carries information about color contrast by comparing the relative rates of quantum catch in the M- and L-cones. Roughly, it can be said that the three subsystems allow three kinds of discriminations: light from dark, yellow from blue, and red from green.

The dimensionality of the color information transmitted by these postreceptoral subsystems is, in the first instance, limited by the number of available cone photopigments. If one or more of the three normal cone photopigments is absent, then the dimensionality

is correspondingly reduced from trichromacy to dichromacy or monochromacy. On the other hand, if an extra, fourth cone photopigment is present, as occurs in certain heterozygotic carriers of color blindness, full four-dimensional or tetrachromatic color vision does not seem readily possible (see page 38). The limitation may be the inability of the postreceptoral subsystems to convey more than three independent color signals (see Chapter 6).

Molecular genetics of the opsin genes

The spectral sensitivity of the cone photopigments is intimately related to the structure of the cone pigment molecules. These are concentrated in the photoreceptor outer segment, a specialized cilium containing the phototransduction machinery (see Fig. 1.2 and Chapter 3). Each pigment molecule consists of a transmembrane opsin (or apoprotein) covalently linked to the same, small conjugated chromophore (11-*cis*-retinal), which is an aldehyde derivative of vitamin A.

All opsins are heptahelical proteins, composed of seven transmembrane helices that are linked together by intra- and extracellular loops. Structural work on the opsin of the rod pigment rhodopsin (Unger & Schertler, 1995), about which we have the most information, indicates that the membrane-embedded helices form a barrel around a central retinal binding pocket (see Fig. 1.2). The binding site of the chromophore in both the cone and rod opsins is located in helix 7, a region that has been relatively conserved during the process of divergent evolutionary change (see the following section).

Photon absorption by the pigment molecules initiates visual excitation by causing an 11-*cis* to all-*trans* isomerization of the chromophore, which activates a transducin G-protein (see Chapter 3). Opsins absorb maximally in the ultraviolet region of the spectrum below 300 nm, whereas retinal absorbs maximally at about 380 nm (Knowles & Dartnall, 1977). It is only by binding together that a broad absorbance band (known as the α-band) in the visible spectrum is created. The λ_{max} of the α-band depends on the geneti-
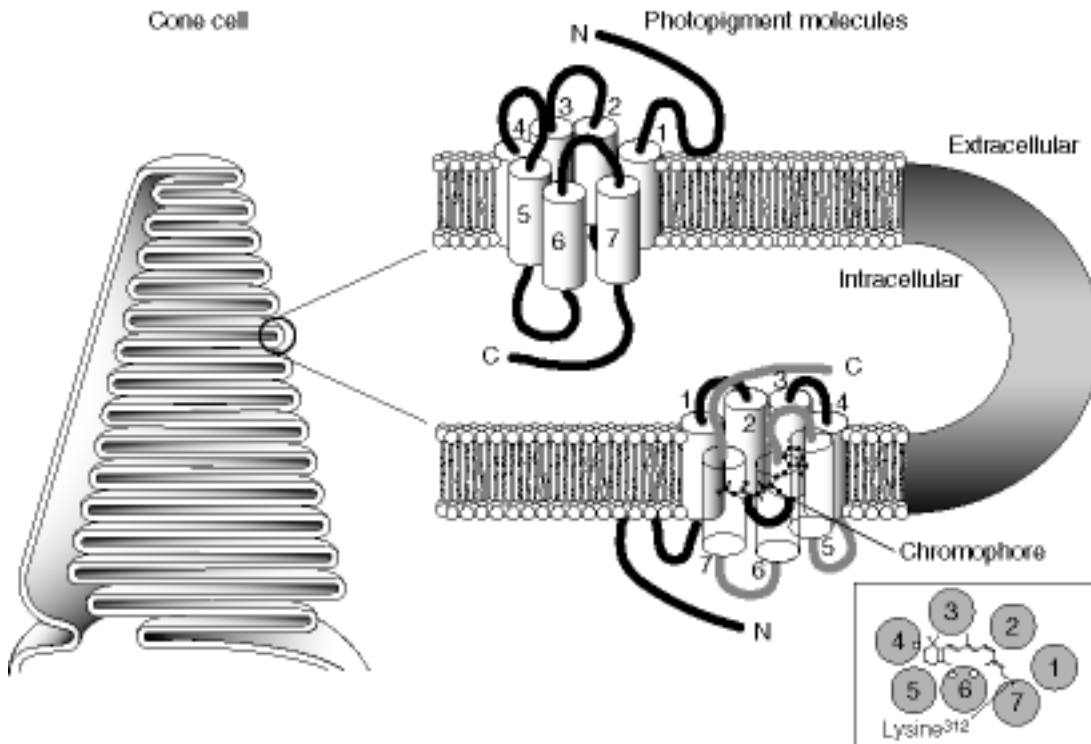
Figure 1.2: Cutaway view of the photopigment molecules (right) packing within the enfolded membrane discs in the outer segments of the cone photoreceptor cells (left). Each molecule consists of a transmembrane opsin bound to a chromophore, 11-*cis*-retinal. The opsin or protein portion of the molecule is a chain of amino acids, running from an amino-terminal end (N), exposed on the external aqueous surface of the membrane discs, to a carboxyl terminal region (C), exposed on the internal aqueous surface of the discs. The chain has seven coils, termed α-helices, spanning the membrane (Hargrave et al., 1984). Linked together by loops in the rest of the chain, the α-helices encircle the chromophore (right, lower cutaway view). The loops are distinguished by whether they occur in the luminal (extracellular) or cytoplasmic (intracellular) face of the cell. The view is from the intracellular surface. It indicates the approximate position of the α-helices and of the three amino acids (open circles) that are believed to have the major influence on the λ_{max} of the pigment (see Fig. 1.5B). The tail of the chromophore is attached by a protonated Schiff base to a charged lysine amino acid residue lying at nucleotide position 312 in the chain of the L- and M-cone opsins (filled circle, see Fig. 1.5B) and at position 293 in the chain of the S-cone opsin (corresponding to position 296 in rhodopsin). Features critical to the function of the opsin are well conserved in all known mammalian species, with the interhelical loops being, on average, more conserved than the transmembrane helical regions.

cally determined amino acid sequence of the opsin and the relationship that the opsin establishes with the chromophore. A second, but lower, absorbance band, known as the β-band, may also be present, which is due to the *cis*-band of the chromophore. The upturn of the L- and M-cone photopigment spectra at very short wavelengths has been interpreted as indicating the

presence of such a β-band (see Fig. 1.1B).

The different opsins of the S-, M-, and L-cone photopigments and of the rod photopigment are encoded by four separate genes. These have been formally identified by the HUGO/GDB (genome data base) Nomenclature Committee as the BCP (blue cone pigment), GCP (green cone pigment), RCP (red cone pigment),

and RHO (rhodopsin) genes. Visual psychophysicists, however, often refer to them as the S-, M-, L-cone and rod pigment genes.

The genes encoding the S-cone and rod pigments reside alone as single copies. The former is found on the long or q-arm of chromosome 7 (Nathans, Thomas, & Hogness, 1986) within a cytogenetic location between 7q31.3 and 7q32 (Nathans, Thomas, & Hogness, 1986; Fitzgibbon et al., 1994) and the latter on the q-arm of chromosome 3, between 3q21.3 and 3q24 (Nathans, Thomas, & Hogness, 1986). In contrast, the genes encoding the L- and M-cone pigments reside on the q-arm of the X-chromosome at location Xq28 (Nathans, Thomas, & Hogness, 1986) within a head-to-tail tandem array (Vollrath, Nathans, & Davis, 1988; Feil et al., 1990), which may contain as many as six gene copies. In general, the L-cone pigment gene is present in only a single copy and precedes the multiple M-cone pigment genes in the array (see the following section). In addition, the array contains the five nested exons of a complete gene (termed TEX28), the first exon of which is located ca. 700 base pairs downstream of the end of the visual pigment gene cluster (see the section on visual pigment gene structure). Extra, truncated (lacking exon 1) nonfunctional copies may be interdigitated between the opsin genes, filling up most of the intervening area. The TEX28 gene is expressed in testes but not in the cone photoreceptor cells (Hanna, Platts, & Kirkness, 1997). It is transcribed in the orientation opposite to the cone opsin genes (see mRNA transcription).

Pigment gene evolution. The reason for the separate chromosomal locations of the opsin genes is unknown, and their evolutionary development is subject to speculation based on comparisons of their sequence homologies (see the section on opsin gene sequence homologies). One plausible alternative, although by no means the only one, is that the three cone opsin genes and human trichromacy evolved in the following steps (see also Chapters 6 and 7; Goldsmith, 1991):

1. The emergence of a primordial opsin gene on the X-chromosome that encodes a pigment with its λ_{max} in

the region conferring the greatest sensitivity to the quantum-intensity–based spectral distribution of sunlight (Dartnall, 1962, but see Lynch & Soffer, 1999) and reflectance of green plants (Lythgoe, 1972). This system formed the basis of the contrast or luminance subsystem of vision, which has a λ_{max} near 555 nm.

2. The emergence of a second opsin gene, about 500 million years ago (Nathans, 1987; Chiu et al., 1994; Hisatomi et al., 1994), located on chromosome 7. Through the accumulation of DNA sequence changes (see the section on opsin gene sequence homologies), it encoded a pigment with its λ_{max} placed at short wavelengths (the S-cone pigment) and expressed it in a different subset of (anatomically distinct) cones from that in which the primordial opsin gene was expressed. The subsequent development of second-order neurons (the yellow-blue opponent color subsystem), which is sensitive to differences in the excitations of the two sets of cones, enabled the discrimination of many forms of natural green vegetation, differing mainly in their reflectances of short-wave light (Hendley & Hecht, 1949; Mollon, 1996).

3. The emergence of a third opsin gene, about 30 to 40 million years ago (Nathans, 1987; Yokoyama & Yokoyama, 1989; Yokoyama, Starmer, & Yokoyama, 1993), as a result of the duplication of the ancestral opsin gene on the X-chromosome. The event copied the transcription unit, but not the locus control region (see the following section). According to one view, the accumulation of DNA sequence changes in the duplicated genes resulted in them encoding distinct M- and L-cone pigments and being expressed in different subsets of (anatomically similar) cones. An alternative view is that the duplication event resulted from unequal crossing over (involving Alu[2] repeats) between two alleles of the ancestral gene that had different spectral sensitivities, so that trichromatic color

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[2]An Alu element is a dispersed repetitive DNA sequence that is about 300 bp in length. The name derives from the restriction endonuclease Alu I that cleaves it. The sequence occurs in about 300,000 copies in the human genome and is believed to have no coding value. An Alu repeat element at the site of insertion of the duplicated opsin gene may have been important in promoting crossing over within the array.

vision did not have to await the accumulation of mutations. Initially, changes in the relative excitations of the two pigments caused by changes in wavelength were undifferentiated, but the subsequent recruitment of existing second-order neurons or the development of new ones (the red-green opponent color subsystem) enabled discriminations in the yellow-green to orange-red spectral region. This duplication event may have occurred in our arboreal ancestors, after the divergence of the Old- and New-World monkeys (see Chapters 6 and 7), as an adaptation to frugivory, assisting the detection of fruit amid foilage (Mollon, 1989, 1991; Osorio & Vorobyev, 1996).

This story is necessarily complicated by the coevolution of the rhodopsin gene, which is similar in structure and sequence to the cone opsin genes. It appears to have derived from the S-cone opsin gene, after the divergence of the latter from the common ancestral gene (Okano et al., 1992). The tight clustering of the λ_{max}'s of almost all vertebrate rhodopsins near 500 nm – the human rod spectral sensitivity measured in vivo peaks at 507 nm and the absorbance spectrum at 493 nm – has so far eluded easy explanation (Goldsmith, 1991). It does not directly correspond to the λ_{max} of starlight, moonlight, or twilight (Lythgoe, 1972).

Visual pigment gene structure. Structurally, each visual pigment gene is a large deoxyribonucleic acid (DNA) molecule, consisting of thousands of subunits – nucleotides – linked together. These are the nucleotide base sequences. Each comprises a nitrogenous base (adenine, guanine, thymine, or cytosine), a phosphate molecule, and a sugar molecule (deoxyribose). Owing to the double-helical structure of DNA, the nucleotide in one DNA strand has a complementary nucleotide in the opposite strand. The two are held together, in nucleotide or base pairs (bp), by weak hydrogen bonds. Adenine (A) conjoins with thymine (T) and guanine (G) with cytosine (C); no other combinations are possible. The base sequences can be divided into promoter, noncoding (intron), and coding (exon) sequences (see Fig. 1.3A).

(i) Transcription unit: The term "transcription unit" is often used to refer to the exons and the intervening

introns to indicate the region that is actually synthesized into messenger ribonucleic acid (mRNA) before being translated into the opsin. The transcription region begins at the start or cap site at the 5' (upstream or head) end of the gene. It is followed by a short leader sequence – 6 bp long in the S-cone pigment gene and 40 bp long in the M- and L-cone pigment genes – that is not translated into the opsin. Downstream of the leader sequence is the start codon, a trinucleotide sequence, ATG, which specifies the initiation of opsin translation. It is paired with a stop codon, TGA, at the 3' (downstream or tail) end, which specifies the termination of opsin translation. In the transcription region, the stop codon is followed by untranslated tail sequences. These include a signal – the polyadenylation or poly (A) site – for the addition of a string of adenosine residues. The exact function of the residues is unknown, but there is evidence that mRNA degradation occurs from the 3' end and that the poly (A) tail together with the poly (A)–specific RNA-binding proteins increases the half-life of the mRNA during translation (see the section on opsin translation).

(ii) Promoters: Promoters are specific regulatory sequences or boxes upstream of the transcription start site. They bind the enzyme (RNA polymerase) that catalyzes the synthesis of the RNA chain, a reaction that is referred to as transcription (see Fig. 1.3). The first promoter sequence, the TATA regulatory box, is ca. 25 bp upstream of the transcription start site. It is involved in binding RNA polymerase via a TATA binding protein. Another promoter sequence, the CCAAT box, is ca. 70–90 bp upstream of the transcription start site. The promoter sequences also interact with transcriptionally active sequences contained in the upstream locus control region (LCR) to regulate the rate of DNA transcription into RNA and hence the amount of opsin gene expression.

(iii) Introns: The intron sequences are silent or noncoding sequences usually believed to have no apparent function (but see the section on intergenic recombination). The possibility that they contain regulatory sequences involved in gene expression, however, cannot be ruled out. They are delimited by recognition
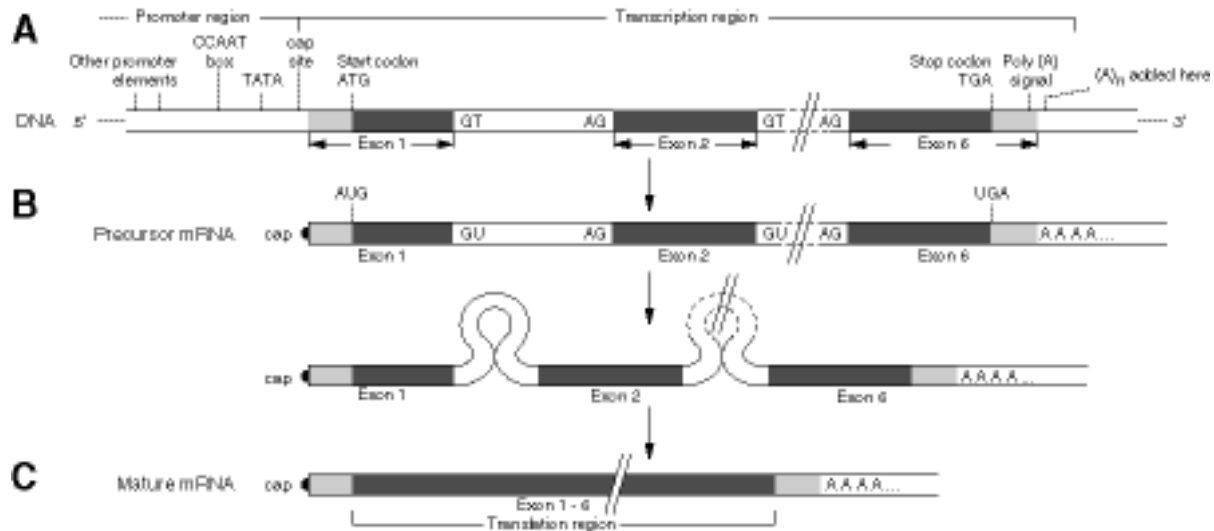
Figure 1.3: Schematic representation of an opsin gene and its transcription into mRNA. (A) The structure of an L-cone opsin gene (DNA), indicating the promoter region, and the untranslated regions at the 5' and 3' ends (gray) the coding regions (black) and the intervening introns (white). (B) and (C) The transcription of the DNA into precursor mRNA and the splicing out of the introns to form mature mRNA. The structure of the mRNA is similar to that of the DNA, except that the sugar molecule is ribose instead of deoxyribose, uracil (U) replaces thymine (T), and the molecule is single-stranded. mRNA is capped or blocked with 7-methylguanosine at its 5' end. There follows a short untranslated region and then the start codon, ATG (AUG), which specifies the initiation of translation. A stop codon, TGA (UGA), indicates the termination of the translated region. Capping and tailing of precursor mRNA precede the splicing out of introns.

sites, which are necessary for identifying and splicing them out from the mRNA precursor (see Fig. 1.3). Introns typically begin with the dinucleotide GT (the splice donor; GU in the precursor mRNA) and end with the dinucleotide AG (the splice acceptor).

(iv) Exons: The opsin-coding sequences are divided into exons, which are separated by the introns and numbered according to their proximity to the 5' end (see Fig. 1.3A). Within the exons, the nucleotide sequences are grouped into triplets – the 3-base sequences or codons – each of which specifies a constituent amino acid (monomer) of the polypeptide chain of the visual pigment opsin (see Table 1.1). There are 64 possible codons (the possible combinations of the four nucleotides), but only 20 unmodified amino acids in the opsin. Thus each amino acid may have more than one codon. Many of the different codons for single amino acids differ only in the third nucleotide of the 3-base sequence.

(v) Gene length: The S-cone pigment gene comprises 5 exons (1,044 bp of which are protein coding) and 4 introns (total length: 2,200 bp). The length of the gene from its mRNA start or cap site (nucleotide base sequence 403) to its poly (A) site (nucleotide base sequence 1,510) is 3,308 bp. (The extra base pairs occur because the exons include 5' and 3' untranslated, nonprotein-coding, sequences that also end up in the mature mRNA; see Fig. 1.3). The M- and L-cone pigment genes each comprise six exons (1,092 bp of which are protein coding) and five introns (total length: 12,036 bp and 14,000 bp, respectively, for the M- and L-cone pigment genes). The length of the M-cone pigment gene is 13,300 bp and that of the L-cone pigment gene is 15,200 bp.

A small, extra exon, encoding only 38 amino acid residues (114 bp), is found at the beginning of the L- and M-cone pigment genes. It may have been added at some point during evolution to the primordial visual

pigment gene to facilitate transcription of more than one gene copy in the tandem array.

mRNA transcription and opsin translation. The base sequences in the DNA are transcribed into RNA, which is subsequently translated to produce the opsin. The primary product in transcription – the mRNA precursor, often called pre-mRNA – contains all of the base sequences, those defining the introns as well as the exons (see Fig. 1.3B). It is blocked or capped with 7-methylguanosine at its 5' end and tailed by a string of adenosine residues at its 3' end. The capping and tailing are believed to permit the export of mRNA from the cell nucleus.

During processing in the cell nucleus, the introns are spliced out, so that the final product in transcription – the mature mRNA (Fig. 1.3C) – only contains the exon sequences. The mature mRNA is exported to the cytoplasm of the photoreceptor cells, where it serves as a template for the synthesis of the opsin from its constituent amino acid residues (see Fig. 1.4). The translation process is complex, involving several stages and a family of transfer RNAs (tRNA), the role of which is to bond with amino acids and transfer them to the ribosome (the site of protein synthesis). The amino acids are assembled sequentially in the growing polypeptide chain of the opsin, from the amino-terminal end to the carboxyl-terminal end, according to the order of codons carried by the mRNA. In the chain, the amino acids are linked by the carboxyl group (COOH) of one amino acid and the amino group (NH_2) of another. Hence, opsins can be identified by their NH_2 (or N) and COOH (or C) ends.

Opsin gene sequence homologies. The nature of the pigment defined by an opsin gene depends on the nucleotide sequences of its exons, which are grouped into triplets (codons) encoding amino acid residues and numbered sequentially beginning with the first codon.

The S-cone pigment gene comprises 348 codons divided over its 5 exons, while the M- and L-cone pigment genes comprise 364 codons divided over their 6 exons. The S-cone pigment gene shows only 43 ± 1%

DNA codons	Amino acid	Abbreviations		Class
GCA, GCC, GCG, GCT	Alanine	ala	A	(1)
AGA, AGG, CGA, CGC, CGG, CGT	Arginine +	arg	R	(4)
GAC, GAT	Aspartic acid -	asp	D	(3)
AAC, AAT	Asparagine	asn	N	(2)
TGC, TGT	Cysteine	cys	C	(1, 2)
GAA, GAG	Glutamic acid -	glu	E	(3)
CAA, CAG	Glutamine	gln	Q	(2)
GCA, GGC, GGG, GGT	Glycine	gly	G	(1)
CAC, CAT	Histidine (+)	his	H	(4, 6)
ATA, ATC, ATT	Isoleucine	ile	I	(1)
TTA, TTG, CTA, CTC, CTG, CTT	Leucine	leu	L	(1)
AAA, AAG	Lysine +	lys	K	(4)
ATG	Methionine (Start)	met	M	(1)
TTC, TTT	Phenylalanine	phe	F	(1, 5)
CCA, CCC, CCG, CCT	Proline	pro	P	(1)
AGC, AGT, TCA, TCC, TCG, TCT	Serine	ser	S	(2)
ACA, ACC, ACG, ACT	Threonine	thr	T	(2)
TGC	Tryptophan	trp	W	(1, 5, 6)
TAC, TAT	Tyrosine	tyr	Y	(2, 5)
GTA, GTC, GTG, GTT	Valine	val	V	(1)
TAA, TAG, TGA	(Stop)			

Table 1.1: The genetic code and the chemical properites of amino acids. The four types of nucleotides forming the deoxyribonucleic acid (DNA) codons are adenylic (A), guanylic (G), cytidylic (C), and thymidylic (T) acid. In ribonucleic acid (RNA) codons, uridylic (U) replaces thymidylic (T) acid. Amino acids belonging to the same class (1–6) are considered homologous and their substitution conservative. (+ or - indicates those amino acids most likely to be positively or negatively charged. The charge of histidine (+) depends on the local environment, and is, therefore, indicated in parentheses.)
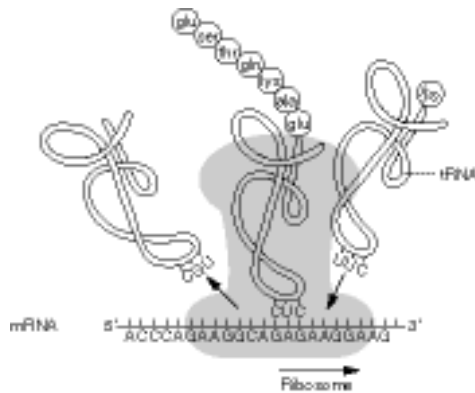
Figure 1.4: The mechanism of translation of the mRNA (from exon 5) into the L-cone opsin. Translation involves tRNA, each of which has a recognition site or triplet nucleotide sequence (anticodon) that is complementary to the triplet nucleotide coding sequence (codon) of mRNA. For example, the tRNA at the right carries lysine and its (RNA) anticodon CUU recognizes the lysine (RNA) codon AAG. Amino acids attach serially to the growing polypeptide chain and the respective tRNAs are jettisoned (as shown at left). The process is mediated by ribosomes (one is shown in outline) moving along the mRNA in a 5' to 3' direction.

amino acid identity with either the M- or L-cone pigment gene (see Fig. 1.5), which is about the same amount of homology with the rod pigment gene (41 ± 1%). In contrast, the M- and L-cone pigment genes show 96% mutual identity for the 6 exons (they are 98% identical at the DNA sequence level if the introns and 3'-flanking sequences are included). From the sequence homologies, it is possible to estimate the evolutionary divergence of the genes: The greater the identity, the more recent the divergence (see the section on pigment gene evolution). A curiosity is that the noncoding intron sequences of M- and L-cone pigment genes are more homologous than the coding exon sequences, even though the former should be freer to diverge than the latter during the course of evolution (see Mollon, 1997).

(i) L- and M-cone exon sequences: There are only 15 codon differences between the L- and M-cone pigment genes (Fig. 1.5B). They are confined to exons 2–5, which encode the seven membrane-embedded α-

helices that together form the chromophore binding pocket (see Fig. 1.2). Three sites are in exon 2 (at codons 65, 111, and 116; for numbering system, see Nathans, Thomas, & Hogness, 1986); two in exon 3 (at codons 153 and 180), three in exon 4 (at codons 230, 233, and 236), and seven in exon 5 (at codons 274, 275, 277, 279, 285, 298, and 309). Six of these differences involve conservative substitutions of hydrophobic residues, which do not influence the interaction of the opsin with the chromophore. Of the remaining nine, one of the sites (codon 116) lies in the first extracellular loop of the molecule; it is therefore unlikely to be involved in a direct interaction with the chromophore. Seven sites, however, lie in the transmembrane helices and may contact the chromophore. These are codons 65, 180, 230, 233, 277, 285, and 309. They involve the substitution of an amino acid residue that lacks a hydroxyl group (a nonpolar or uncharged amino acid) by one that carries a hydroxyl group (a polar or charged amino acid). On theoretical grounds, amino acid substitutions that change the number or locations of polar side chains (e.g., those carrying an hydroxyl group) in the retinal-binding pocket could alter the spectral tuning of the 11-*cis*-retinal chromophore by readjusting its three-dimensional packing or by changing the electrical properties of its immediate environment (Kropf & Hubbard, 1958; Mathies & Stryer, 1976; Hays et al., 1980).

The effect of replacing polar amino acids by nonpolar ones is supported by the results of site-directed mutagenesis experiments in bovine rhodopsin (Chan, Lee, & Sakmar, 1992) and in human hybrid cone pigments (Merbs & Nathans, 1992b; Asenjo, Rim, & Oprian, 1994), as well as by comparisons between electroretinographic (ERG) measurements of primate cone pigment spectral sensitivities and corresponding amino acid sequences (Neitz, Neitz, & Jacobs, 1991). The largest shifts in λ_{max} are produced by substituting alanine for threonine at codon 285 (ca. -14 nm), phenylalanine for tyrosine at position 277 (ca. -7 nm), and alanine for serine at position 180 (ca. -4 nm) (Merbs & Nathans, 1992a). By contrast, substitutions at positions 65, 230, 233, and 309 produce shifts of approximately 1 nm or less at λ_{max} (Merbs & Nathans, 1993).
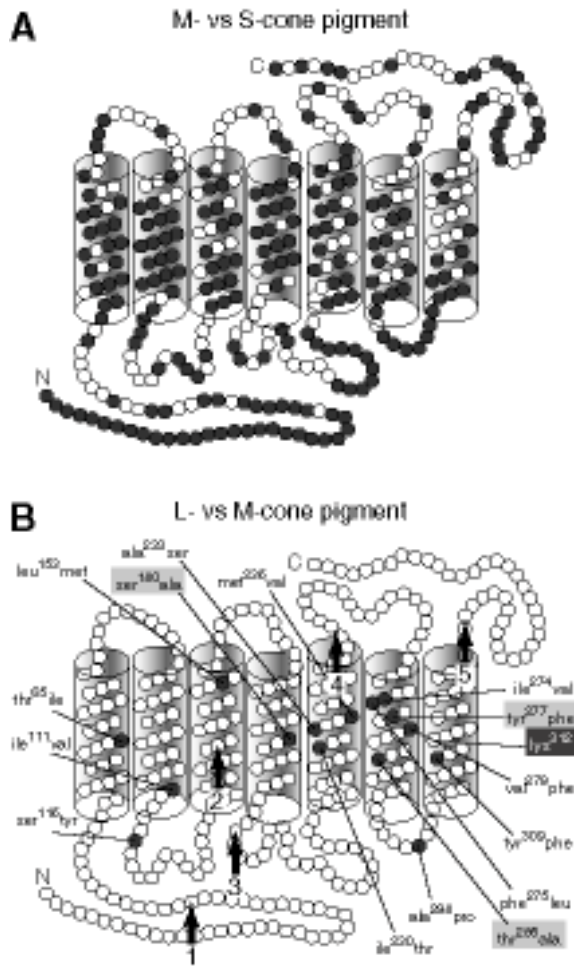
Figure 1.5: Pairwise comparisons of human visual pigment molecules showing amino-acid identities (open circles) and differences (filled circles) (after Nathans, Thomas, & Hogness, 1986). In each representation, the seven α-helices are arranged in a line. When intramembrane regions are optimally aligned, the amino-proximal tails (extracellular face) of the M- (or L-) cone pigments are 16 amino acids longer than for the S-cone pigment. The alignment can be improved by inserting into the M- (or L-) cone pigment sequences gaps of two amino acids and of one amino acid, respectively, at positions 4 residues and 29 residues from the carboxyl terminus. (A) Identity between the M- and S-cone pigments. (B) Identity between the L- and M-cone pigments. The location of lysine[312], the site of covalent attachment of 11-*cis* retinal, and the 15 amino acid substitutions are indicated. The start of each of the 5 intron positions are indicated by numbered vertical arrows. The subsitutions at codons 180, 277, and 285 (highlighted) are believed to contribute the majority of the spectral difference between the M- and L-cone pigments.

Such single amino acid substitution data, however, do not explain the nonadditive shifts in λ_{max} that are observed when more than one hydroxyl group is simultaneously substituted (Merbs & Nathans, 1992b; Asenjo, Rim, & Oprian, 1994), nor do they take into account the influence of multiple aliphatic amino acid differences on side-chain packing (Merbs & Nathans, 1993).

(ii) L- and M-cone 5' and intron sequences: L- and M-cone pigment genes differ not only in their coding sequences, but also in their 5' and intron sequences (Nathans, Thomas, & Hogness, 1986; Vollrath, Nathans, & Davis, 1988). In the vast majority of color normal arrays sequenced in Caucasian males, there is only one L-cone pigment gene, which is longer than all of the other gene copies (see the section on the arrangement of the gene array). It is located at the 5' (upstream) end of the array and abuts single copy DNA sequences, which are not found in front of the other, downstream genes. The length difference arises because its intron 1 *typically* contains 1,612 bp extra sequences (comprising 1,284 bp of three Alu elements and 328 bp of intervening unique-sequence DNA), which are also not found in the downstream genes (cf. the L- and M-cone pigment genes in Fig. 1.7A).

Although the extra intron sequences are found in >99% of Caucasian males, in ca. 45, 35, and 2.5 of African, Afro-American, and Japanese males, respectively, the most proximal gene in the array lacks the extra sequences in intron 1 and is the same size as the downstream M-cone pigment genes (Jørgensen, Deeb, & Motulsky, 1990; Meagher, Jørgensen, & Deeb, 1996). A reason for this may be that it contains inserted (exon 2) M-cone pigment-specific sequences (see the next section).

Normal and hybrid pigment genes. The S-cone opsin gene sequence seems to be nearly invariant in the human population. In contrast, the M- and L-cone opsin genes are diversiform, owing to hybrid variants and shared polymorphisms (see Fig. 1.6).

Hybrids are fusion genes containing the coding sequences of both L- and M-cone pigment genes. They are produced by intragenic crossing over: the breaking

during meiosis of one maternal and one paternal chromosome at the opsin gene locus; the exchange of the corresponding sections of nucleotide sequences; and the rejoining of the chromosomes (see the section on intragenic recombination and Fig. 1.15C).

Intragenic crossing over between the M- and L-cone pigment genes is much more likely to occur within intron sequences than within exon sequences, owing to the approximately tenfold greater size of the introns compared with the exons and the paradoxically greater DNA sequence similarity of the M- and L-cone pigment gene introns compared with the exons (Shyue et al., 1995). Thus, in general, hybrid genes contain some number of contiguous exons from one end of an L-cone pigment gene joined to the remaining exons from the other end of an M-cone pigment gene. Those beginning with L-cone exon sequences are known as 5'L-3'M (or 5'red-3'green) hybrid or fusion genes, and those beginning with M-cone exon sequences as 5'M-3'L (or 5'green-3'red) hybrid genes. The 5'L-3'M hybrid genes encode M or M-like anomalous pigments; whereas the 5'M-3'L hybrid genes encode L or L-like pigments. Therefore, a convenient shorthand terminology for referring to normal and hybrid genes is to identify their exon sequences as being M-cone or L-cone pigment-specific (see Fig. 1.6). Two factors, however, complicate this simple picture.

First, exon 3 is more variable than exons 2, 4, or 5 in its amino acid residues (Winderickx, Battisti, et al., 1993; Sharpe et al., 1998), owing to the existence of several shared genetic polymorphisms between the M- and L-cone pigment genes. Genetic polymorphisms (or dimorphisms, if confined to two forms) are allelic variants of a gene occurring with a frequency greater than 1%. Most of the polymorphisms in exon 3 are confined to a dimorphic subsitution of a single nucleotide sequence. These alter the encoded amino acid without apparently affecting the properties of the photopigment. However, one – the substitution of a serine for alanine residue at codon 180 (the only one involving the substitution of a hydroxyl group) – produces a phenotypic variation. It causes a slight red shift (see below). Current estimates in normal observers suggest that the polymorphism is not equally distributed (see
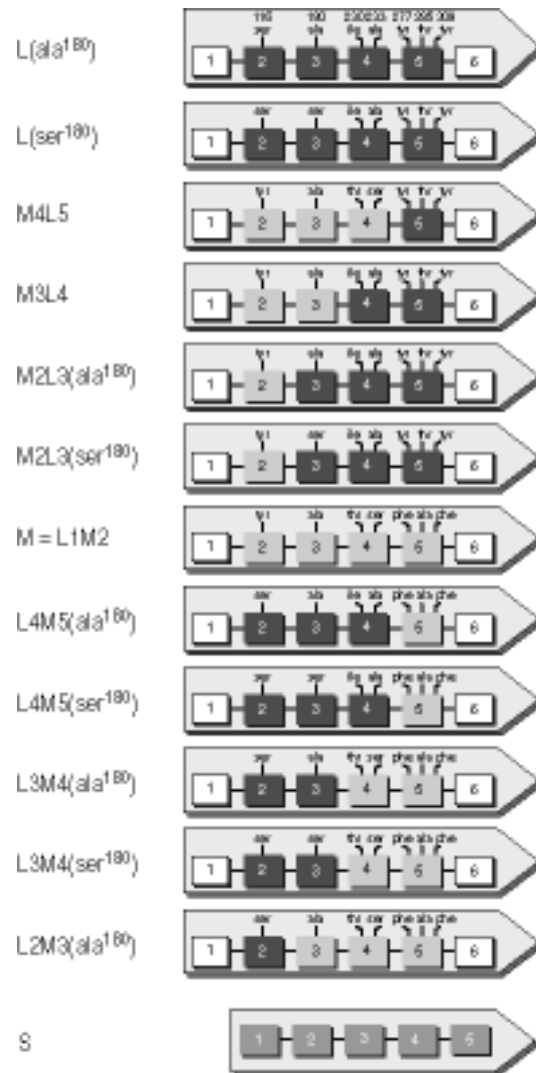
Figure 1.6: Exon arrangement of the S-, M-, L-, 5'L-3'M-hybrid, and 5'M-3'L-hybrid pigment genes. The S-cone pigment gene has one fewer exon, missing from its 5' end, than the X-linked pigment genes. There are no sequence differences between X-linked genes in exons 1 and 6. The 7 amino acid residues indicated above exons 2 to 5 are those responsible for the spectra shift between the normal and anomalous pigments. Dark gray indicates an L-cone pigment gene-specific sequence; light gray, an M-cone pigment gene-specific sequence.

Table 1.2): Among human L-cone pigment genes approximately 56.3% have serine and 43.7% have alanine at position 180, whereas, among M-cone pigment genes approximately 6% have serine and 94% have alanine (Winderickx et al., 1992b; Winderickx, Battisti, et al., 1993; Neitz & Neitz, 1998; Sharpe et al., 1998; Schmidt et al., 1999). However, large variability may occur between groups of different ethnic origin. In one report, 80% of African (N = 56), 84% of Japanese (N = 49), and 62% of Caucasian (N = 49) males had serine at codon 180 (Deeb & Motulsky, 1998). Therefore, it is useful to designate an M- or L-cone pigment gene, by an abbreviation that reflects the identity of the polymorphic residue at position 180 in exon 3.

A further complication is that frequently pigment gene sequences reveal an M-cone pigment gene exon 2 embedded within an L-cone pigment gene or a 5'L-3'M hybrid gene (Sharpe et al., 1998), indicating a complicated history of recombination events, and, therefore, making it pertinent to designate whether exon 2 is derived from an M- or L-cone pigment gene.

Thus, each normal or hybrid gene is more properly referred to by an abbreviation that reflects not only the origin of its various exons, but also the identity of the polymorphic residue at position 180. For example, L-cone pigment genes can be designated $L(ala^{180})$ or $L(ser^{180})$ to indicate the presence of alanine or serine, respectively, at position 180. $L4M5(ala^{180})$ is a hybrid pigment encoded by a gene in which exons 1–4 are derived from an L-cone pigment gene, exons 5 and 6 are derived from an M-cone pigment gene (i.e., the point of crossing over is in intron 4), and position 180 is occupied by alanine. $L(M2;ser^{180})$ is an L-cone pigment gene in which exon 2 is derived from an M-cone pigment gene and position 180 is occupied by serine. The fact that sequence differences between L- and M-cone pigment genes are confined to exons 2–5 implies that an L1M2 hybrid gene encodes a de facto M-cone pigment and, likewise, that an M1L2 hybrid gene encodes a de facto L-cone pigment.

Protein sequence variation and spectral sensitivity. Several in vitro and in vivo techniques have been

Study	Population	Polymorphic residue	Allele frequency
L-cone opsin genes			
Winderickx, Battisti, et al. (1993)	74 normals	ala^{180}	0.440
	35 deutans	ser^{180}	0.560
Sjoberg et al. (1997), see Neitz & Neitz (1998)	130 normals	ala^{180}	0.485
		ser^{180}	0.515
Sharpe et al. (1998)	27 deuteranopes	ala^{180}	0.259
		ser^{180}	0.741
Schmidt et al. (1999)	36 normals	ala^{180}	0.395
	2 deuteranopes	ser^{180}	0.605
Mean	304	ala^{180}	0.437
		ser^{180}	0.563
M-cone opsin genes			
Winderickx, Battisti, et al. (1993)	52 normals†	ala^{180}	0.959
	12 deutan	ser^{180}	0.041
	8 protans		
Sjoberg et al. (1997), see Neitz & Neitz (1998)	130 normals	ala^{180}	0.930
		ser^{180}	0.070
Mean		ala^{180}	0.940
		ser^{180}	0.060

Table 1.2: Polymorphisms in the L- and M-cone opsin genes at codon 180 in human males. For descriptions of deutans, protans, and deuteranopes, see section on color blindness. (†Selected from a larger population of 72; only those were included who had one type of M-cone opsin gene or two types differing by only a single polymorphism.)

applied to studying the variation in normal and, less frequently, hybrid pigment spectral sensitivities (for a review, see Stockman et al., 1999). A partial list, emphasizing studies that have investigated the hybrid pigments, is given in Table 1.3.

The in vitro measurements include ERG, single photoreceptor suction electrode action spectra (see Chapter 4), microspectrophotometry (MSP), and photobleaching difference absorption spectra measurements of recombinant cone pigments produced in tissue culture cells. The in vivo measurements include reflection densitometry, linear transforms of psycho-

Genotype	In vivo	In vitro	
	Sharpe et al. (1998)	Merbs & Nathans (1992a, 1992b)	Asenjo, Rim, & Oprian (1994)
S	418.9 ± 1.5†	426.3 ± 1.0	424.0*
$M(ala^{180}) =$ $L1M2(ala^{180})$	527.8 ± 1.1	529.7 ± 2.0	532 ± 1.0
$L2M3 (ala^{180})$	528.5 ± 0.7	529.5 ± 2.6	532 ± 1.0
$L3M4 (ser^{180})$	531.5 ± 0.8	533.3 ± 1.0	534 ± 1.0
$L4M5 (ala^{180})$	535.4	531.6 ± 1.8	—
$L4M5 (ser^{180})$	534.2	536.0 ± 1.4	538 ± 1.0
$M2L3 (ala^{180})$	—	549.6 ± 0.9	—
$M2L3 (ser^{180})$	—	553.0 ± 1.4	559 ± 1.0
$M3L4$	—	548.8 ± 1.3	555 ± 1.0
$M4L5$	—	544.8 ± 1.8	551 ± 1.0
$L (ala^{180})$	557.9 ± 0.4	552.4 ± 1.1	556 ± 1.0
$L (M2, ala^{180})$	556.9	—	—
$L (M2, ser^{180})$	558.5	—	—
$L (ser^{180})$	560.3 ± 0.3	556.7 ± 2.1	563 ± 1.0

Table 1.3: Absorbance spectrum peaks ($\lambda_{max} \pm$ SD) of the human normal and hybrid cone pigments. (†Value from Stockman et al., 1999; *value from Oprian et al., 1991.)

physical color matching functions (CMFs), and spectral sensitivity measurements in normal and color-deficient observers of known genotype under conditions chosen to isolate preferentially a single cone pigment (see Chapter 2 for a review of measurements of the normal cone absorption spectra).

(i) S-cone pigment: The λ_{max} ($\pm$ the standard deviation) of the human S-cone pigment, measured at the retina (see legend to Fig. 1.1), has been placed at: (i) 419.0 ± 3.6 nm by in vitro MSP of human cones (Dartnall, Bowmaker, & Mollon, 1983); (ii) 424 (Oprian et al., 1991) or 426 nm (Merbs & Nathans, 1992a) by in vitro absorption spectroscopy of recombinant cone pigments; (iii) 419.0 and 419.7 nm by in vivo central

and peripheral spectral sensitivity measurements, respectively, in normal and blue-cone monochromat (see page 41) observers (Stockman, Sharpe, & Fach, 1999); and (iv) 420.8 nm by transforms of the Stiles and Burch 10-deg CMFs (Stockman, Sharpe, & Fach, 1999). Determination of the λ_{max} of the S-cone pigment in solution is complicated by short-wavelength–absorbing bleaching products that partially overlap the pigment absorbance and must be subtracted from it. On the other hand, the in vivo determinations are complicated by several factors, including individual differences in the absorption of the lens and macular pigment (see Chapter 2). Some variability in the λ_{max} of the S-cone pigment has been suggested (Stockman, Sharpe, & Fach, 1999; see p. 44).

(ii) L- and M-cone pigments: The λ_{max}'s of the normal M-, L(ala^{180})-, and L(ser^{180})-cone pigments have been placed, respectively, at: (i) 530.8 ± 3.5, 554.2 ± 2.3, and 563.2 ± 3.1 nm (558.4 ± 5.2 nm for the mixed L-cone pigments) by MSP[3] of human cones (Dartnall, Bowmaker, & Mollon, 1983); (ii) 529.7 ± 2.0, 552.4 ± 1.1, and 556.7 ± 2.1 nm (Merbs & Nathans, 1992a) or 532 ± 1.0, 556 ± 1.0, and 563 ± 1.0 nm (Asenjo, Rim, & Oprian, 1994) by in vitro spectroscopy of recombinant cone pigments; (iii) 530 and 560 nm (mixed L-cone pigments; Schnapf, Kraft, & Baylor, 1987) or 531 (Kraft, private communication), 559.2, and 563.4 nm (Kraft, Neitz, & Neitz, 1998) by suction electrode data; (iv) 528.6 ± 0.5, 557.5 ± 0.4, and 560.2 ± 0.3 nm by foveal spectral sensitivity measurements in dichromat observers (Sharpe et al., 1998, 1999; Stockman & Sharpe, 2000a); and (v) 530.6 and 559.1 nm (mixed L-cone pigments) by transforms of the Stiles and Burch 10-deg CMFs (Stockman & Sharpe, 2000a).

The in vivo spectral sensitivity measurements obtained from dichromats (Sharpe et al., 1998) show a mean separation of ca. 2.7 nm between the L(ala^{180})- and L(ser^{180})-cone pigments. This value is somewhat less than that which has been obtained from site-directed mutagenesis experiments and other tech-

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[3]These values are based on ad hoc subgrouping according to whether the individual λ_{max} lies above or below the group mean and not according to genotype.

niques. However, all of the previous reported values, whether based on inferences from Rayleigh matches (Winderickx et al., 1992b; Sanocki et al., 1993; Sanocki, Shevell, & Winderickx, 1994; He & Shevell, 1994), spectral sensitivities (Eisner & MacLeod, 1981), cloned pigment (Merbs & Nathans, 1992a, 1992b; Asenjo, Rim, & Oprian, 1994), or on the ERG (Neitz, Neitz, & Jacobs, 1995), have shown that serine-containing pigments are red-shifted with respect to alanine-containing pigments. The in vivo estimates accord with other psychophysical measures of the variability of the L-cone λ_{max} in the normal population, based on the analysis of color-matching data by Stiles and Burch (1959), which preclude shifts greater than 3.0 nm (Neitz & Jacobs, 1989, 1990; Webster & MacLeod, 1988; Webster, 1992).

As of yet, no reliable in vivo spectral sensitivity data exist for the two polymorphic variants of the M-cone pigment. However, in vivo comparisons between subjects with a L2M3(ala[180]) pigment and those with a L3M4(ser[180]) pigment, for whom the only important amino acid difference is at position 180, show a mean shift of 3.0 nm (see Table 1.3). Further, the spectral shift between the λ_{max}'s of the M(ala[180])- and M(ser[180])-cone pigments has been estimated provisionally (awaiting in vivo confirmation) at: (i) 5.9 nm by ad hoc analysis of MSP data (Dartnall, Bowmaker, & Mollon, 1983); and (ii) 4.3 to 4.4 nm (Merbs & Nathans, 1992b) and 2 nm (Asenjo, Rim, & Oprian, 1994) by in vitro spectroscopy of recombinant normal and 5'M-3'L hybrid cone pigments.

(iii) Hybrid cone pigments: Estimates of the λ_{max}'s of the hybrid pigments encoded by 5'L-3'M and 5'M-3'L hybrid genes, which presumably underlie anomalous trichromacy (see color blindness), are summarized in Table 1.3. Differences between the in vivo and in vitro estimates probably reflect the limitations of the measuring techniques (see Chapter 2). For instance, the absorption measurements of visual pigment in vitro are accurate within only about 0.5 to 1.0 log unit of the λ_{max} and thus encompass only a limited range of wavelengths. Additionally, they will differ from the in vivo measurements because they do not account for waveguiding in the photoreceptor.

Nonetheless, the in vivo and in vitro data support one another in indicating that both 5'L-3'M and 5'M-3'L hybrid genes encode a range of pigments with spectral sensitivities, which, in every case so far examined, lie between those of the normal L- and M-cone pigments. The data further indicate that the spectral sensitivity of the hybrid pigment depends on the position of the crossing-over and on the identity of the polymorphic amino acids at position 180. For each exon, the set of amino acids normally associated with the L- or M-cone pigments produce, respectively, spectral shifts to longer or shorter wavelengths, thus producing a monotonic relationship between the λ_{max} and the fraction of the hybrid pigment derived from the L and M parental pigments (see Sharpe et al., 1998).

The primary determinants of the spectral shift are located in exon 5, as seen by the clustering of the λ_{max} of all of the pigments encoded by 5'L-3'M genes within 8 nm of the maxima of the normal M pigments. Further, a comparison of the in vivo measured λ_{max}'s in Table 1.3 indicates that the L/M sequence differences in exon 5 – principally the residues at 277 and 285 – result in spectral shifts of 15–25 nm, the exact value depending on sequences in exons 2–4. The in vivo measured data further suggest that substitutions at the sites confined to exons 2–4 produce much smaller spectral shifts: Exon 2 contributes at most 0–2.0 nm; exon 3, 1.0–4.0 nm; and exon 4, 2.5–4.0 nm. These results are in approximate agreement with the in vitro results (Merbs & Nathans, 1992b; Asenjo, Rim, & Oprian, 1994) and with inferences based on a comparison of primate visual pigment gene sequences and cone spectral sensitivity curves (Neitz, Neitz, & Jacobs, 1991; Ibbotson et al., 1992; Williams et al., 1992).

(iv) Sequence variation and opsin viability: Amino acid substitutions may have other consequences than shifting the spectral sensitivity of the X-chromosome–linked opsins. They also could alter the quantum efficiency or the optical density of the pigment. As Williams et al. (1992) point out, in vitro expression studies have noted that some hybrid pigments may be unstable or of reduced optical density (Merbs & Nathans, 1992b; Asenjo, Rim, & Oprian, 1994). Further, both
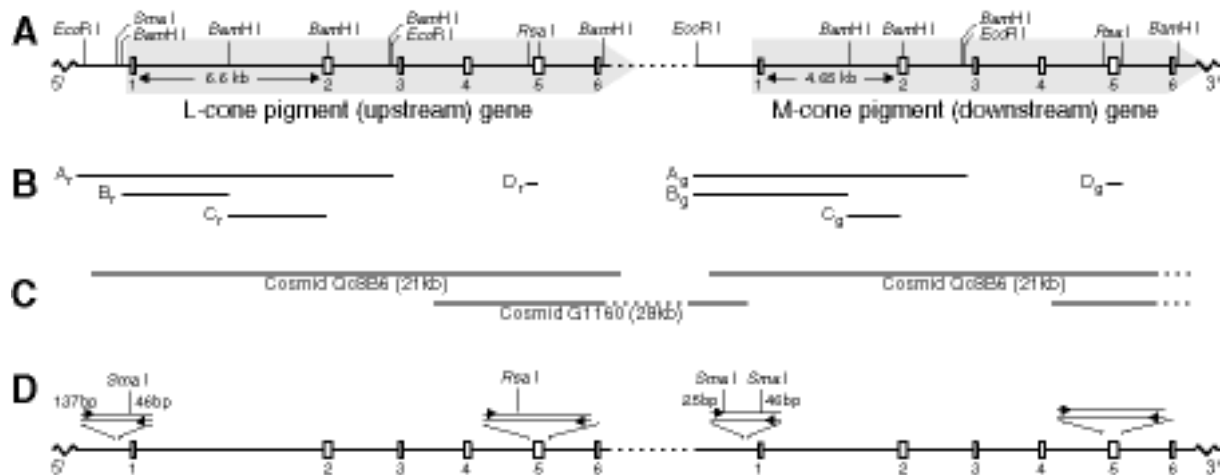
Figure 1.7: A schematic representation of the restriction enzymes and probes used to quantitate or directly visualize opsin genes in the X-chromosome linked visual pigment gene array. (A) The recognition or cleavage sites of the restriction enzymes, *Bam*H I, *Eco*R I, *Hin*d III, *Rsa* I, and *Sma* I, used to define fragment sequences. The exons and intervening introns of a single L-cone pigment gene (upstream) and a single M-cone pigment (downstream) gene are indicated. (B) The L-cone pigment gene restriction fragments, A_r, B_r, C_r, and D_r, and the M-cone pigment gene restriction fragments, A_g, B_g, C_g, and D_g used in the conventional gel electrophoresis/Southern blot hybridization method to estimate the ratios and numbers of L- and M-cone pigment genes in the array (Nathans, Thomas, & Hogness, 1986). (C) Positions of the hybridization pattern of the cosmids, Qc8B6 (Gene Bank Accession number Z68193), and G1160 (Accession number Z46936), used to directly visualize the opsin genes by in situ hybridization. (D) Primer sequences from a 183-bp fragment containing promoter sequences (ca. 50 bp upstream exon 1) and from a ca. 300-bp fragment containing the sequences of exon 5, used by the endlabeled PCR-product method to count the number of genes and the ratio between L-cone and M-cone pigment genes, respectively, in the opsin gene array (Neitz & Neitz, 1995).

psychophysical (Miller, 1972; Smith & Pokorny, 1973; Knau & Sharpe, 1998) and retinal densitometric (Berendschot, van de Kraats, & van Norren, 1996) data suggest that the L- and M-cone pigments, as estimated in deuteranopes and protanopes, respectively (see section on color blindness), differ in optical density.

It is unlikely that other differences between the opsin genes, in particular the extra sequence in intron 1 of the L-cone opsin gene, could contribute to levels of expression and optical density. Although some genes have enhancer sequences in their introns or even 3' of the transcription unit, the available transgenic data for M- and L-cone opsin genes indicate that cone-specific expression is only regulated by the promoter and LCR (however, transgenic expression in the absence of introns does not preclude a role in level of expression). Moreover, most of the extra sequence in

intron 1 of the L-cone opsin gene is made up of Alu repeat elements.

The size of the opsin gene array. The opsin gene array on the X-chromosome varies in size, typically containing more than two opsin genes (Nathans, Thomas, & Hogness, 1986). Its variability in the normal population is a subject of controversy; the differences between investigators have been used to challenge the Young–Helmholtz trichromatic theory of color vision as well as current models about the evolution of the human photopigments (Neitz & Neitz, 1995). At the heart of the controversy are the different quantitation and direct techniques (see Figs 1.7 and 1.8) used to assess the total copy number and ratio of L- and M-cone pigment genes within the array (see Table 1.4).
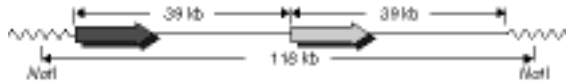
Figure 1.8: The *Not* I fragment that carries the entire opsin gene array, used to determine the total number of genes (Vollrath, Nathans, & Davis, 1988; Macke & Nathans, 1997). It comprises: (i) 40 kb of nonrepeated single-copy flanking DNA; and (ii) repeating units of ca. 39-kb length, each unit consisting of one complete opsin gene (13.2–15.2 kb) and the 24-kb highly conserved flanking region. Thus, the size of the *Not* I fragment will vary in steps of ca. 39 kb; which can be resolved by pulsed field gel electrophoresis. The total gene number will be equal to: (the size of the *Not* I fragment - 40 kb)/39 kb. In the example shown, the 118-kb-long *Not* I fragment contains two genes.

(i) Techniques: The techniques rely on bacterial restriction enzymes (endonucleases) that cleave the opsin gene at specific base sequences to produce fragments that differ between the M- and L-cone photopigment genes (Nathans, Thomas, & Hogness, 1986). When isolated on a suitable filter gel, the restriction fragments can be electrically separated according to molecular weight by conventional gel electrophoresis and visualized by Southern blotting by hybridizing them with radioactively labeled probe DNA that recognizes specific nucleotide sequences.

Targeted regions of interest are several restriction fragment length polymorphisms (RFLP). These are pairs of small fragments cleaved by the *Eco*R I, *Bam*H I, and *Rsa* I enzymes (Fig. 1.7A). They include A_r/A_g, B_r/B_g, C_r/C_g, and D_r/D_g (Fig. 1.7B), one of which is specific to the L- (subscript r for red) and the other to the M- (subscript g for green) cone pigment gene. Their labeled intensities can be quantitated by autoradiography or phosphor-imaging to provide information about the relative number of L- and M-cone pigment genes in the array and, by inference, about the total number of gene copies (Nathans, Thomas, & Hogness, 1986).

A targeted large fragment, which is cleaved by the *Not* I enzyme, carries the entire opsin gene array (see Fig. 1.8). It can be resolved by pulsed field gel electrophoresis and visualized by Southern blotting to pro-

vide direct information about the total number of genes in the array (Vollrath, Nathans, & Davis, 1988; Macke & Nathans, 1997). *Not* I fragments differing in length will separate in an electrophoresis gel according to the number of genes that they contain. The lengths can then be measured in kilobases relative to size standards (e.g., concatamers of bacteriophage λ DNA).

Alternatively, fragments cleaved by the *Sma* I and *Rsa* I enzymes (Fig. 1.7D) can be amplified by using polymerase chain reaction (PCR). Opposite ends of the targeted region of the fragments that differ between the L- and M-cone opsin genes are annealed with primer pairs that recognize specific nucleotide sequences (end-product labeling). The primers are then extended in opposite directions by using a DNA polymerase (an enzyme that catalyzes the synthesis of DNA) to add nucleotide bases to cover the entire targeted area. Repetition of the cycle generates copies of the target DNA between the primers in an exponential manner. Amplified 183-bp fragments from the M- and L-cone gene promoter sequences and ca. 300-bp fragments from exon 5 of the M- and L-cone pigment genes (Fig. 1.7D) can be resolved by gel electrophoresis and quantitated to provide information about the number of genes and the ratio of genes, respectively, in the array (Neitz & Neitz, 1995).

(ii) Results: The techniques involving either quantitation of RFLPs detected by Southern blot hybridization after conventional gel electrophoresis (Nathans, Thomas, & Hogness, 1986; Drummond-Borg, Deeb, & Motulsky, 1989; Schmidt et al., 1999) or quantitation of sequence differences by denaturing electrophoresis that resolves DNA fragments based on nucleotide sequence as well as size (single-strand conformation polymorphism electrophoresis; SSCP) after PCR of M- and L-cone pigment gene promoter sequences (Yamaguchi, Motulsky, & Deeb, 1997) yield smaller copy number estimates than those relying on quantitation of end-labeled restriction products after PCR amplification (Neitz & Neitz, 1995; Neitz, Neitz, & Grishok, 1995; Schmidt et al., 1999). The former studies report an average of three pigment genes, with only a single L-cone pigment copy present and not more than five M-cone pigment copies,

Study	Method	Technique	No.	Mean (± SD)	Range
Nathans, Thomas, & Hogness (1986)	RFLP quantitation	gel electrophoresis/ Southern blotting	18	3.1 ± 0.6	2–4
Drummond-Borg et al. (1989)	RFLP quantitation	gel electrophoresis/ Southern blotting	134	3.2 ± 1.0	2–6
Neitz & Neitz (1995)	RFLP quantitation	endlabeled PCR products	27	4.3 ± 1.9	2–9
Neitz, Neitz, & Grishok (1995)	RFLP quantitation	endlabeled PCR products	26	4.5 ± 1.9	2–9
Yamaguchi et al. (1997)	SSCP quantitation	PCR and SSCP	51	2.9 ± 0.8	2–5
Macke & Nathans (1997)	sizing of the *Not* I fragment	pulsed field gel electrophoresis	67	2.9 ± 0.9	1–5
Wolf et al. (1999)	*in situ* visualization	fiber FISH	8	$3.5 \pm 1.7†$	1–6
Schmidt et al. (1999)	sizing of the *Not* I fragment	pulsed field gel electrophoresis	35*	3.3 ± 1.0	2–6
	RFLP quantitation	gel electrophoresis/ Southern blotting	35*	3.9 ± 1.0	2–6
	RFLP quantitation	endlabeled PCR products	35*	4.3 ± 1.4	2–9

Table 1.4: Typical number of genes in the X-chromosome–linked visual pigment gene arrays of white Caucasian males of unselected or color normal (*) phenotype. Some of the methods overestimate the number of genes (see text). († The population was skewed to include extremes.)

whereas the latter studies (Neitz & Neitz, 1995; Neitz, Neitz, & Grishok, 1995) suggest that nearly 50% of all subjects carry two or more (up to four) L-cone pigment genes, or 5'M-3'L hybrid genes, with some having as many as nine gene copies in total (Table 1.4).

Light on the controversy has been shed, however, by recent developments: (i) the application of direct visualization techniques, including pulsed field gel electrophoretic sizing of *Not* I fragments (Macke & Nathans, 1997; Schmidt et al., 1999; see Fig. 1.8) and the fiber FISH (fluorescent in situ hybridization; Parra & Windle, 1993) protocol (Wolf et al., 1999; see Fig. 1.7C); and (ii) comparisons between the various methods in the same population of individuals (Wolf et al., 1999; Schmidt et al., 1999). The two direct procedures, which agree exactly in their results on the same individuals (Wolf et al., 1999), demonstrate that on average a typical array contains three pigment genes. Further, they suggest that reports of frequent occurrences of larger arrays, including those with two or more (up to four) L-cone-pigment genes (Neitz & Neitz, 1995; Neitz, Neitz, & Grishok, 1995), may reflect technical artefacts that are inherent in PCR

methods (Macke & Nathans, 1997; Yamaguchi et al., 1997; Schmidt et al., 1999) and ambiguities arising from an inability to distinguish 5'M-3'L hybrid genes from L-cone pigment genes.

Examples of visual pigment gene arrays differing widely in gene number are shown in Fig. 1.9. It presents digitized images of single DNA fibers that have been subjected to dual-color FISH, using the cosmids[4] Qc8B6 and G1160 as probes (see Fig. 1.7C; Wolf et al., 1999). In the images, identification by gene or exon type is not possible because each gene is pseudocolored red and each intergenic region – the ~25-kb region at the downstream or 3' end of each gene – is pseudocolored green. The first (upper) fiber is from a deuteranope (see color blindness). His hybridized fibers exhibit one red and one green signal, indicating the occurrence of a single gene copy. The second, third, and fourth fibers are from trichromats, whose hybridized fibers display two, three, and six gene cop-

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[4]Cosmids are artificially constructed cloning vectors containing the cos site of bacteriophage λ. They permit cloning of larger DNA segments than can be introduced into bacterial hosts in conventional plasmid vectors.
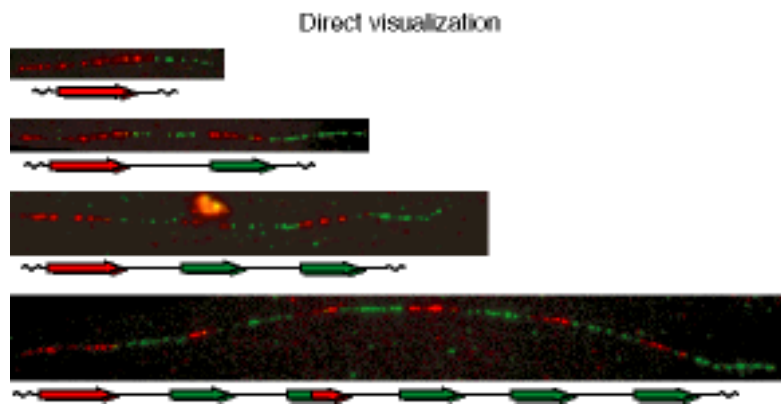
Direct visualization

Figure 1.9: Opsin gene array. Single hybridized DNA fibers of four individuals with differently sized opsin gene arrays, containing one, two, three, and six genes (top to bottom). The genes appear red; the intergenic regions, green. The magnification factor is reduced for the six-gene array. For clarity, the location of the genes (arrows) and intergenic regions (straight lines) in each array are schematically depicted below the hybridized images. The color coding has been altered so that red now refers to the L-cone pigment gene, green to the M-cone pigment gene, and red-green to a 5'M-3'L hybrid gene.

ies, respectively. In each array, the upstream (5') end can be clearly identified because of the characteristic greater length of the most proximal gene.

The arrangement of the gene array. Precise sizing of the number of genes in the array is the important first step in determining the composition of the array. The next steps are to determine the L- to M-cone pigment gene ratio and order of the array. Owing to the high (96%) sequence homology of the L- and M-pigment genes, it is not yet possible to do either of these by direct procedures.

(i) Techniques: Information about the L- to M-cone pigment gene ratio can be obtained by RFLP quantitation of the relative band intensities of fragment pairs. However, the interpretation of these procedures is complicated by the difficulty in distinguishing normal from hybrid genes on the basis of limited restriction fragment pairs (it often requires looking at the D_r/D_g fragments, which encompass exon 5; see Fig. 1.7B) and by potential artefacts such as the background level of radioactivity in the gels and the separation of peaks for the fragment pairs. No procedure is currently available for strictly determining the order of genes in the array, regardless of the array size. A prerequisite for nucleotide sequencing would be the stable propagation in *E. coli* or yeast of large cloned segments with multiple pigment genes. This has not yet been demonstrated. However, very recently Hayashi et al. (1999),

using long-range PCR amplification of a 27.4 kb opsin gene fragment, have been able for the first time to completely define the order of gene types in a three gene array (see section on deuteranomaly). This feat is achieved by employing standard techniques to define the most 5' (upstream) gene and also to define the types of genes present without regard to their order in the array; and by employing the long-range PCR to define the most 3' (downstream) gene in the array.

(ii) Results: Generally, the RFLP quantitation methods support the interpretation that there is only a single L-cone pigment gene in the array, occupying the most proximal position, followed by one or more M- or 5'M-3'L hybrid pigment genes (see Yamaguchi et al., 1997; Schmidt et al., 1999). In the Caucasian male population, the range appears to be one to five M-cone pigment gene copies, with a mean of two (Macke & Nathans, 1997; Schmidt et al., 1999). In non-Caucasian populations, both the range and mean are smaller: About one-half of Japanese (48.5%) and Afro-American (42%) males have a single downstream M-cone pigment gene as opposed to about one-fifth (22%) of Caucasian (Jørgensen et al., 1990; Deeb et al., 1992).

The reason for multiple M-cone pigment genes in the array is unclear. It has been speculated that variations in the L- to M-cone ratio in the photoreceptor mosaic (see Fig. 1.1C and Chapter 6) may be related to the number of M-cone pigment genes in the array: The higher the number of M-cone pigment genes, the

greater the number of M-cones. However, Nathans et al. (1992) failed to find any correlation between the relative sensitivities to red and green spectral lights and the number of M-cone pigment genes.

Examples of the variation in pigment gene arrangment are shown in Fig. 1.9 below the hybridized images. The inferred arrangments are based on the FISH results combined with direct sequencing or indirect RFLP quantitation. In the first array, sequencing of exons 2 to 5 establishes that the single gene has only the L-cone pigment-specific sequences (Sharpe et al., 1998). The presence of a single L-cone pigment gene accords with the phenotype, deuteranopia (see page 27). In the second (two gene copies) and third (three gene copies) arrays, RFLP quantitation (based on the A, B, and C fragment pairs) establishes a 1:1 and 1:2 L- to M-cone pigment gene ratio, respectively (Schmidt et al., 1999). Further, the phenotype in both is normal. Therefore, it is reasonable to infer that in both a single L-cone pigment gene is followed by normal M-cone pigment genes. In the fourth (six gene copies) array, RFLP quantitation (based on A and C fragment pairs) establishes a 1:3 L- to M-cone pigment gene ratio; and examination of the B and D fragment pairs reveals a 5'M-3'L hybrid gene (Schmidt et al., 1999). Thus, an L-cone, a 5'M-3'L hybrid, and four M-cone pigment genes are probably present. Because the phenotype is normal, the hybrid gene has been placed at an arbitrary position downstream from a normal M-cone pigment gene (its actual position cannot yet be determined). The placement is consistent with models of gene expression in the array that are described below.

Gene expression in the array. Which opsin genes in the array are actually expressed in the cone photoreceptor cells, quite apart from the number of genes available in the array, can be determined by assessing the ratio of gene mRNA transcripts in extracts of retina. This involves reverse-transcribing cellular RNA into cDNA and then amplifying and quantitating different sequence variants.

mRNA analysis, however, is limited not only by problems inherent to quantitation, but also by two other factors. First, the method can only differentiate whether genes of different types are expressed. That is, if there are five M-cone pigment genes in the array, all with the same sequences, it is not possible to say whether only one or all five are expressed. Second, the method cannot take into account variations in the levels of expression of the gene among individual cone photoreceptor cells. For instance, a difference in the ratio of mRNA transcripts may reflect differences in the number of cones containing L-pigment as compared with the number containing M-pigment, or it may reflect differences in the amount of pigment contained in L-cones as compared with the amount contained in M-cones (e.g., a difference in photopigment optical density; see Chapter 2).

Two issues have been investigated by mRNA transcript analysis: (i) selective expression of the visual pigment genes (Are all genes in complex arrays expressed?) and (ii) differential transcription of the expressed genes (Are L-cone pigment genes more frequently expressed than M-cone pigment genes in the cone photoreceptors?).

(i) Selective expression: Winderickx et al. (1992a) detected in male donor eyes only two retinal mRNA transcripts: one coding for an L-cone pigment and the other for an M-cone pigment. In those donors who had two or more M-cone (or 5'M-3'L hybrid) pigment genes, only one allele was represented in the retinal mRNA. Yamaguchi, Motulsky, and Deeb (1997) and Hayashi et al. (1999) subsequently confirmed and extended these findings. In donors of unknown phenotype with a 5'M-3'L hybrid gene in addition to normal L- and M-cone pigment genes in their array, either (i) the normal L- and M-cone pigment genes were expressed, but not the hybrid gene, or (ii) the normal L-cone and the hybrid pigment genes were expressed, but not the normal M-cone pigment gene. What is decisive is the position of the M and 5'M-3'L hybrid pigment genes in the array. None of these studies found evidence of the presence or expression of more than one L-cone pigment gene (see deuteranomaly). On the other hand, Sjoberg, Neitz, Balding, and Neitz (1998) reported that about 10% of men express more than one L-cone pigment gene. It is unclear, however,

whether the authors, when they refer to extra L-cone pigment genes, are describing L-cone pigment genes or 5'M-3'L hybrid pigment genes that express an L-cone–like pigment. Regardless, they reject the hypothesis that only two opsin genes from one X-chromosome array can be expressed.

(ii) Differential transcription: Yamaguchi, Motulsky, and Deeb (1997) found, in extracts of whole retina, that the ratio of expressed L- to M-cone opsin retinal mRNA varies widely (from unity to ten times greater L-cone opsin expression, with a mode of four) and is not correlated with the ratio of L- to M-cone opsin genes (Yamaguchi et al., 1997). Hagstrom, Neitz, and Neitz (1997), looking at 6-mm-diameter patches of retina (corresponding to about 20 deg of visual angle), reported that the average ratio of L- to M-cone opsin mRNA in patches centered on the fovea was roughly 1.5:1.0, whereas in patches centered at 12 mm (ca. 41 deg) eccentricity it increased to 3.0:1.0 (see also Hagstrom et al., 1998). There were, however, large individual differences among eyes examined: The L- to M-cone opsin mRNA ratios in the fovea patches differed by a factor of greater than 3.

A favoring of L- over M-cone pigment expression, in both the fovea and retinal periphery, is supported by other indirect evidence, including psychophysical spectral sensitivity measurements (e.g., DeVries, 1948a; Brindley, 1954a; Vos & Walraven, 1971; Kelly, 1974; Walraven, 1974; Smith & Pokorny, 1975; Cicerone & Nerger, 1989; Vimal et al., 1989, 1991; Pokorny, Smith, & Wesner, 1991; Wesner et al., 1991; Cicerone et al., 1994), retinal densitometry (Rushton & Baker, 1964), flicker electroretinography (Shapley & Brodie, 1993; Usui et al., 1998), and MSP of human cones (Bowmaker & Dartnall, 1980; Dartnall, Bowmaker, & Mollon, 1983). Taken together, the mean ratios yielded by these methods suggest that there are roughly twice as many L- as M-cones in the central fovea. However, it should be pointed out that individual ratios that are estimated by such methods are highly variable between observers (ranging from 0.33:1 to 10:1) and further that each method, other than MSP, has serious problems of interpretation (see Chapters 2 and 4).
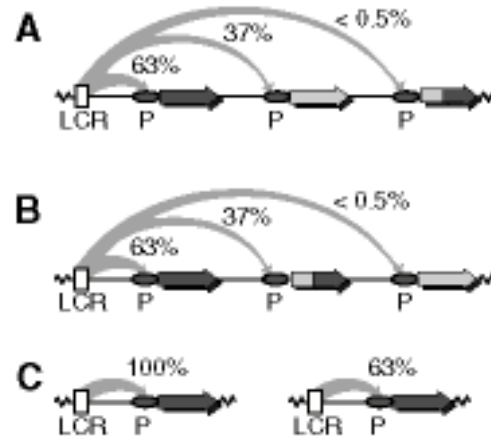

Figure 1.10: Model of opsin gene expression (after Nathans et al., 1989; Winderickx et al., 1992a). (A) Gene expression in an opsin gene array in which a 5'M-3'L hybrid gene (encoding an anomalous L-cone–like pigment) occupies a distal position relative to the normal L- and M-cone pigment genes. An individual with such an array would test as color normal. The expression ratios shown are based on the presumed 1.5 L- to M-cone ratio in the foveal photoreceptor mosaic (see Fig. 1.1C and Chapter 5). (B) Gene expression in an opsin gene array in which the 5'M-3'L hybrid gene occupies a proximal position relative to the normal M-cone pigment gene. An individual with such an array would test as deuteranomalous or deuteranopic, depending on the fusion point of the hybrid gene. This assumes that if the third gene in the array is expressed ($< 0.5\%$), it is not expressed in sufficient amounts to predominate over the upstream genes to enable normal color vision. (C) Two possibilities for gene expression in the reduced, single-gene array of a deuteranope (see section on color blindness). The alternatives are related to the missing cone and replacement cone models in dichromats. (LCR = locus control region; P = promoter

(iii) A model: A possible model of gene expression that incorporates selective expression and differential transcription is shown in Fig. 1.10 (based on Nathans et al., 1989; Winderickx et al., 1992a). The presence of a locus control region (LCR, see blue-cone monochromacy) located between 3.1 and 3.7 kb upstream (5') of the transcription inititation (cap) site of the most proximal gene in the array is known to be required for cone photoreceptor-specific expression (Nathans et al., 1989; Wang et al., 1992; Nathans et al., 1993). LCRs