The EMBO Journal vol.11 no.6 pp.2039-2045, 1992

The polymorphic photopigments of the marmoset: spectral tuning and genetic basis

Andrew J.Williams, David M.Hunt, James K.Bowmaker[1] and John D.Mollon[2]

School of Biological Sciences, Queen Mary and Westfield College, University of London, [1]Department of Visual Science, Institute of Ophthalmology, University of London and [2]Department of Experimental Psychology, University of Cambridge, UK

Communicated by B.Boycott

The marmoset (*Callithrix jacchus jacchus*), a South American monkey, is polymorphic for the middle- to long-wave cone photopigments: the three variant pigments have spectral peaks at 543, 556 and 563 nm. Comparisons of the deduced amino acid sequences of these pigments indicate that the variations in spectral sensitivity are associated with the presence or absence of hydroxyl-bearing residues at sites 180 and 285; but, in contrast to the additive hypothesis of Neitz *et al.* (1991), we propose that adjustments at site 233 may also be required to produce viable long-wave and middle-wave pigments. Within a family group of monkeys, we find that a restriction site polymorphism in the photopigment gene segregates in a way that is consistent with the single X-linked gene hypothesis previously proposed on the basis of the photopigment types present in male and female marmosets.

Key words: colour vision/photopigment genes/primates

Introduction

Animal vision is mediated by a class of proteins, the opsins, which bind retinal, the aldehyde of vitamin A, to produce light-sensitive visual pigments. Opsins are integral membrane proteins composed of a single polypeptide chain (consisting of 364 amino acids in the long-wave and middle-wave cone opsins in primates) that is thought to form seven α-helical, hydrophobic membrane-spanning regions linked by straight-chain extra-membrane hydrophilic loops. These seven helices form a bundle or palisade within the membrane, creating a pocket in which retinal is bound by a Schiff's base linkage to a lysine residue in the centre of the seventh helix (Applebury and Hargrave, 1986). The wavelength at which retinal absorbs maximally is displaced by the opsin, and can lie anywhere from the near ultraviolet to the red spectral region (Bowmaker, 1991).

In Old World primates including man, three distinct photopigments with maximal sensitivities at 420–430 nm (blue-sensitive, short-wave pigment), 535 nm (green-sensitive, middle-wave pigment), and 565 nm (red-sensitive, long-wave pigment) are present in separate populations of cones (Dartnall *et al.*, 1983; Bowmaker *et al.*, 1991) and trichromatic vision is achieved by the neural comparison of the quantum catches in these different cones. The opsin component of each of the three pigments is coded by a separate gene: the gene for the short-wave photopigment is on chromosome 7 whereas the middle-wave (MW) and long-wave (LW) photopigments are coded by closely linked genes on the X chromosome (Vollrath *et al.*, 1988; Feil *et al.*, 1990). The juxtaposition and close homology of the MW and LW genes (Nathans *et al.*, 1986) suggests a relatively recent duplication from a single ancestral gene and supports the idea that trichromacy in Old World primates evolved from a dichromatic form of colour vision in which there was only a single photopigment sensitive in the red/green spectral region (Ladd-Franklin, 1892; Jacobs, 1981; Bowmaker, 1991; Mollon, 1991).

A form of colour vision that more closely resembles this ancestral type is seen in New World primates. In all platyrrhine monkeys examined so far (Mollon *et al.*, 1984; Jacobs and Neitz, 1985, 1987; Jacobs *et al.*, 1987), the males possess only a single photopigment sensitive in the red/green region of the spectrum. However, within a species the pigment is polymorphic (Bowmaker *et al.*, 1984, 1985; Jacobs, 1984; Travis *et al.*, 1988; Neitz *et al.*, 1991), with at least three spectrally different forms present. Individual male monkeys possess only one of the three forms of the photopigment whereas females may similarly have only one or may possess any two of the three. To account for these observations, it has been proposed (Mollon *et al.*, 1984) that, in contrast to the situation in Old World monkeys, these pigments are encoded by a single opsin gene on the X chromosome. Random X inactivation (Lyon, 1962) of one or other of the two X chromosomes in females will account for the presence of only a single pigment in the outer segments of individual cones. Thus females homozygous at the locus will possess only a single spectral class of MW/LW cones whereas heterozygous females will exhibit two spectrally distinct classes of MW/LW photoreceptor.

Only a limited number of amino acids in the inner face of the helices are thought to be critical in determining the spectral characteristics of these photopigments (Kosower, 1988), so that it should be possible to correlate their spectral differences with specific amino acid substitutions. In man, the MW and LW opsins differ at 15 sites (Nathans *et al.*, 1986), though probably only seven of these are candidates for a role in spectral tuning, since they are located in transmembrane regions and exhibit non-homologous amino acid substitutions in which a hydroxyl group is present in one opsin and absent in the other. These hydroxyl groups are thought to either stabilize or destabilize the ground state relative to the excited state and thus shift the maximum absorbance of the pigment to shorter or longer wavelengths. Neitz *et al.* (1991) have recently put forward the hypothesis that substitutions at only three of these sites are responsible for the spectral difference between the human MW and LW pigments.

The marmoset *Callithrix jacchus jacchus* is an example of a New World monkey that exhibits three photopigments sensitive in the MW/LW spectral region, with absorbance

maxima at 543, 556 and 563 nm (Travis *et al.*, 1988; Tovée *et al.*, 1992). In this study, we relate amino acid substitutions in the opsins to the spectral characteristics of the three photopigments, and from the presence of a restriction enzyme site polymorphism in a family group, we show that the segregation of the opsin component of the photopigment is consistent with the presence of a single polymorphic X-linked gene.

Results

Spectral phenotypes

Spectral phenotypes were assigned to animals after microspectrophotometric determination of the wavelength of maximal absorbance of the long-wave cones (Table I). Marmosets A1, A3, A5, A6, A7, B2, B4, B5 and C3 were used in a previous behavioural study where full details of the absorbance spectra are given (Tovée *et al.*, 1992). CN58 and CN59 were additional animals of the 543 phenotype since no male animals of that phenotype were present in the family groups of Tovée *et al.* (1992). The curves presented in Figure 1 are the mean absorbance spectra of the male records in Table I.

Amplication and sequencing of the three spectral phenotypes

The MW and LW opsin genes in man comprise six exons with exons 2–5 coding for the seven transmembrane regions of the protein (Figure 2). Since it is believed that only amino acids in these regions are important in determining the spectral differences between the two pigments (Nathans *et al.*, 1986), sequencing of the marmoset genes was restricted to these exons. The polymerase chain reaction (PCR; Mullis *et al.*, 1986) was used to amplify regions of the exons from the male animals shown in Table I, and the resulting nucleotide sequences were used to deduce the amino acid sequences of the three opsin variants. The results are shown in Figure 3, together with the equivalent regions of the human MW and LW opsin sequences.

Amino acid differences between the three sequences are present at eight sites (Table II). Of these, five are unlikely to be involved in spectral tuning: positions 115 and 116 are not in a transmembrane region (Kosower, 1988) and the substitutions at positions 97 (isoleucine or valine), 173 (valine or isoleucine) and 229 (phenylalanine or isoleucine) are considered to be homologous (Lehninger, 1982). Non-homologous substitutions in transmembrane regions are found at positions 180, 233 and 285. In each case, a hydroxyl-bearing amino acid is either present or absent in one or other pigment.

Transmission of the opsin gene in a marmoset family group

A family group of monkeys segregating for the 543 and 563 variants was used to examine the pattern of inheritance of the photopigment gene. Animals 1, 3, 5, 6 and 7 of the family group shown in Figure 4 are identified with an A prefix in Table I. The members of this family group have been the subject of a previous study (Tovée *et al.*, 1992) where details of their behaviourally determined colour vision and the spectral characteristics of their photopigments are reported.

The presence of a unique restriction enzyme site in the DNA sequence of one of the spectral phenotypes has enabled us to correlate the segregation of the photopigment with a particular DNA haplotype within the family group. In exon 5 of the 543 phenotype an *Rsa*I restriction enzyme site is created at position 831 by the substitution of a C for a T (Figure 4); the presence or absence of this site in PCR-amplified fragments of exon 5 was used to determine the presence or absence of the 543 gene. Exon 5 was amplified from genomic DNA of five members of the family group and the resulting fragments digested with *Rsa*I. In the presence of the *Rsa*I site, two fragments of 129 and 61 bp are produced whereas, in its absence, a single undigested fragment of 190 bp is seen.

As shown in Figure 4, the *Rsa*I site is always associated with the presence of a 543 pigment. Thus, in animal 5 with the 543 phenotype, only the smaller *Rsa*I-digested fragments are present whereas in animals 6 and 7 with the 563 phenotype, the site is absent and only the larger undigested fragment is seen. In females 1 and 3, classified by microspectrophotometry as possessing both pigments (in separate receptors), both digested and undigested fragments are seen,

Table I. Microspectrophotometry of long-wave cones in individual marmosets

Animals	Number of cones analysed	λ_{max}	Standard deviation	Spectral phenotype
Males				
A6	16	562.0	2.7	563
A7	21	563.4	3.3	563
B5	27	564.2	3.4	563
B2	24	556.0	4.0	556
B4	22	556.7	3.0	556
C3	28	555.7	3.3	556
CN58	10	543.3	2.5	543
CN59	11	541.7	2.3	543
Females				
A1	11	540.2	3.6	543/563
	3	566.3	1.0	
A3	16	543.4	2.9	543/563
	20	563.1	2.7	
A5	42	543.6	3.2	543

The λ_{max} value is an average for all the records that passed the selection criteria of that class of cone for each animal. Data for animals A1, A3, A5, A6, A7, B2, B4, B5 and C3 are from Tovée *et al.* (1992).

confirming the presence of both the 543 and 563 forms of the gene, whereas female 5 is homozygous for the 543 allele. From this, the genotype of the male parent 2 can be inferred as 543 (although this animal died before any data could be obtained). The overall pattern of segregation is consistent with the presence of a single X-linked opsin gene, and indicates that the different spectral phenotypes are determined by different allelic variants of this gene.

Discussion

Marmosets possess visual pigments maximally sensitive in the middle to long-wave region of the spectrum that are thought to be coded by different allelic forms of a single gene (Mollon *et al.*, 1984). Individual male animals have dichromatic colour vision and express only one of the three spectral forms of the pigment, with maximum sensitivity at either 543, 556 or 563 nm (Travis *et al.*, 1988; Tovée *et al.*, 1992). The sequencing of a substantial part of the corresponding opsin gene from animals expressing one or other of these three spectral phenotypes has confirmed that each is coded by a different form of the gene. The deduced amino acid sequences show that the 563 and 556 opsins differ at five sites, and the 556 and 543 at three sites.

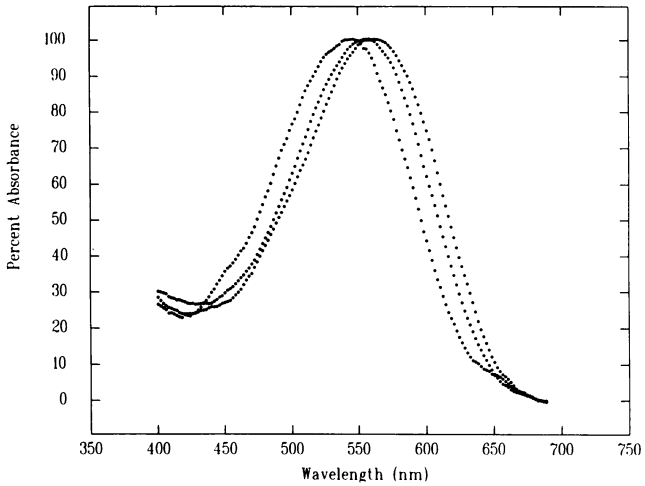

Fig. 1. Microspectrophotometric absorbance spectra for the three marmoset visual pigments. The three MW/LW photopigments depicted show peaks at 543, 556 and 563 nm.

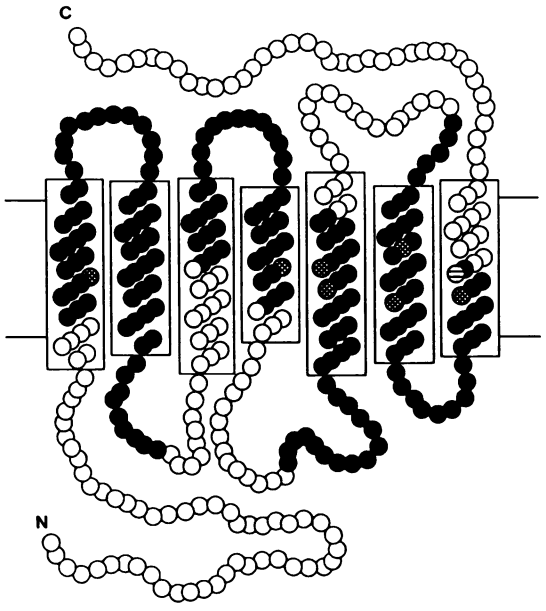

Fig. 2. A two-dimensional model of the cone visual pigment. The regions of the molecule that correspond to the DNA regions sequences in this study are identified by filled circles. The position of the chromophore binding site is indicated by a lined circle and the seven candidate sites for spectral tuning by hatched circles.

It has been proposed (Nathans *et al.*, 1986) that the spectral differences between photopigments depend on the presence or absence of hydroxyl-bearing amino acids at certain positions relative to the chromophore. In the marmoset, the only non-homologous substitutions in membrane-spanning regions present amongst the three opsin alleles are at sites previously identified in the human MW and LW genes as potential sites for spectral tuning. It would appear therefore that spectral tuning depends on relatively few sites, perhaps as a result of structural restrictions that limit interactions with the chromophore.

In the marmoset, the substitution of alanine for threonine at position 285 differentiates the 543 opsin from the 556 and 563 opsins and may account for the 13 nm shift from 543 to 556 nm. A similar substitution at position 285 differentiates the 556 and 541 opsins in another callitrichid, the saddle-backed tarmarin, *Saguinus fuscicollis* (Neitz *et al.*, 1991). The 563 opsin is distinguished from the other two by non-homologous substitutions at two sites, position 180 (serine for alanine) and position 233 (glycine for serine) so that the 7 nm shift from 563 and 556 would appear to depend on either one or both substitutions. However, since the closely related saddle-backed tamarin lacks the glycine/serine difference at position 233 (Neitz *et al.*, 1991), this site may not be important for spectral tuning, the entire 7 nm shift from 556 to 563 nm being achieved by substitution at position 180. This conclusion is consistent with the hypothesis put forward by Neitz *et al.*, (1991) from a study of the amino acid sequences of opsins from two other species of South American monkey and a human deuteranope.

Contrary to this hypothesis, however, and suggestive of a role for site 233 in spectral tuning, is the degree of conservation of the amino acids present at this site in the MW and LW photopigments of different species. In the case of man, alanine is present at site 233 in the LW pigment and serine in the MW pigment. All six species of Old World monkey examined by Ibbotson *et al.* (1992) similarly exhibit a non-polar amino acid at site 233 in the LW pigment and a hydroxyl-bearing amino acid at the corresponding position in the MW pigment, although in the latter case the hydroxyl-bearing residue is threonine, rather than serine as in man. It is interesting that similar differences are present in the sequences inferred from the MW- and LW-like genes of the blind cave fish, *Astyanax fasciatus* (Yokoyama and Yokoyama, 1990). In the case of those New World monkeys that do exhibit a variation at site 223 (*Saimiri sciureus* and *Callithrix jacchus jacchus*), it is a non-polar amino acid that is present in the cone opsin with the longest λ_{max} value and a hydroxyl-bearing residue in the opsin with the shortest. Thus, the 561 and 532 nm pigments of *Saimiri* exhibit respectively alanine and serine at site 233 (Neitz *et al.*, 1991), whereas the present study shows the same difference between the 563 and 543 nm pigments of *Callithrix*. One rule does emerge from all ten of the primate species so far

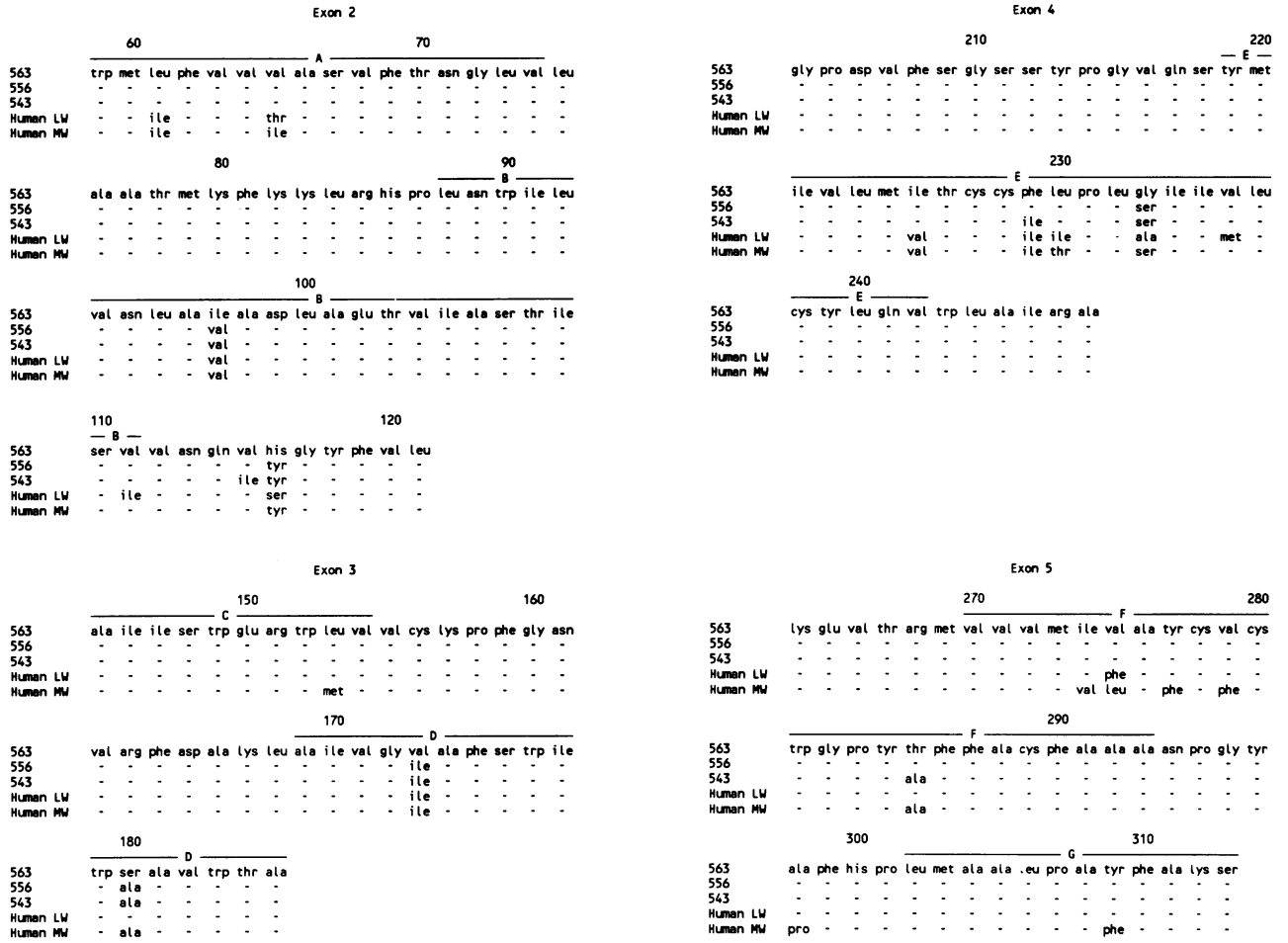

Fig. 3. Deduced amino acid sequence of the three marmoset spectral phenotypes. The horizontal lines correspond to transmembrane regions A–G. PCR fragments were isolated from low melting point agarose and sequenced by the dideoxy method using Sequenase version II and [^{35}S]dATP either after blunt-end ligation into M13mp18, after TA cloning (Invitrogen) into plasmid pCR^{TM}1000 or directly by a method adapted from Winship (1989).

sequenced: a non-polar (non-hydroxyl-bearing) residue is always found at site 233 in pigments with a λ_{max} >560 nm. Conversely, in the case of all the eight species that exhibit a pigment with a λ_{max} <541 nm, the latter pigment has a hydroxyl-bearing amino acid at site 233. Pigments with intermediate values of λ_{max} may or may not exhibit a hydroxyl-bearing residue at this site (Table III).

If indeed the spectral tuning of marmoset photopigments is dependent on substitutions at three rather than two amino acid sites, then recombination might be expected to yield additional spectral variants to the three pigments so far described, particularly since the three sites are encoded by different exons. But another possibility is that additive models, such as that of Neitz *et al.* (1991), are too simplistic, and that some substitutions require other facilitating or compensating substitutions if the molecule is to retain function. Consider the case of a callitricid opsin with a hydroxyl-bearing amino acid at site 233 but with the substitutions (serine at site 180 and threonine at site 285) that otherwise give a spectral sensitivity peaking at 563 nm. Such an opsin may be eliminated, either because it binds inadequately to retinal or because it is non-viable in some other way.

In discussing the human, squirrel monkey and tamarin gene sequences, Neitz *et al.* (1991) drew attention to the coupling of substitutions at positions 230 and 233; this could have been extended to position 236 where methionine is associated with Ile230 and Ala233, and valine with Thr230 and Ser233 (Table III). These couplings suggest a functional role, possibly in the process of spectral tuning. They are not however present in the marmoset where leucine and valine are found at positions 230 and 236 respectively, with either glycine or serine at position 233. Alternative combinations of amino acids at these positions are also found in Old World monkeys (Ibbotson *et al.*, 1992).

The X-linked inheritance of the opsin gene was originally inferred (Mollon *et al.*, 1984) from the observation that males are limited to only a single photopigment sensitive

Table II. Summary of amino acid differences for the three spectral phenotypes of the marmoset

Amino acid position	Spectral phenotype		
	563	556	543
97	Ile	Val	Val
115	Val	Val	Ile
116	His	Tyr	Tyr
173	Val	Ile	Ile
180	Ser^{OH}	Ala	Ala
229	Phe	Phe	Ile
233	Gly	Ser^{OH}	Ser^{OH}
285	Thr^{OH}	Thr^{OH}	Ala

A

Spectral phenotype	Nucleotide sequence								
		820			830			840	
563	GTG	ATG	ATC	GTG	GCG	TAT	TGC	GTC	TGC
543	GTG	ATG	ATC	GTG	GCG	TAC	TGC	GTC	TGC

RsaI site

B

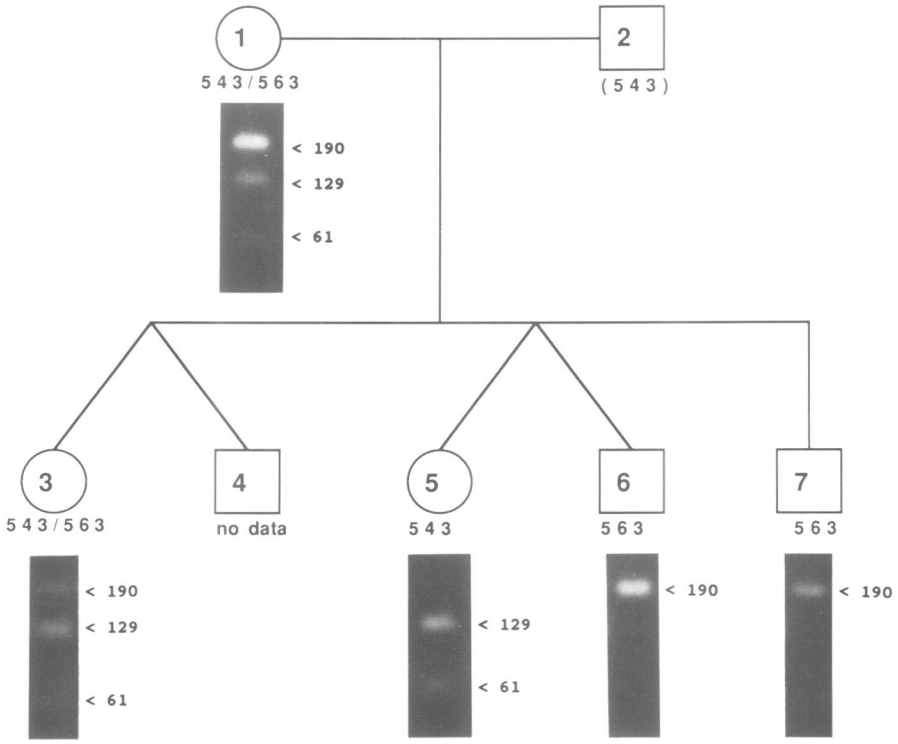

Fig. 4. Segregation of the opsin gene in a family group. (**A**) Partial nucleotide sequence of exon 5 showing the position of the *RsaI* site. (**B**) Genomic DNA was isolated from liver tissue and used for the PCR amplification of exon 5. The PCR products were displayed by electrophoresis and the 190 bp fragment of exon 5 recovered from low melting point agarose. Digests with *RsaI* were then carried out using standard conditions and a 4-fold excess of enzyme, and the products displayed on a 1.5% low melting point agarose gel using 1 × TAE. In the presence of an *RsaI* restriction site, the enzyme cut the amplified region of exon 5 into two fragments of 129 and 61 bp.

in the MW/LW region of the spectrum whereas females may have two different forms of the pigment. This contrasts with the situation of Old World monkeys where two distinct genes code for the separate MW and LW pigments (Vollrath *et al.*, 1988; Feil *et al.*, 1990). In a recent study (Tovée *et al.*, 1992), we were able to follow the inheritance of the photopigments and the forms of colour vision they conferred in a family group of marmosets in which individual males expressed either the 543 or 563 pigments and females either one or both pigments. The presence of an *RsaI* restriction enzyme site in the 543 gene has now enabled us to show that the production of the 543 or 563 photopigment is correlated with the presence of a particular DNA haplotype and hence with the presence of a particular photopigment gene variant. Animals that expressed only a single pigment exhibited the restriction site in PCR-amplified fragments only in association with the 543 pigment, whereas it was absent in animals that expressed only the 563 pigment. However, in females that expressed both pigments, both undigested and digested fragments were seen. The presence or absence of this site therefore identifies the two allelic forms of a single gene: in males (with only a single X chromosome) only a single pigment and DNA haplotype is present, whereas in females (with two X chromosomes) expressing two different pigments, two different DNA haplotypes are found. The process of random X inactivation (Lyon, 1962) is then sufficient to account for the production of only a single pigment in individual cones of such females.

It has recently been shown by behavioural tests that female marmosets possessing two distinct classes of cone sensitive in the red/green region of the spectrum are trichromatic (Tovée *et al.*, 1992). If this trichromatic vision does convey a biological advantage, it is curious that it remains restricted to a subset of females and is totally absent in males. The close proximity (Vollrath *et al.*, 1988; Feil *et al.*, 1990) and sequence homology (Nathans *et al.*, 1986; Ibbotson *et al.*,

Table III. Amino acid substitutions at six sites in primate photopigments

Species	Spectral peak	Amino acid sites						Reference[a]
		180	230	233	236	277	285	
Diana monkey	566	–	Ile	Ala	Val	Tyr	Thr	1
Human	565	Ser	Ile	Ala	Met	Tyr	Thr	2,3
Marmoset	563	Ser	Leu	Gly	Val	Tyr	Thr	
Tamarin	562	Ser	Ile	Ala	Met	Tyr	Thr	3
Squirrel monkey	561	Ser	Ile	Ala	Met	Tyr	Thr	3
Marmoset	556	Ala	Leu	Ser	Val	Tyr	Thr	
Tamarin	556	Ala	Ile	Ala	Met	Tyr	Thr	3
Squirrel monkey	547	Ala	Ile	Ala	Met	Phe	Thr	3
Marmoset	543	Ala	Leu	Ser	Val	Tyr	Ala	
Tamarin	541	Ala	Ile	Ala	Met	Tyr	Ala	3
Human	535	Ala	Thr	Ser	Val	Phe	Ala	2,3
Squirrel monkey	532	Ala	Thr	Ser	Val	Phe	Ala	3
Diana monkey	531	–	Thr	Thr	Val	Phe	Ala	1

[a]Key to references:
1, Ibbotson *et al.* (1992). Note that identical residues are present at these sites in five other species of Old World monkey examined by Ibbotson *et al.* (1992). 2, Nathans *et al.* (1986). 3, Neitz *et al.* (1991).

1992) of the separate MW and LW genes in Old World primates indicate that trichromatic colour vision has evolved by gene duplication from a single ancestral gene. In New World monkeys, it depends on a gene polymorphism that is perhaps maintained in the population by the advantage it confers on heterozygous females. An alternative explanation, discussed in detail by Tovée *et al.* (1992), supposes that there are visual tasks that are better performed by dichromatic observers. For example, the perception of food against certain textured backgrounds might be better performed by dichromatic observers. If this is the case, the polymorphism of photopigments might be maintained by a frequency-dependent advantage of individual dichromats (Clarke, 1979); alternatively, since marmosets forage in a group and the presence of several types of observer within the group may increase foraging success, the polymorphism may be maintained by kin selection.

Materials and methods

Microspectrophotometry

The monkeys were anaesthetized with an intramuscular injection of ketamine hydrochloride and were then killed by an intraperitoneal injection of nembutal. Enucleation of eyes was performed in dim red light and retinal tissue prepared for microspectrophotometry as described by Mollon *et al.* (1984). Retinal samples were taken from the foveal region and the spectral absorbances of the outer segments of individual photoreceptors measured with a dual-beam Liebman microspectrophotometer (Liebman and Entine, 1964; Knowles and Dartnall, 1977).

The λ_{max} of each microspectrophotometry record was estimated by fitting a template curve of rhodopsin (Knowles and Dartnall, 1977) to the absorbance data. The template was expressed on an abscissal scale of log frequency, since Mansfield (1985) has shown that with this transformation the absorbance curves of visual pigments have almost the same shape, independent of λ_{max}. Full details of the curve fitting procedures and the criteria used in the selection of data are given in Bowmaker *et al.* (1991) and Tovée *et al.* (1992).

Amplification of opsin gene fragments

In total, eight male animals were used for the sequencing of the opsin genes, three with the 563 phenotype, three with the 556 phenotype and two with the 543 phenotype. Of these, the 563 and 556 animals were identical to the animals identified as A6, A7, B2, B4, B5 and C3 in a previous behavioural study (Tovée *et al.*, 1992).

Oligonucleotide primers designed from a marmoset cDNA opsin gene sequence were used in the PCR to amplify regions of exon 2 (bases 175–363), exon 3 (bases 433–555), exon 4 (bases 610–744) and exon 5 (bases 790–939) using genomic DNA isolated from liver tissue from animals displaying each of the three visual pigment phenotypes. The sequences of the primers are as follows:

exon 2	+ve	5′-GTGTACCACCTCACCAGTGTC-3′
	−ve	5′-AGGACACACATCGGGTGGCCG-3′
exon 3	+ve	5′-TCACAGGTCTCTGGTCCCTGG-3′
	−ve	5′-CTCCAACCAAAGATGGGCGG-3′
exon 4	+ve	5′-ACGGCCTGAAGACTTCATGC-3′
	−ve	5′-CTCGGATGGCCAGCCACACTT-3′
exon 5	+ve	5′-AATTCCACCCAGAAGGCAGAG-3′
	−ve	5′-ACGGGGTTGTAGATAGTGGC-3′

PCRs contained 200 ng genomic DNA, 200 ng of each primer, 1 U of *Taq* polymerase, 0.2 mM each of dATP, dCTP, dGTP and dTTP, and reaction buffer (Cetus) in a final volume of 50 μl. The following PCR parameters were used: 94°C for 3.5 min, 30 cycles of 60°C for 1 min, 72°C for 1 min, 94°C for 1 min, and a final step of 72°C for 10 min. The PCR products were visualized by electrophoresis in a 1.5% low melting point agarose gel using a 0.09 M Tris–borate, 0.002 M EDTA buffer pH 8.0. Agarose containing the target bands was excised, melted at 68°C for 10 min and extracted with pre-warmed (68°C) buffered phenol after the addition of 0.1 vol of pre-warmed (68°C) 5 M NaCl. After a brief vortex and spin, a second phenol extraction followed by two chloroform–amyl alcohol (24:1) extractions were carried out. DNA was then precipitated at −70°C for 30 min by the addition of 0.5 vol 8 M ammonium acetate and 5 vol ethanol. After centrifuging and vacuum drying, pellets were dissolved in 10 μl water.

Cloning and sequencing

Amplified fragments were sequenced either by a direct method (Winship, 1989) using the oligonucleotides employed in the amplification reaction to prime the sequencing reaction, or after blunt-end cloning into the *Sma*I site of M13mp18 or into plasmid pCR™1000 using the TA cloning system (Invitrogen). Recombinant clones were sequenced by the dideoxy method using Sequenase (USB) version II and [^{35}S]dATP. The products were loaded onto a 6% polyacrylamide gel, separated at 1500 V for ~3 h and autoradiographed overnight.

Digestions of PCR fragments

PCR products were separated by agarose gel electrophoresis and the required fragment was recovered from the gel as described above. Restriction enzyme digestions were carried out under standard conditions in the presence of a 4-fold excess of enzyme and the resulting fragments were separated by electrophoresis in 1.5% low melting point agarose.

Acknowledgements

This work was supported by a Wellcome Trust grant number 17016.

References

Applebury,M.L. and Hargrave,P.A. (1986) *Vision Res.*, **26**, 1881–1895.
Bowmaker,J.K. (1991) In Gregory,R. and Cronly-Dillon,J.R. (eds), *Vision and Visual Dysfunction*. Macmillan, London, Vol. 3, pp. 63–81.
Bowmaker,J.K., Mollon,J.D. and Travis,D. (1984) *J. Physiol.*, **353**, 25P.
Bowmaker,J.K., Jacobs,G.H., Spiegelhalter,D.J. and Mollon,J.D. (1985) *Vision Res.*, **25**, 1937–1946.
Bowmaker,J.K., Astell,S., Hunt,D.M. and Mollon,J.D. (1991) *J. Exp. Biol.*, **156**, 1–19.
Clarke,B.C. (1979) *Proc. Roy. Soc. Lond. B*, **205**, 453–474.
Dartnall,H.J.A., Bowmaker,J.K. and Mollon,J.D (1983) *Proc. Roy. Soc. Lond. B*, **220**, 115–130.
Feil,R., Aubourg,P., Heilig,R. and Mandel,J.L. (1990) *Genomics*, **6**, 367–373.
Ibbotson,R.E., Hunt,D.M., Bowmaker,J.K. and Mollon,J.D. (1992) *Proc. Roy. Soc. Lond. B*, **247**, 145–154.
Jacobs,G.H. (1981) *Comparative Color Vision*. Academic Press, New York.
Jacobs,G.H. (1984) *Vision Res.*, **24**, 1267–1277.
Jacobs,G.H. and Neitz,J. (1985) *Vision Res.*, **25**, 141–143.
Jacobs,G.H. and Neitz,J. (1987) *Vision Res.*, **27**, 1263–1268.
Jacobs,G.H., Neitz,J. and Crognale,M. (1987) *Vision Res.*, **27**, 2089–2100.

Knowles,A. and Dartnall,H.J.A. (1977) In Davson,H. (ed.), *The Physiology of Vision*, Vol. 2B, *The Eye*. Academic Press, London.
Kosower,E.M. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 1076–1080.
Ladd-Franklin,C. (1892) *Second International Congress of Psychology*, (London 1892). Kraus reprint, 1974.
Lehninger,A.L. (1982) *Principles of Biochemistry*. Worth, New York.
Liebman,P.A. and Entine,G. (1964) *J. Opt. Soc. Am.*, **54**, 1451–1459.
Lyon,M. (1962) *Am. J. Hum. Genet.*, **14**, 135–148.
Mansfield,R.J.W. (1985) In Levine,J.S. and Fein,A. (eds), *The Visual System*. Alan Liss, New York, pp. 89–106.
Mollon,J.D. (1991) In Cronly-Dillon,J.R. and Gregory,R.L. (eds), *Vision and Visual Dysfunction*. Macmillan Press, London, Vol. 2, pp. 306–319.
Mollon,J.D., Bowmaker,J.K. and Jacobs,G.H. (1984) *Proc. Roy. Soc. Lond. B*, **222**, 373–399.
Mullis,K., Faloona,F., Scharf,S., Saiki,R., Horn,G. and Erlich,H. (1986) *Cold Spring Harbor Symp. Quant. Biol.*, **51**, 263–273.
Nathans,J., Thomas,D. and Hogness,D.S. (1986) *Science*, **232**, 193–202.
Neitz,M., Neitz,J. and Jacobs,G.H. (1991) *Science*, **252**, 971–974.
Tovée,M., Mollon,J.D. and Bowmaker,J.K. (1992) *Vision Res.*, in press.
Travis,D.S., Bowmaker,J.K. and Mollon,J.D. (1988) *Vision Res.*, **28**, 481–490.
Vollrath,D., Nathans,J. and Davies,R.W. (1988) *Science*, **240**, 1669–1672.
Winship,R.S. (1989) *Nucleic Acids Res.*, **17**, 1266.
Yokoyama,R. and Yokoyama,S. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 9315–9318.

Received on January 14, 1992; revised on February 19, 1992