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Structure and Evolution of the Polymorphic Photopigment Gene of the Marmoset

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The marmoset *Callithrix jacchus jacchus*, is typical of a New World monkey in exhibiting a polymorphism of photopigments in the middlewave to longwave (535–565 nm) region of the spectrum. The single X-linked opsin gene that encodes the protein component of these pigments is present in three allelic forms producing, in marmosets, pigments with maximum sensitivities at about 543, 556 and 563 nm. All male monkeys are dichromats, whereas females may be either dichromats or trichromats. A cDNA sequence corresponding to the 563 form of this gene is reported, together with partial genomic DNA sequences of exons 2, 3, 4 and 5 of all three alleles. The origin of these sequences and their divergence from the middlewave- and longwave-sensitive pigments of man is discussed from both a functional and an evolutionary standpoint.

Vision Visual pigments Primates Evolution

INTRODUCTION

The light-sensitive photopigments of the retina are formed by a class of protein molecule, the opsins, that bind retinal, the aldehyde derivative of vitamin A. Opsins are membrane proteins that are thought to form seven α-helical, hydrophobic transmembrane regions linked by straight-chain extra-membrane hydrophilic loops. The membrane regions form a bundle or palisade within which retinal is bound by a Schiff's base to a lysine residue located in the centre of the seventh helix (Applebury & Hargrave, 1986). The peak absorbance wavelength for retinal is shifted to longer wavelengths by the opsin, and the degree of shift is dependent on particular amino acid substitutions in the different opsins (Neitz, Neitz & Jacobs, 1991; Ibbotson, Hunt, Bowmaker & Mollon, 1992; Oprian, Asenjo, Lee & Pelletier, 1991; Merbs & Nathans, 1992; Williams, Hunt, Bowmaker & Mollon, 1992).

The cone photopigments that provide the basis for colour vision within the vertebrates are thought to have diverged from an ancestral rod opsin about 500 million years ago (Yokayama & Yokayama, 1988; Fryxell & Meyerowitz, 1991) and it is probable that the earliest vertebrates had a form of dichromatic colour vision based on the minimum requirement for colour vision, two spectrally distinct classes of photoreceptor (Bowmaker, 1991). The number of cone pigments differs however amongst present-day species with either trichromacy or tetrachromacy, based on three or four pigments respectively, being common in teleost fish, amphibians, reptiles and birds, and it is likely that these polychromatic colour vision systems evolved independently within each group (Bowmaker, 1991). In contrast, mammals remain largely dichromatic and it is only in the primates that a form of trichromatic colour vision is again found.

In the catarrhine branch of the primate family (which includes man and Old World monkeys), three cone pigments with maximum sensitivites in the violet (420–430 nm), green (535 nm) and yellow-green (565 nm) regions of the spectrum are present. Each opsin is coded by a separate gene, the short-wave (SW) opsin gene on chromosome 7 in man, and the middle-wave (MW) and long-wave (LW) opsin genes closely linked on the X chromosome (Vollrath, Nathans & Davies, 1988; Feil, Aubourg, Heilig & Mandel, 1990). In contrast, platyrrhine monkeys from the New World possess only two loci, one coding for a SW opsin, producing a pigment spectrally similar to the SW pigment in Old World monkeys (Travis, Bowmaker & Mollon, 1988), and a second coding for a pigment that is maximally sensitive in the green-to-red spectral region (Mollon, Bowmaker & Jacobs, 1984; Bowmaker, Jacobs, Spiegelhalter & Mollon, 1985; Jacobs & Neitz, 1987). This fundamentally dichromatic colour vision system is therefore similar to that of non-primate mammals. The presence of a polymorphism for the MW/LW gene means, however, that a mechanism exists for the production of trichromatic colour vision in these species (Mollon, Bowmaker & Jacobs, 1984; Bowmaker *et al.*,

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1985; Bowmaker, Jacobs & Mollon, 1987; Jacobs & Neitz, 1985; Travis *et al.*, 1988): since the gene is X-linked (Mollon *et al.*, 1984; Williams *et al.*, 1992), females can possess two different alleles and, through the process of X-inactivation (Lyon, 1962), can express different pigments in individual cone cells. Female marmosets of this type have been shown behaviourally to be trichromats, whereas male monkeys are always dichromats (Tovée, Bowmaker & Mollon, 1992).

The genetic event that gave rise to separate MW and LW genes in catarrhine monkeys must have occurred after their separation from platyrrhine monkeys some 30–40 million years ago (Martin, 1990); the polymorphism of cone pigments in the New World monkeys may be an intermediate stage therefore in the evolution of catarrhine trichromacy (Bowmaker *et al.*, 1987), or the duplication of an ancestral gene may have preceded the divergence of MW and LW pigments in the catarrhine line. In order to examine the structural and evolutionary relationships of these photopigment genes, we have determined the sequence of the MW/LW gene in the marmoset, *Callithrix jacchus jacchus*, a New World monkey. In this species, three forms of the MW/LW pigment with absorbance peaks at about 543, 556 and 563 nm are found, each coded by a different allelic variant of the photopigment gene (Williams *et al.*, 1992). The particular amino acid substitutions that govern these spectral characteristics (Williams *et al.*, 1992) are also used in the spectral tuning of MW and LW cone pigments in man, in two other New World species (Neitz *et al.*, 1991) and in Old World monkeys (Ibbotson *et al.*, 1992). In order to extend the comparison of gene sequences, we have determined the cDNA sequence of one of the allelic forms of the marmoset gene, together with the regions of the genomic gene of all three alleles that code for the non-homologous amino acid substitutions present in the human (Nathans, Thomas & Hogness, 1986) and Old World monkey (Ibbotson *et al.*, 1992) opsins.

MATERIALS AND METHODS

Microspectrophotometry

Microspectrophotometry was used to determine the spectral absorbance of the cone pigments in individual male monkeys in order to assign them to one of the three classes of spectral phenotype with maximal absorbances at 543, 556 and 563 nm. Full details of the methodology are given in Tovée *et al.* (1992).

Isolation of RNA and DNA

Animals were narcotized with ketamine hydrochloride and then given a fatal dose of sodium pentabarbitone. Liver tissue was rapidly removed, frozen in liquid nitrogen and stored at -70°C. Total RNA was isolated from fresh retinal tissue of a male animal showing the 563 phenotype by a method described in Hunt, Wake, Mercer and Danks (1986). cDNA was synthesized by AMV reverse transcriptase and the product used as template in a polymerase chain reaction (PCR) using oligonucleotide primers corresponding to the 5 and 3′ coding region of the human LW photopigment gene (Nathans *et al.*, 1986).

Genomic DNA was isolated from the liver tissue of seven male animals, 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).

Tissue was homogenized in 5 ml of buffer containing 150 mM NaCl, 25 mM EDTA pH 8.0, and DNA isolated by the method described in Ibbotson *et al.* (1992).

Amplification of opsin gene fragments

The polymerase chain reaction (Mullis, Faloona, Scharf, Saiki, Horn & Erlich, 1986) was used to amplify opsin gene fragments. Genomic DNA isolated from liver tissue was used to amplify regions of exon 2 (from base 175 to 363), exon 3 (from base 433 to 555), exon 4 (from base 610 to 744) and exon 5 (from base 790 to 939). The primers were designed from the marmoset cDNA sequence. Each reaction contained approx. 200 ng of template DNA, 200 ng of each primer, 1 unit of *Taq* polymerase, 0.2 mM each of dATP, dCTP, dGTP and dTTP, and reaction buffer (Cetus) in a final volume of 50 μl. Either 30 or 35 cycles were used with an annealing temperature of 60°C, elongation temperature of 72°C, and denaturing temperature of 94°C. The products of the reaction 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 1/10 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 addition of $\frac{1}{2}$ vol 8 M ammonium acetate and 5 vol ethanol. After centrifuging and vacuum drying, pellets were dissolved in 10 μl water.

Cloning and sequencing

A number of additional oligonucleotides were used to amplify a series of partially overlapping fragments from the marmoset cDNA (Fig. 1). These fragments were then isolated and sequenced by a direct method (Winship, 1989) using the PCR-oligonucleotides to prime the sequencing reactions.

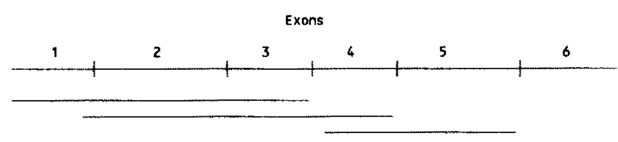

FIGURE 1. Use of PCR primers for production of overlapping fragments for direct sequencing of the marmoset cDNA. The horizontal lines indicate the regions of the cDNA amplified by different combinations of primers.

The exon sequences are based on amplifications from at least two animals of the same spectral phenotype. These fragments were sequenced either by the direct method of Winship (1989) with oligonucleotides used in the amplification reaction to prime the sequencing reaction, or after blunt-end cloning into the *SmaI* 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-labelled dATP. The products of the sequencing reaction were loaded on to a 6% polyacrylamide gel, separated at 1500 V for about 2 hr, and autoradiographed overnight.

Data analyses

The DNA sequences of the marmoset photopigment gene, the human MW and LW genes (Nathans *et al.*, 1986), and the chicken iodopsin gene (Kuwata, Imamoto, Okano, Kokame, Kojima, Matsumoto, Morodome, Fukada, Shichida, Yasuda, Shimura & Yoshizawa, 1990) were compared. The number of nucleotide substitutions for each pair of genes (n) was determined and the total number of nucleotide substitutions per site (d), corrected for multiple substitutions, was calculated by $d = -(\frac{3}{4})\ln[1 - (\frac{4}{3})n]$ (Jukes & Cantor, 1969). The phylogenetic trees were constructed by the neighbour-joining method of Saitou and Nei (1987) from a computer program kindly supplied by Dr M. Nei of the Center for Demographic and Population Genetics, The University of Texas Health Science Center at Houston, Texas, U.S.A.

M

FIGURE 2. PCR-amplified fragment obtained from marmoset retinal cDNA. The PCR amplification was carried out with oligonucleotide primers designed from the human LW gene sequence (Nathans *et al.*, 1986), and the products loaded on to a 1.5% agarose gel and separated by electrophoresis. M, molecular size markers—ϕX174/*HaeIII* digest.

RESULTS

Sequencing of marmoset photopigment cDNA

Retinal RNA from a male marmoset classified by microspectrophotometry as possessing the 563 nm pigment was used for the synthesis of cDNA, and this was then used in a PCR with oligonucleotide primers designed from the human LW opsin gene (Nathans *et al.*, 1986) to amplify a DNA sequence corresponding to the marmoset opsin mRNA (Fig. 2). The nucleotide and deduced amino acid sequence are shown in Fig. 3, together with the amino acid sequence of the human MW and LW pigments. No sequence gaps are necessary for alignment: the marmoset pigment is identical in length (364 amino acids) to the human pigment.

The percentage of amino acids that are either identical or homologous (Lehninger, 1982) to the human MW or LW pigments is shown in Table 1. In both cases, the

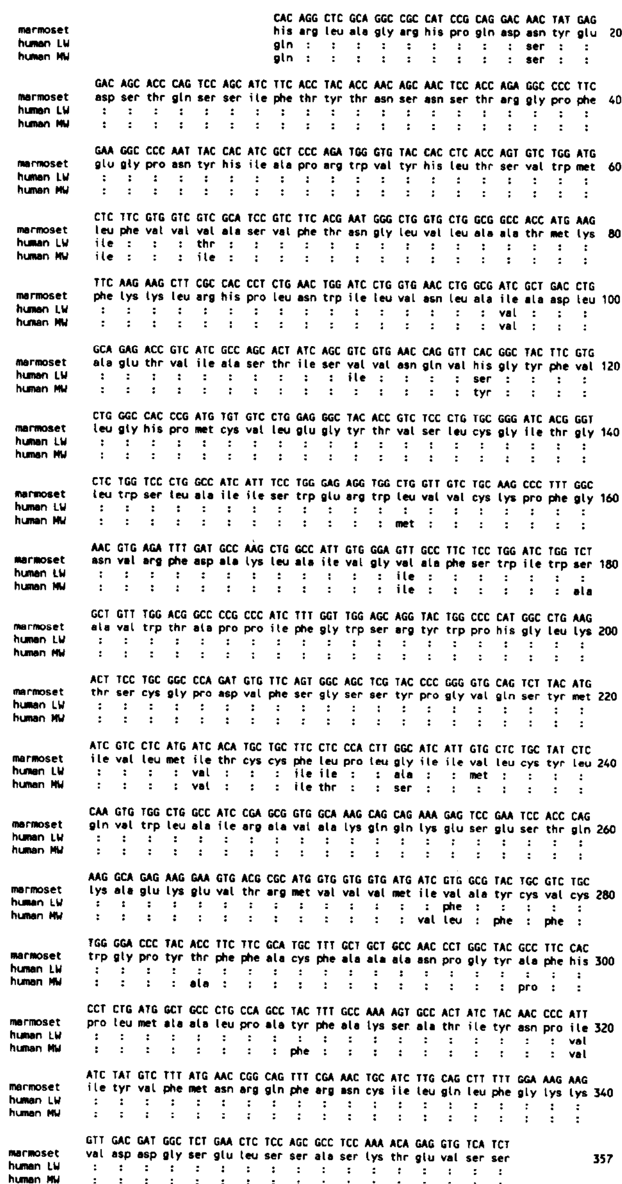

FIGURE 3. Nucleotide and deduced amino acid sequence of photopigment cDNA obtained from a male marmoset displaying the 563 spectral phenotype. The sequence has been aligned with the human MW and LW sequences.

TABLE 1. Percentage sequence homology and identity of the human and marmoset photopigments

	Human		Marmoset
	LW	MW	563
Human LW	100	96.0	95.7
Human MW	98.6	100	94.3
Marmoset 563	98.9	98.3	100

The values above the diagonal represent the percentage of amino acids that are identical and those below the diagonal represent the percentage of amino acids that are identical or homologous.

TABLE 2. Comparison of the amino acid sequence of the marmoset photopigment with the human MW and LW pigments

	Number of differences	
	LW	MW
Transmembrane regions	12	17
Luminal loops	3	4
Cytoplasmic loops	0	0
Total	15	21

marmoset pigment is more like the human LW than the human MW pigment.

A number of amino acid residues that are considered to be important for the overall function of the photopigment are conserved in the marmoset. A lysine residue forms the retinal binding site at position 312, with glutamate at position 129 providing the Schiff counterion (Sakmar, Franke & Khorana, 1989; Nathans, 1990). The N-terminal regions of other opsins are known to be glycosylated (Applebury & Hargrave, 1986) and an Asn-Serine-Thr glycosylation site at position 34–36 in the first luminal loop formed by the amino terminal part of the molecule is present in marmoset and human pigments. The positions of cysteine residues are also invariant, reflecting their role in the structural integrity of the molecule (Karnik & Khorana, 1990).

The distribution of amino acid substitutions across the transmembrane and loop regions of the marmoset and human pigments is shown in Fig. 4. Most substitutions fall in the transmembrane regions, particularly the fourth and fifth regions, and there is a complete absence of substitutions in any of the cytoplasmic loops. The total number of differences and their distribution across the transmembrane and loop regions is summarized in Table 2.

Nucleotide sequences of the different spectral phenotypes

The MW and LW opsin genes of man are comprised of six exons, with exons 2–5 coding for the seven transmembrane regions of the protein. Since only amino acids in these regions are thought to be important in determining the spectral differences between the two pigments (Kosower, 1988), PCR-amplification was restricted to these four exons of the marmoset gene, using genomic DNA from at least two animals of each spectral phenotype. The resulting fragments were then cloned and sequenced: the nucleotide sequences for the three alleles are shown in Fig. 5, together with the equivalent regions of the human MW and LW genes.

The photopigment polymorphism is presumably maintained in the population by balancing selection (Mollon et al., 1984) acting on the coding changes that confer the spectral differences on the three photopigments. The key sites are probably those at amino acids 180, 233 and 285 (Williams et al., 1992) and the coding changes arise from single base pair substitutions at base 538 in exon 3, base 697 in exon 4, and base 856 in exon 5. Silent site substitutions in the vicinity of these sites are unlikely to be the result of recent inter-allelic recombination events and may therefore give some indication of the origin of the three marmoset alleles. The nearest silent site substitutions to sites 538 and 697 are at positions 516, 546 and 552 in exon 3, and position 696

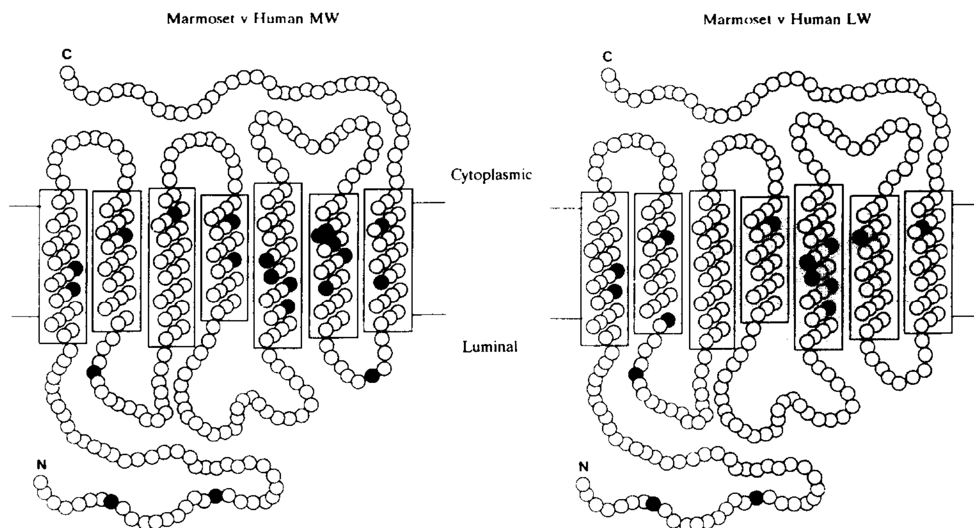

FIGURE 4. A two-dimensional model of the cone photopigment. Amino acid residues that differ in the marmoset and human pigments are indicated by solid circles. The model is based on the diagram of bovine rhodopsin devised by Hargrave et al. (1983).

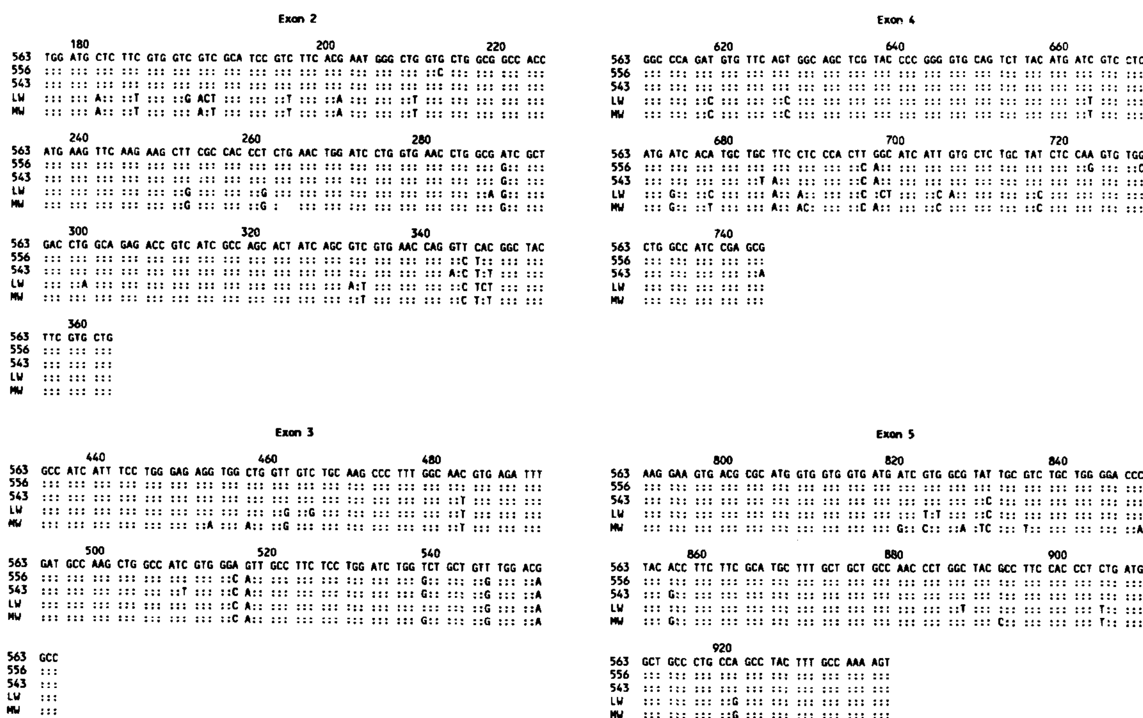

FIGURE 5. Nucleotide sequence of exons 2, 3, 4 and 5 of the three marmoset photopigment alleles. PCR amplifications were carried out with oligonucleotide primers designed from the marmoset cDNA sequence. Amplified exon fragments were then isolated from a 1.5% agarose gel, cloned and sequenced. The three marmoset sequences have been aligned with the human MW and LW sequences.

in exon 4, and in each case, it is the 563 allele that differs from the other two suggesting that the appearance of the 563 allele preceded the separation of the 556 and 543 alleles. Substitution at amino acid position 285 is limited to the 543 photopigment. In contrast however to amino acids 180 and 233, there are no adjacent silent site substitutions to this coding change; the coding change responsible for the amino acid substitution at position 285 in the 543 allele would appear therefore to be more recent than the other spectrally-significant amino acid substitutions, suggesting that the 543 allele may be the most recent addition to the polymorphism.

Phylogenetic trees

Phylogenetic relationships of the primate opsin gene sequences were examined by the neighbour-joining method of Saitou and Nei (1987). The number of nucleotide substitutions per site were estimated by the Jukes and Cantor (1969) method which corrects for multiple changes.

Using the alignment in Fig. 3, the proportion of silent-site substitutions for the pairwise comparisons of the marmoset, human MW and LW (Nathans *et al.*, 1986), chicken LW or iodopsin (Kuwata *et al.*, 1990) and blind cave fish (Yokayama & Yokayama, 1990) genes is shown in Table 3. The derived phylogenetic tree (Fig. 6) shows that the rate of divergence of the human and marmoset genes from the branch point of the Old World/New World lineages is approximately the same. The separate human MW and LW genes appear after this branch point, and subsequent to this event, the rate of accumulation of silent-site changes in the two human genes is approximately the same.

DISCUSSION

The marmoset cDNA sequence presented in this paper is the first for a New World monkey photopigment to be published. Compared to the human (Nathans *et al.*, 1986) and Old World monkey (Ibbotson *et al.*, 1992)

TABLE 3. Average number of silent substitutions per site for marmoset, human, chicken, and blind cave fish photopigment genes

	Human MW	Human LW	Chicken LW	Fish LW-like
Marmoset 563	0.039 ± 0.006	0.041 ± 0.006	0.149 ± 0.012	0.188 ± 0.013
Human MW		0.011 ± 0.003	0.148 ± 0.012	0.190 ± 0.013
Human LW			0.150 ± 0.012	0.186 ± 0.013
Chicken LW				0.198 ± 0.014

The number of nucleotides compared for each gene is 1047. Values have been corrected for multiple substitutions by the method of Jukes and Cantor (1969). The standard errors are calculated as $\sqrt{K^c/N}$ where K^c is the corrected value for silent site substitutions and N is the number of nucleotides compared.

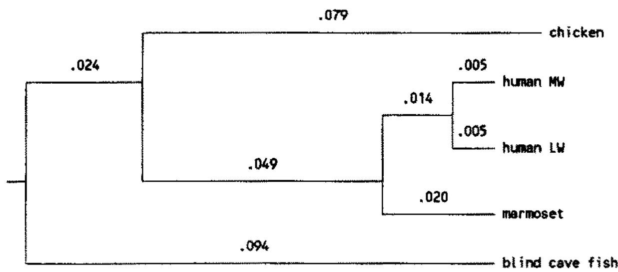

FIGURE 6. Phylogenetic tree obtained from sequence divergence of the marmoset 563 cDNA, the human MW and LW genes, the chicken iodopsin gene, and the blind cave fish LW-like gene. The trees were constructed by the neighbour-joining method of Saitou and Nei (1987). The average number of substitutions per site for each branch of the tree is indicated. The human MW and LW sequences are from Nathans *et al.* (1986), the chicken iodopsin sequences are taken from Kuwata *et al.* (1990) and blind cave fish LW-like sequences from Yokayama and Yokayama (1990).

MW and LW pigments, the marmoset photopigment shows only a limited number of differences and these are confined to the transmembrane and luminal loop regions. No differences are found in any of the cytoplasmic loops, indicating a strong functional constraint on divergence in these regions that may be imposed by their role in the binding of G-proteins and other effector molecules that control the cascade of reactions that arises after photon capture (Applebury & Hargrave, 1986).

Spectral tuning of human (Neitz *et al.*, 1991) and marmoset (Williams *et al.*, 1992) photopigments has been shown to depend on substitution at a limited number of amino acid sites within the transmembrane regions; the closer similarity of the marmoset 563 nm pigment to the human LW (565 nm) than to the human MW (535 nm) pigment is at least partially the result of common residues at these key sites. However, substitutions in transmembrane regions are not restricted to these key sites and the fifth transmembrane region in particular appears to have diverged quite considerably from the equivalent regions of the human pigments.

Using a value of 400 million years (Myr) ago (Yokoyama & Yokoyama, 1990) for the divergence of fish and bird/mammal lineages, and assuming a constant rate of substitutions, an estimate of about 300 Myr for the divergence of the reptilian ancestors of birds and mammals is obtained, in close agreement with estimates obtained from the fossil record (Dickerson, 1971; Moore, Goodman, Callahan, Holmquist & Moise, 1976; Wilson, Carlson & White, 1977) and from the β-globin gene (Efstratiadis, Posakony, Maniatis, Lawn, O'Connell, Spritz, DeRiel, Forget, Weissman, Slighthom, Blechl, Smithies, Baralle, Shoulders & Proudfoot, 1980). The same approach produces a value of about 85 Myr for the branching of catarrhine and platyrrhine primates, considerably earlier therefore than the estimate of 35–40 Myr for the separation of the New and Old World monkeys obtained from other sources (Koop, Goodman, Chan & Slighton, 1986; Martin, 1990). Although it is possible that the marmosets and tamarins may represent a primitive primate group (Hershkovitz, 1977) that had a separate origin from the ancestral primate stock and therefore form a separate lineage from the Cebidae or "true" New World monkeys, the size of the discrepancy indicates that other factors are responsible. In particular, there is evidence that the rate of gene evolution is significantly slower in primates than in other mammals (Koop *et al.*, 1986), primarily as a result of longer generation times (Li & Tanimura, 1987), and this is reflected in the present finding that silent-site mutations in the marmoset MW/LW opsin gene have accumulated at a rate of only 0.57×10^{-9} ($0.02/35 \times 10^{-6}$) substitutions per site per year in the 35 Myr since the divergence of the catarrhine and platyrrhine branches. Such a rate contrasts with the value of 5×10^{-9} substitutions per site per year obtained by Li, Gojobori and Mei (1981) for the neutral mutation rate of mammalian globin pseudogenes.

In contrast to the over-estimate of the antiquity of the catarrhine/platyrrhine branch, the phylogenetic tree appears to under-estimate, at only 22 Myr, the date of first appearance of separate MW and LW genes of catarrhine primates. Distinct MW and LW pigments are present in all catarrhine species so far examined (Hárosi, 1987; Bowmaker, Astell, Hunt & Mollon, 1991); so it is probable that the duplication event that gave rise to separate MW and LW genes occurred at about the time of establishment of the catarrhine lineage between 35 and 40 Myr ago (Martin, 1990). A factor that may account for the discrepant estimate of the age of the MW/LW gene duplication is the presence of gene conversion between the MW and LW genes of both the humans and Old World monkeys (Ibbotson *et al.*, 1992), since this will have the effect of reducing the apparent age of the MW/LW duplication.

Some indication of the order of appearance of the three marmoset alleles can be obtained from a comparison of silent site substitutions that are adjacent to the coding changes that form the functional basis of the polymorphism (Kreitman, 1991). Coding changes at amino acid positions 180 and 233 distinguish the 563 allele from the other two, and silent site substitutions adjacent to both these sites in the 563 allele suggest that the separation of this allele preceded the separation of the 556 and 543 variants. Position 285 differentiates the 556 and 543 variants, but in this case, there is a lack of adjacent silent site substitutions, indicating that the coding change that gave rise to the 543 allele occurred more recently.

Since recombination between allelic genes will tend to homogenize sequence differences, it is not possible to estimate the antiquity of the marmoset photopigment gene polymorphism. However, a polymorphism similar to the one in marmosets may have been present in the ancestral primate to the platyrrhine and catarrhine lineages, and the separate MW and LW genes of catarrhine primates could therefore have arisen from an unequal crossover that placed two different forms of the gene on to a single chromosome. If such an event took place, then some evidence of it may still be present in the pattern of silent site substitutions that flank the key codons for spectral tuning. The informative sites are 516,

546 and 552 in exon 3, and 696 in exon 4, and in each case, the human MW and LW genes are similar to both the 556 and 543 marmoset alleles but differ from the 563 variant. In the original unequal exchange therefore, the transfer of a 556-like and a 543-like variant on to a single chromosome is the more likely, and this would have been followed by the displacement of the spectral peaks of the two pigments in opposite directions to give rise to the spectral locations of the present-day pigments of catarrhine primates. An alternative hypothesis is that the duplication involved a single form of the ancestral pigment gene and that the differences between the present-day MW and LW pigments are entirely the result of mutation and selection subsequent to gene duplication. Since a common set of amino acid sites is responsible for spectral tuning in both Old and New World primates, this would suggest that the spectral differences seen in the photopigments of these species can only be achieved by substitution at a limited number of sites.

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