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Evolution

Sequence divergence of the red and green visual pigments in great apes and humans

(higher primates/red and green opsins)

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ABSTRACT **We have determined the coding sequences of red and green visual pigment genes of the chimpanzee, gorilla, and orangutan. The deduced amino acid sequences of these pigments are highly homologous to the equivalent human pigments. None of the amino acid differences occurred at sites that were previously shown to influence pigment absorption characteristics. Therefore, we predict the spectra of red and green pigments of the apes to have wavelengths of maximum absorption that differ by <2 nm from the equivalent human pigments and that color vision in these nonhuman primates will be very similar, if not identical, to that in humans. A total of 14 within-species polymorphisms (6 involving silent substitutions) were observed in the coding sequences of the red and green pigment genes of the great apes. Remarkably, the polymorphisms at 6 of these sites had been observed in human populations, suggesting that they predated the evolution of higher primates. Alleles at polymorphic sites were often shared between the red and green pigment genes. The average synonymous rate of divergence of red from green sequences was ≈1/10th that estimated for other proteins of higher primates, indicating the involvement of gene conversion in generating these polymorphisms. The high degree of homology and juxtaposition of these two genes on the X chromosome has promoted unequal recombination and/or gene conversion that led to sequence homogenization. However, natural selection operated to maintain the degree of separation in peak absorbance between the red and green pigments that resulted in optimal chromatic discrimination. This represents a unique case of molecular coevolution between two homologous genes that functionally interact at the behavioral level.**

Vision in animals is mediated by light-sensitive visual pigments composed of a protein moiety (opsin) and a covalently linked chromophore (retinaldehyde). The opsins belong to the family of proteins characterized by seven transmembrane α-helical domains that form a pocket in which the chromophore is embedded (1). The wavelength of maximum absorption (λ_{max}) of the chromophore is shifted to longer wavelengths as a result of interaction with specific amino acid residues within the transmembrane pocket. The λ_{max} of a variety of animal photopigments ranges from the near ultraviolet (335 nm) to the red region (625 nm) of the spectrum (2, 3) depending on the amino acid sequence of the opsin. In catarrhine primates, which include Old World monkeys, great apes, and humans, color vision is normally trichromatic and is based on the presence, in separate populations of cones, of three classes of photopigments that are maximally sensitive to red (560–565 nm), green (530–535 nm), and blue (420–430 nm) light (4–10). The opsins of the red and green pigments are encoded by genes located on the long arm of the X chromosome (q28), while that of the blue pigment is on chromosome 7. The highly homologous red and green opsin genes are arranged in head-to-tail tandem arrays composed of one red opsin gene 5′ of one or more green opsin genes (11). The extensive homology and close proximity of the red and green opsin genes predispose this locus to unequal recombination, which may lead to the deletion or addition of green opsin genes or to the formation of red/green hybrid genes (12–14). In contrast, New World primates have a single X chromosome-linked gene that specifies a photopigment with sensitivity in the red/green region of the spectrum (15–18). Thus, the New World primates resemble nonprimate mammals in having generally dichromatic vision, with trichromacy present in females heterozygous for alleles of the X chromosome-linked red/green opsin gene that encode pigments with different spectral sensitivities (15, 16, 19–22).

The cone pigments are believed to have diverged from an ancestral rod opsin ≈500 million years ago (23, 24). It is believed that duplication of an ancestral red/green opsin gene occurred shortly after separation of the Old and New World lineages 30–40 million years ago (25). Subsequent divergence resulted in the present red and green opsin genes in the Old World primates.

The red and green photopigments of eight species of fruit-eating Old World monkeys that inhabit different regions of Asia and Africa exhibit relatively homogeneous (a 5-nm variation in both pigments) λ_{max} values of 565 and 535 nm, respectively (10). This is in contrast to the relative heterogeneity of λ_{max} values of red/green pigments among New World primates.

The human red and green opsin genes are each composed of six exons. The sequence of the encoded proteins differ at either 15 or 16 positions (11) depending on which polymorphic alleles are compared. However, at only seven of these positions (which are distributed across exons 2–5) are there critical differences involving a hydroxyl-bearing vs. a nonpolar amino acid. These sites, therefore, are the only ones likely to play a role in tuning the absorption spectrum of the pigments (26, 27). Comparisons of the spectral sensitivities of red/green photopigments of Old and New World monkeys (28) with the corresponding amino acid sequences implicated three of the seven sites as the major determinants of spectral sensitivity. These amino acid differences are at residues 180 in exon 3 and 277 and 285 in exon 5. Microspectrophotometric studies of the polymorphic pigments of the marmoset confirmed these results and, in addition, implicated substitutions at residue 230 in spectral tuning (29). Further evidence for the importance of the 180, 277, and 285 residues in spectral tuning was provided by substitutions of residues corresponding to these positions in bovine rhodopsin (30). Rhodopsin contains the nonhydroxyl-bearing amino acid residues found in the green opsin at these positions. Substitution of the hydroxyl-bearing amino acids found in the red

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pigment resulted in spectral shifts of 2, 10, and 14 nm, respectively.

Merbs and Nathans (31) expressed *in vitro* the nine most common red–green hybrid genes found in the human population and measured their photobleaching difference spectra. Hybrids in which exons 3, 4, or 5 were exchanged produced pigments with spectral peaks that were intermediate between those of the normal red and green pigments. In agreement with results of molecular and psychophysical studies (32), exon 5 was shown to play a major role in determining the difference in absorption between the red and green pigments.

A common polymorphism at position 180 of the red opsin was discovered in the Caucasian population (33). In 62% of males the position is occupied by serine and in 38% it is occupied by alanine. The distribution of these alleles in the population is significantly correlated with the Rayleigh match range, such that individuals with serine require less red light in the mixture of red and green to match the standard yellow light. Merbs and Nathans (34) expressed these two alleles *in vitro* and showed them to differ λ_{max} by $\approx$5 nm.

Ibbotson *et al.* (29) determined the gene organization and sequence of exons 4 and 5 of the red and green pigment genes of six species of Old World monkeys. The genomic structure appears to be similar to that of the human red and green opsin genes, and, at least in one species (*Ceropithecus talapoin*), the numerical polymorphism typical of the green opsin gene in humans was observed.

The sequence and spectral sensitivities of the visual pigments of the great apes have not been determined. Psychophysical experiments conducted by Grether (35) indicated that color vision in chimpanzees is very similar to that in humans. There have been no studies on gorillas or orangutans. We have deduced sequences of the red and green opsins of the chimpanzee (*Pan troglodytes* and *Pan paniscus*), gorilla (*Gorilla gorilla*), and orangutan (*Pongo pygmaeus*) from nucleotide coding sequences and assessed the extent of inter- and intraspecies polymorphisms.

MATERIALS AND METHODS

Subjects. Peripheral blood was obtained from eight male and seven female common chimpanzees (*P. troglodytes*), one male pygmy chimp (*P. paniscus*), two male and five female gorillas (*G. gorilla*), and three male and one female orangutans (*P. pygmaeus*). DNA from peripheral leukocytes of four unrelated subjects each of chimpanzees, gorillas, and orangutans were obtained from the Yerkes Regional Primate Center at Emory University (Atlanta). The rest of the chimpanzee specimens were obtained from the University of Washington Primate Center.

DNA Amplification and Sequencing. The coding sequences of the red and green opsin genes of the higher primates were amplified by the PCR using oligonucleotide primers complementary to sequences of the corresponding human genes. Genomic DNA extracted from peripheral blood leukocytes by the proteinase K/SDS phenol protocol was used as a template in the amplification reactions. The amplified exon sequences were cloned into plasmid vector pGEM3 (Promega) or pCRII using the TA cloning system (Invitrogen) and were sequenced by the dideoxy-nucleotide chain-termination method using the Sequenase kit (United States Biochemical) and [α-^{32}P]dCTP (New England Nuclear). At least four independent clones were sequenced from each amplified exon. When appropriate, the amplified products were sequenced directly after electrophoresis on a low melting point agarose gel as described (36). The primer sequences as well as the strategies and conditions used in amplification have been described in detail elsewhere (36). Primers specific for red or green gene sequences in the 5′ upstream region as well as in exons 2, 4, and 5 were used in amplification of gene-specific segments. In some instances, gene-specific amplification was accomplished by two nested PCR amplification steps. For example, genomic segments encompassing exon 2, intron 2, and exon 3 were first amplified by using either the red or green exon 2 primers with the nonspecific exon 3 primer. Each of these gene-specific fragments was subsequently used as a template in a second round of amplification with the nonspecific exon 3 primers. The same strategy was used for gene-specific amplification of segments encompassing exons 3–4 and 4–5.

Within-species sequence differences were determined by single-strand conformation differences (SSCP) as detailed by Winderickx *et al.* (36), which is a powerful technique in comparing exon sequences of a number of subjects even when a mixture of red and green gene-specific sequences are simultaneously amplified.

RESULTS

Sequence of the Red and Green Pigment Opsins of the Great Apes. All six exons of the red and green pigment genes of the common chimpanzee, pygmy chimpanzee, gorilla, and orangutan were successfully amplified from genomic DNA by the PCR with primers (36) complementary to the corresponding human coding sequences. Exon DNA segments were sequenced either directly or after cloning into a bacterial plasmid. Sequence polymorphisms among members of the same species were detected by SSCP analysis and all variant exons were sequenced.

Amino acid sequence differences among the pigments of the great apes and humans are shown in Fig. 1*A*, and silent nucleotide substitutions are shown in Fig. 1*B*. The coding sequences of the red and green opsin genes of the apes are highly homologous to those of humans. The numbers of synonymous and nonsynonymous substitutions that differentiate the coding sequences of the red from green pigment genes among the various species are shown in Table 1. As expected, the number of synonymous differences was greater than that of nonsynonymous differences. Pigment coding sequences of gorillas and orangutans diverged farther from the human sequences than did those of chimpanzees. The orangutan and gorilla sequences are as different from each other as they are from those of chimpanzees and humans.

Amino acid residues unique to the apes were found at 7 positions of the red and 9 positions of the green pigments. At 8 (4 in each pigment) of these 16 positions, the differences were functionally conservative and therefore are unlikely to influence spectral characteristics. Among the nonconservative amino acid differences, those at positions 71, 85, and 338 are located on the cytoplasmic face of the membrane, whereas those at positions 29, 124, and 298 are on the luminal (extracellular) surface. Differences at positions 65 (polymorphic for isoleucine/threonine in the red and green opsins of gorillas and orangutans) and 113 (lysine for asparagine substitution in one of the orangutans) are located within the transmembrane domain and are therefore in a position to potentially influence absorption spectra.

The numbers of synonymous and nonsynonymous differences between the red and green opsin coding sequences within each species are shown in Table 2. The number of synonymous within-species differences between the red and green pigment coding sequences ranged between 1 and 6 (average, 3.6), and that of nonsynonymous substitutions ranged between 12 and 18 (average, 15.6). Amino acid residues at positions 180, 233, 277, and 285, which had been shown to account for spectral differences between red and green pigments (28, 29), are completely conserved among apes and humans. In addition, red–green opsin differences at residues 65, 230, 233, and 309 are hydroxyl-bearing in one pigment and nonpolar in the other. In fact, all differences

A

	29	65	71	85	97	111	113	115	116	124	153	171	174	178	180	225	230	233	236	274	275	277	279	285	298	309	338
HUM R	Thr	Thr	Asn	Arg	Val	Ile	Asn	Val	Ser	Pro	Leu	Val	Ala	Ile	Ser	Val	Ile	Ala	Met	Ile	Phe	Tyr	Val	Thr	Ala	Tyr	Gly
CHM R-1						Val						Ile															
CHM R-2												Ile							Val								
PYG R				Leu		Val						Ile							Val								
GOR R-1		Ile				Val						Ile				Ile			Val								
GOR R-2		Ile				Val						Ile							Val								
GOR R-3						Val						Ile				Ile			Val								
GOR R-4						Val						Ile							Val								
ORG R-1		Ile				Val	Lys					Ile															Arg
ORG R-2		Ile										Ile															Arg
ORG R-3						Val						Ile															Arg

	Thr	Ile	asn	Arg	Val	Val	Asn	Val	Tyr	Po	Met	Ile	Ala	Ile	Ala	val	Thr	Ser	Val	Val	Leu	Phe	Phe	Ala	Pro	Phe	Gly
HUM G																											
CHM G-1			Asp																								
CHM G-2			Asp						Ser				Val	Val													
PYG G					Leu								Val	Val											Thr		
GOR G-1	Ile	Thr											Val	Val		Ile											
GOR G-2	Ile												Val	Val		Ile											
GOR G-3	Ile									Ala			Val	Val		Ile											
ORG G-1		Thr			Ile			Ile					Val	Val													
ORG G-2								Ile					Val	Val													

B

	52	67	68	78	81	86	96	97	100	103	105	122	123	155	161	184	194	206	209	233	278	283	284	287	296	307	337	362	363
HUM R	GTG	TCC	GTT	ACC	TTC	CAC	GCA	GTC	CTA	ACC	ATC	GGC	CAC	GTG	AAT	ACA	TAC	GAC	AGC	GCT	TGC	CCC	TAC	TTC	GGT	CCG	TTC	TCG	CCT
CHM R-1	GTA	TCT					GCG			ACT															GGC				
CHM R-2	GTA	TCT	GTG				GCG			ACT	ATT														GGC				
CHM R-3	GTA	TCT	GTG				GCG			ACT	ATT			GTC											GGC				
PYG R		TCT					GCG			ACT															GGC				
GOR R-1		TCT	GTC		TTT		GCG								AAC			GAT	AGT	GCC					GGC		TTT	TCA	CCG
GOR R-2		TCT	GTC		TTT		GCG								AAC			GAT	AGT	GCC					GGC		TTT	TCA	CCG
GOR R-3		TCT	GTC		TTT		GCG								AAC					GCC					GGC		TTT	TCA	CCG
GOR R-4		TCT	GTC				GCG								AAC					GCC					GGC		TTT	TCA	CCG
ORG R-1			GTC	ACT		CAT							CAT	GTC	AAC	ACG	TAT	GAT			TGT	CCT			GGC	CCA			
ORG R-2			GTC	ACT		CAT	GCG						CAT	GTC	AAC	ACG		GAT			TGT	CCT			GGC	CCA			

	GTG	TCC	GTC	ACC	TTC	CAC	GCG	GTC	CTG	ACC	ATC	GGC	CAC	GTG	AAT	ACA	TAC	GAC	AGC	AGC	TGC	CCA	TAC	TTC	GGC	CCG	TTC	TCG	CCT
HUM G																													
CHM G-1	GTA	TCT							CTA	ACT	ATT				AAC		TAT					CCC	TAT						
PYG G		TCT							CTA		ATT				AAC														
GOR G-1		TCT			TTT			GTT	CTA						AAC			GAT	AGT			CCC					TTT	TCA	CCG
GOR G-2		TCT			TTT				CTA						AAC							CCC					TTT	TCA	CCG
GOR G-3		TCT							CTA						AAC							CCC					TTT	TCA	CCG
ORG G-1				ACT		CAT			CTA				CAT		AAC	ACG							TAT	TTA					
OGG G-2				ACT		CAT	GCA		CTA			GGT	CAT		AAC	ACG							TAT	TTA					

FIG. 1. Differences in nucleotide and amino acid sequences of red and green pigment genes of higher primates. Sequences of the common chimpanzee (CHM), pygmy chimpanzee (PYG), gorilla (GOR), and orangutan (ORG) have been aligned with the corresponding human (HUM) sequences. (*A*) Amino acid differences. (*B*) Synonymous substitutions. Top group of rows are of red (R) pigment sequences and bottom group of rows are of green (G) pigment sequences. R-1, R-2, etc., and G-1, G-2, etc., represent different red and green sequences, respectively, found in each species of apes. Codon numbers at which nucleotide or amino acid differences from the human sequence were observed are indicated above the sequence. Blank spaces indicate identity with the human sequence.

between the human red and green opsins are conserved in the apes except for that at position 111, which is valine in pigments of apes and in the human green pigment only.

Polymorphisms in the Coding Sequences of the Red and Green Pigment Genes of Apes. Within-species differences in the coding sequences of both red and green opsin genes were observed among the gorillas (two males and five females) and orangutans (three males and one female) and the common chimpanzees (eight males and seven females). The nature and locations of these polymorphisms are given in Fig. 1 and Table 3. All 17 polymorphisms were located in exons 2, 3, and 4, and at eight positions at least one allele in one pigment was shared with the other pigment. Five of these polymorphisms had been observed in human populations.

The Tyr116Ser polymorphism observed in exon 2 of the green pigment gene of the common chimpanzee, and previously observed in humans (11), is unlikely to influence the absorption spectrum of the pigment since it is located on the luminal face of the outer segment membrane. In gorillas, three of the seven polymorphisms were nonsynonymous. Of particular interest is the Thr65Ile polymorphism observed in both the red and green opsin genes. The Ile65 allele was also observed in the green opsin gene in 4 of 120 Caucasian males (33) but not in 56 African–Americans and 49 Japanese–Americans (unpublished observation). In orangutans, 4 of 7 polymorphisms were nonsynonymous, including the Ile65Thr observed in gorillas and humans. The synonymous GCA96GCG polymorphism was also observed (GCG in 3 of 84 males) in the red opsin gene of African–Americans (unpublished observations), and the Ile111Val substitution was observed in Caucasians (11). The Ser180Ala polymorphism in the red pigment observed in human populations (Alanine allele frequency of 0.38, 0.2, and 0.16 in Caucasians, African–Americans, and Japanese–Americans, respectively) was not observed among the apes studied. It is very likely that additional polymorphisms will be uncovered in the apes as more unrelated individuals are examined.

DISCUSSION

A high degree of homology was observed in coding sequences of the green and red opsin genes among the great apes and humans. Differences in sequence between the human green and red pigments at positions 180, 230, and 285, previously shown to account for the difference of ≈30 nm in peak absorption values (530 nm for green vs. 560 nm for red)

Table 1. Pairwise comparisons between red and green coding sequences of apes and humans

	Chimpanzee											
	Common			Pygmy			Gorilla			Orangutan		
	S	N	T	S	N	T	S	N	T	S	N	T
Green opsin												
Human	8	2	10	4	2	6	7	2	9	8	1	9
Chimpanzee												
Common				3	3	6	8	3	11	10	2	12
Pygmy							8	4	12	5	3	8
Gorilla										11	3	14
Red opsin												
Human	5	0	5	3	2	5	8	1	9	9	2	11
Chimpanzee												
Common				1	1	2	8	0	8	13	1	14
Pygmy							7	1	8	13	3	16
Gorilla										13	2	15

Shown are number of synonymous (S), nonsynonymous (N), and total (T) differences in nucleotide sequence for green and red opsins. Polymorphisms in which at least one allele is shared between the two species have been excluded.

between these photopigments (28–31, 45), are completely conserved in the great apes. Therefore, identity at these positions predicts absorption spectra with peak absorption values that differ by <2 nm from each other and from those of the human green and red pigments. No microspectrophotometric data on any of the great apes are available for comparison with these predictions. Several species of Old World monkeys have red and green photopigments with peak absorption values that are also very similar to those of the equivalent human pigments (2, 6–9). Few studies have been done to assess color vision phenotypically among the great apes. The only investigation was that conducted on the common chimpanzee (*P. troglodytes*) with the conclusion that color vision in this species was similar to that in humans (35). Macaque monkeys were shown by several tests to have color vision that is very similar, but not identical, to that of humans (37–40). It would therefore be of interest to determine whether color vision in the Old World monkeys and great apes is identical or very similar to that in humans. Small differences in color perception could result from small differences in pigment peak absorption and in the ratio of red to green cones in the retina (22, 41).

A total of 17 biallelic polymorphic sites were observed among the apes. This number represents a minimum since a relatively small number of subjects from each species was studied. Polymorphisms were observed in exons 2, 3, and 4 only. Remarkably, 5 of these polymorphisms had been observed in human populations, suggesting that they are of ancient origin and predate radiation of the higher primates. In comparison, 11 and 8 polymorphic sites were observed in the human red and green coding sequences, respectively (36). As was the case for the human polymorphisms, alleles at some

Table 2. Intraspecies differences between red and green opsin coding sequences

	S	N	T
Human	3	12	15
Common chimpanzee	4	14	18
Pygmy chimpanzee	3	17	20
Gorilla	1	17	18
Orangutan	6	18	24

Shown are number of synonymous (S), nonsynonymous (N), and total (T) nucleotide sequence differences between red and green pigment coding sequences within each of the species of higher primates. Polymorphisms that involve sharing of at least one allele between the two pigments have been excluded.

Table 3. Polymorphisms in red and green pigment genes of great apes

	Polymorphism	Gene
Common chimpanzee	Ile111Val*†	Red
	Tyr116Ser*†	Green
	Met236Val*†	Red
	GTG68GTT	Red
	ATT105ATC*	Red
	GTG155ATC	Red
Gorilla	Thr65Ile*†	Red
	Pro124Ala	Green
	Ile225Val*	Red
	TTC81TTT‡	Red and green
	GTT97GTC	Green
	GAC206GAT‡	Red and green
	AGC209AGT‡	Red and green
Orangutan	Thr65Ile†‡	Red and green
	Ile97Val	Green
	Ile111Val*†	Red
	Asn113Lys	Red
	GCA96GCG†‡	Red and green
	GGC122GGT‡	Green
	TAC194TAT	Red

The two alleles are shown flanking the amino acid residue number.
*Polymorphisms in which one of the alleles is shared between red and green coding sequences.
†Polymorphisms that had been observed in human populations.
‡Polymorphisms in which both alleles are identical between red and green sequences.

of the polymorphic sites in the chimpanzee, gorilla, and orangutan were shared between the red and green gene lineages (Table 3). Considering only synonymous substitutions, the red and green coding sequences from each species are more similar to each other than to the corresponding genes of the other species. Furthermore, the average synonymous rate of divergence between the red and green coding sequences since duplication of the ancestral gene ≈35 million years ago (1.0×10^{-10} per site per year) is much lower than that of a number of proteins in Old World monkeys (2.3×10^{-9}) and higher primates (1.1×10^{-9}) (42).

The juxtaposition and high degree of homology of the red and green pigment genes of the catarrhine primates has predisposed this locus to illegitimate recombination/gene conversion between these two genes. Such processes will tend to homogenize sequences of these two genes, as evidenced by the sharing of alleles at polymorphic sites and result in an apparent low rate of synonymous substitution. Strong evidence for gene conversion/recombination between the red and green pigment genes in the lineages leading to Old World monkeys and humans has been observed (11, 12, 32, 36, 43, 44). The apparent absence of gene conversion at sites (for example, residues 277 and 285) that play a major role in spectral tuning of the photopigments could be the result of natural selection. We propose that, since duplication of the ancestral gene, natural selection operated to maintain the degree of separation in peak absorption between the red and green pigments that allowed for optimal chromatic discrimination in a particular environment. Thus, certain combinations of sequences of the red and green pigment genes of a particular X chromosome-linked gene array afford a selective advantage to the individual by interacting at the neuronal level of color perception. This represents a unique case in which sequence homogenization and natural selection played an important role in the coevolution of tandemly repeated genes.

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