The “Five-Sites” Rule and the Evolution of Red and Green Color Vision in Mammals

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Amino acid changes S180A (S → A at site 180), H197Y, Y277F, T285A, and A308S are known to shift the maximum wavelength of absorption (λmax) of red and green visual pigments toward blue, essentially in an additive fashion. To test the generality of this “five-sites” rule, we have determined the partial amino acid sequences of red and green pigments from five mammalian orders (Artiodactyla, Carnivora, Lagomorpha, Perissodactyla, and Rodentia). The result suggests that cat (Felis catus), dog (Canis familiaris), and goat (Capra hircus) pigments all with AHYTA at the five critical sites have λmax values of ~530 nm, whereas rat (Rattus norvegicus) pigment with AYYTS has a λmax value of ~510 nm, which is accurately predicted by the five-sites rule. However, the observed λmax values of the orthologous pigments of European rabbit (Oryctolagus cuniculus), white-tailed deer (Odocoileus virginianus), gray squirrel (Sciurus carolinensis), and guinea pig (Cavia porcellus) are consistently more than 10 nm higher than the predicted values, suggesting the existence of additional molecular mechanisms for red and green color vision. The inferred amino acid sequences of ancestral organisms suggest that the extant mammalian red and green pigments appear to have evolved from a single ancestral green–red hybrid pigment by directed amino acid substitutions.

Introduction

Hominoids, Old World monkeys, and some New World monkeys have trichromatic color vision, while many other mammals are dichromatic (Jacobs 1993; Jacobs et al. 1996). Mammalian color vision is mediated by visual pigments present in the outer segments of cone photoreceptor cells. Each visual pigment consists of a transmembrane protein, an opsin, and a chromophore 11-cis retinal (Wald 1968), whose spectral sensitivity is measured by the maximum wavelength of absorption (Amax). It has been proposed that the principal difference between middle-wavelength-sensitive (MWS; green) and long-wavelength-sensitive (LWS; red) visual pigments is due to amino acids AFA (amino acids A, F, and A at sites 180, 277, and 285, respectively) in the green pigment and SYT at the corresponding sites in the red pigment (Yokoyama and Yokoyama 1990). In Primates, this “three-sites” rule has been well established (Neitz, Neitz, and Jacobs 1991; Asenjo, Rim, and Oprian 1994). In humans, the red and green pigments also require amino acid differences at sites 116, 230, 233, and 309 for further minor adjustments (Asenjo, Rim, and Oprian 1994). Thus, it is widely accepted that amino acid differences at the three sites, especially at 277 and 285, cause the major difference between green and red color vision of humans and other primates.

This may give the impression that the molecular mechanism of red and green color vision is now completely understood. However, a goldfish visual pigment with SYT at the three critical sites has a λmax value of 525 nm and is most sensitive to green (Johnson et al. 1993). The inconsistency of this SYT-containing pigment with green sensitivity has gone unnoticed. Recently, however, it has been shown that a mouse pigment with AYT has a λmax value of 510 nm and that this blue shift in λmax can be fully explained by two additional amino acid changes, H197Y (H → Y at site 197) and A308S (Sun, Macke, and Nathans 1997). Thus, the molecular mechanisms of red and green color vision are more complicated than previously thought. One effective way in elucidating the molecular bases of color vision is to identify potentially important amino acid substitutions that may cause λmax shifts and test these hypotheses by using mutagenesis experiments (Yokoyama 1997). Toward this end, the partial nucleotide sequences of the red and green pigment genes of eight mammalian species representing five different orders (Artiodactyla, Carnivora, Lagomorpha, Perissodactyla, and Rodentia) were sequenced. The data show that the λmax values of the visual pigments of these species cannot always be explained by the amino acid differences at the five critical sites, suggesting the existence of additional molecular mechanisms for red and green color vision. The data also provide insight on how the red and green color vision of mammals evolved during the last 80 Myr.

Materials and Methods

Mammalian Species

In addition to their short-wavelength-sensitive (SWS; blue) pigments, dogs (Canis familiaris) (Jacobs 1993), cats (Felis catus) (Guenther and Zrenner 1993), and goats (Capra hircus) (Jacobs 1993) have red pigments with λmax values of 550–555 nm. Similarly, in addition to blue pigments, white-tailed deer (Odocoileus virginianus), European rabbits (Oryctolagus cuniculus), guinea pigs (Cavia porcellus), gray squirrels (Sciurus carolinensis), and rats (Rattus norvegicus) have green pigments with λmax values of 537 (Jacobs et al. 1994), 523 (Nuboer, van Nuys, and Wortel 1983), 529 (Jacobs and Deegan 1994), 543 (Blakeslee, Jacobs, and Neitz 1988), and 510 nm (Jacobs, Neitz, and Deegan 1991), respectively. Using reverse transcription–polymerase chain reaction (RT-PCR) amplification (see below), we
sequenced exons 2–5 of the red and green opsin complementary DNAs (cDNAs) of these species. Although its \( \lambda \text{max} \) value is not known, the orthologous pigment of horses (\textit{Equus caballus}) has also been included in the analysis, because horses belong to a different order, Perissodactyla, and add further variation to the data.

Background on the Mammalian Phylogeny

Recent molecular phylogenetic analyses provide a reasonable resolution of the mammalian phylogeny. Two Carnivora (cats and dogs) and Artiodactyla (goats and deer) are closely related within each order and the two sets of organisms form a sister taxon (Cao, Adachi, and Hasegawa 1994; Cao et al. 1994a, 1994b). Lagomorpha (rabbit) may be more closely related to Primate than to Rodentia (Kuma and Miyata 1994; Cao, Okada, and Hasegawa 1997) and Rodentia may be most distantly related to other mammals (Cao, Adachi, and Hasegawa 1994; Cao et al. 1994a, 1994b; Cao, Okada, and Hasegawa 1997).

Guinea pigs have been classified into the order Rodentia by taxonomists (e.g., Novacek 1992), but this phylogenetic position has been debated by molecular systematists (Graur, Hide, and Li 1991; Hasegawa, Cao, and Yano 1992; Cao et al. 1994a, 1994b; Cao, Hasegawa, and Miyata 1994; D’Erchia et al. 1996; Graur, Duret, and Gouy 1996; Cao, Okada, and Hasegawa 1997). However, considering all the data available to date, the rodent monophyly appears to be most likely. Using this information, the composite phylogenetic tree for the red and green pigments under consideration is given in figure 1A (see fig. 1 in Cao, Okada, and Hasegawa 1997).

RT-PCR Amplification and Sequencing

Cat (\textit{F. catus}), dog (\textit{C. familiaris}), European rabbit (\textit{O. cuniculus}), rat (\textit{R. norvegicus}), horse (\textit{E. caballus}), and guinea pig (\textit{C. porcellus}) retinas were obtained from Pel-Freez (Rogers, AR), while gray squirrel (\textit{S. carolinensis}) and white-tailed deer (\textit{O. virginianus}) retinas were isolated from road-killed animals.

Total RNA was prepared from one retina each (two retinas from a rat) by acid guanidine thiocyanate–phenol–chloroform extraction (Chomczynski and Sacchi 1987). The cDNA clones were obtained by RT-PCR amplification using forward primer (5\(^\prime\)–T(T/C)GAAGGGCC(G/C)AATT(A/T)CCACAT-3\(^\prime\); from the second position of codon 40 to that of codon 47), reverse primer (5\(^\prime\)–TGCGGTTGATGAAGACAGAT-3\(^\prime\); from the first position of codon 321 to the second position of codon 328), and \textit{Taq} DNA polymerase (Promega). For the rat, the guinea pig, and the gray squirrel, a different forward primer (5\(^\prime\)–GCATCTT(T/C)ACCTA(T/C)ACCAA(T/C)AG-3\(^\prime\); from the second position of codon 26 to that of codon 33) was used. For each set of primers, cDNA was reverse transcribed at 42°C for 1 h and at 95°C for 5 min, and then PCR was carried out for 30 cycles at 94°C for 45 s, 55°C for 1.5 min, and 72°C for 2 min. PCR products were gel-isolated and subcloned into the T-tailed EcoRV-digested Bluescript plasmid vector (Hadジェb and Berkowitz 1996). The DNA sequence was determined from both strands using primer-directed dideoxynucleotide chain-termination sequencing (Sambrook, Fritsch, and Maniatis 1989). The amino acids were then deduced from these sequences. The nucleotide sequences of the pigment genes of the European rabbit, the white-tailed deer, the rat, the guinea pig, the gray squirrel, the horse, the cat, and the dog will appear in GenBank with the accession numbers AF031526, AF031527, AF031528, AF031529, AF031530, AF031531, AF031532, and AF031533, respectively.

Data Analyses

Using gecko green (GenBank accession number M92036), American chameleon red (U08131), and chicken red (M62903) pigments, the rooted phylogenetic tree for the mammalian visual pigments was constructed by applying the neighbor-joining (NJ) method (Saitou and Nei 1987) to the 15 nucleotide sequences with multiple substitutions (Kimura 1980). The tree was tested by the bootstrap method (Felsenstein 1985) with 1,000 replications.

The ancestral amino acid sequences of the opsins were inferred by Yang, Kumar, and Nei’s (1995) likelihood-based Baysian method using a modified version of Jones, Taylor, and Thornton’s (1992) empirical-substitution model (JTT model). The Dayhoff model (based on Dayhoff, Schwartz, and Orcutt 1978) and the equal-input model were also used to observe the effects of different substitution models.

Results and Discussion

Red and Green Visual Pigments of Mammals

Amino acids at sites 48–320 of the eight mammalian pigments, together with those of human red (GenBank accession numbers M13300–M13305) and green (M13306, K03490–K03494) pigments, goat red pigment (U67999–U68004), and mouse green pigment (AF011389), are shown in figure 2. The data show that the proportions of identical amino acids per site for the two human pigments, for the horse, goat, deer, cat, and dog pigments, and for the rat and mouse pigments are always higher than 93% within each group (table 1), suggesting that they form three separate clusters.

The rooted NJ tree for the 12 pigments based on the pairwise Poisson distance is given in figure 1B. In this tree, clusterings of the pigments from Carnivora (cats and dogs), Artiodactyla (deer and goats), and Perissodactyla (horses), those from humans, and those from Rodentia (mice, rats, and squirrels) are reasonably well established and consistent with the current view of mammalian phylogeny (see fig. 1A). However, having low bootstrap values, the deeper phylogenetic relationships of these pigments cannot be well established (fig. 1B).

The Ancestral Pigment of Mammals

The human red and green pigments differ at 15 sites (Nathans, Thomas, and Hogness 1986). The amino acids at these sites of the mammalian ancestor (node a in fig. 1A) were inferred by using the JTT model of amino acid substitution (fig. 3). When other models of
Fig. 1.—Phylogenetic tree topologies of the mammalian red and green pigments and ancestral amino acids inferred from JTT model (Yang, Kumar, and Nei 1995). A, Composite tree topology inferred by considering organismal relationships (see fig. 1 in Cao, Okada, and Hasegawa 1997). The numbers after P refer to observed \( \lambda_{\text{max}} \) values, whereas those beside branches are inferred values assuming that S180A, H197Y, Y277F, T285A, and A308S reduce the \( \lambda_{\text{max}} \) value of a visual pigment by 7, 28, 10, 16, and 18 nm, respectively. The circles and underlines indicate mutated amino acids and significant differences between the observed and predicted \( \lambda_{\text{max}} \) values. The five residue numbers are based
amino acid substitution (the equal-input and Dayhoff models) are used, identical results are obtained, with the exception of site 275, where the ancestral amino acid cannot be determined unequivocally (fig. 3). From this, it is clear that the ancestral pigments had the green-pigment-like composition in the N-terminal one-third, the red-pigment-like amino acids in the C-terminal one-third, and a mixture of the two types in the middle segment. This structure resembles the green–red hybrid genes generated by unequal homologous recombinations between the extant green and red pigment genes in human populations (Nathans, Thomas, and Hogness 1986; Neitz, Neitz, and Jacobs 1989; Merbs and Nathans 1992; Neitz, Neitz, and Grishok 1995).

The mammalian ancestor (node a in fig. 1A) appears to have had only one green–red hybrid gene in its genome. Three observations support this notion. First, the nucleotide sequences of the red or green cDNA clones isolated from one individual show at most five nucleotide differences, suggesting that these differences are most probably due to allelic differences rather than multiple loci. Second, when exons 2–5 of the rod or green pigment genes of the horse, the deer, the cat, and the dog were PCR-amplified separately and directly sequenced, the resulting sequences were virtually identical to those derived from the cDNA clones from separate individuals of the corresponding species. Third, from isolated genomic clones and genomic Southern blot analyses of the goat, the rabbit, and the rat, it is strongly suggested that they have single-copy genes rather than multiple loci (Radlwimmer and Yokoyama 1997; unpublished data). Thus, most extant mammalian red and green pigments must have evolved from the green–red hybrid pigments by directed amino acid substitutions. Hominoids, Old World monkeys, and some New World monkeys have distinct blue, green, and red genes (Jacobs 1993; Jacobs et al. 1996; Nei, Zhang, and Yokoyama 1997). These red and green genes might have been derived either by duplication of the hybrid gene followed by directed nucleotide substitutions or by the formation of the red and green pigment allelic sequences followed by the duplication of the two loci by unequal recombination (Windericks et al. 1992; Shyue et al. 1995). From the existing data, we cannot reject either one of these possibilities (Nei, Zhang, and Yokoyama 1997). Even in these species, however, directed amino acid substitutions must have played an important role during the evolution of the red and green pigments.

The “Five-Sites” Rule of Red and Green Color Vision

As mentioned earlier, amino acids at five critical sites 180, 197, 277, 285, and 308, are now known to control red and green color vision (Yokoyama and Yokoyama 1990; Neitz, Neitz, and Jacobs 1991; Asenjo, Rim, and Oprian 1994; Sun, Macke, and Nathans 1997). We inferred the amino acids at the five sites of the pigments at all interior nodes in figure 1A, where the results obtained using the JTT model are also presented. The inferred amino acids of ancestral pigments always have posterior probabilities higher than 90% and are highly reliable. When the other models of amino acid substitutions are used, only two differences can occur. When the Dayhoff model is used, the amino acid at site 197 is Y with a probability of 59% and H with a probability of 41% for both node c and node d. However, when the equal-input model is used, the probabilities of Y and H are 31% and 69%, respectively, for nodes c and d. Thus, our inference of amino acid at site 197 for nodes c and d is not clear-cut. This ambiguous inference is caused because human, horse, cat, dog, goat, and deer pigments have H197 (H at site 197), but the rabbit pigment has Y at the corresponding site (fig. 1A). To explain the data, H197 for nodes c and d requires one change, H197Y in rabbit, whereas Y197 requires two independent changes, Y197H at both node e and node f. Thus, for nodes c and d, H197 is more likely than Y197, as indicated by the JTT and equal-input models.

The amino acid changes S180A, H197Y, Y277F, T285A, and A308S are known to reduce λmax values of the human red pigment by approximately 7, 28, 10, 16, and 18 nm, respectively, and the effects of these amino acid substitutions on a λmax value are considered to be more or less additive (Neitz, Neitz, and Jacobs 1991; Asenjo, Rim, and Oprian 1994; Sun, Macke, and Nathans 1997). If we apply this rule directly to the inferred ancestral amino acid sequences in figure 1A, the mammalian ancestor at node a appears to have had a green pigment with a λmax of ~530 nm. This ancestral green pigment has been transmitted directly to the guinea pig and rodent ancestors and, furthermore, the pigment of the ancestral murine species at node i has subsequently achieved further blue shift by two amino acid substitutions, S180A and A308S (fig. 1A). The ancestor of primates, rabbits, horses, cats, dogs, goats, and deer achieved their red color vision at node e primarily by one amino acid change, Y197F. The extant green pigments of humans and deer seem to have evolved from the ancestral red pigment at node e and node g, respectively, by two independent amino acid substitutions, Y277F and T285A. Finally, the extant green pigment of rabbits appears to have evolved from the ancestral red pigment at node d by H197Y and A308S. Since the molecular basis of red and green color vision is not firmly established yet (see below), the details of this evolutionary scenario may require further revision. However, both sites 197 and 308 of the red and green pigments of hominoids and Old World and New World monkeys are monomorphic and consist of H197 and A308, where the strict three-sites rule (see Introduction)
**Fig. 2.**—Amino acid sequences of the red and green pigments in mammals. The numbering system of amino acid sites used is the same as that of the human red and green pigments. Dots indicate the identity of the amino acids with those of the human red pigment. The seven transmembrane regions (Hargrave et al. 1983) are boxed. A = alanine, C = cysteine, D = aspartic acid, E = glutamic acid, F = phenylalanine, G = glycine, H = histidine, I = isoleucine, K = lysine, L = leucine, M = methionine, N = asparagine, P = proline, Q = glutamine, R = arginine, S = serine, T = threonine, V = valine, W = tryptophan, and Y = tyrosine. ND, λ max not determined. Mutated amino acids are underlined.
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Table 1
Proportions of Identical Nucleotides per Site (above the diagonal) and Those of Identical Amino Acids per Site (below the diagonal) \((\times10^2)\)

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**Note.**—819 nucleotides and 273 amino acids were compared. ND \(= \lambda_{\text{max}} \) not determined.

FIG. 3.—Amino acid compositions at 15 sites where the human red (bold letters) and green (outlined letters) pigments differ. The ancestral amino acids that have a probability of less than 90% are underlined. ND, \(\lambda_{\text{max}} \) not determined.
considering either sites 180, 277, and 285 or sites 197 and 308 separately. Such interactions might have been important in causing the higher observed $\lambda_{\text{max}}$ values, which needs to be tested experimentally. Second, there may exist currently unknown amino acids at sites other than 180, 197, 277, 285, and 308 that shift the $\lambda_{\text{max}}$ values. To identify such amino acids, we can first determine potentially important amino acids that may shift the $\lambda_{\text{max}}$ values. The effects of these amino acid changes on $\lambda_{\text{max}}$ shifts can be studied by isolating full-length green opsin cDNAs of the four species and constructing appropriate mutants. These opsins can then be expressed in cultured cells and reconstituted with 11-cis retinal, and $\lambda_{\text{max}}$ values of the resulting pigments must be measured (for examples, see Sakmar, Franke, and Khorana 1989; Zhukovsky and Oprian 1989; Nathans 1990a, 1990b; Oprian et al. 1991; Chan, Lee, and Sakmar 1992; Yokoyama, Knox, and Yokoyama 1995; Sun, Macke, and Nathans 1997). For example, from the data in figure 3, we can infer the amino acid sequence of the mammalian ancestor (at node a in fig. 1A; results not shown) and identify amino acid replacements at reasonably conserved sites of the rabbit, deer, guinea pig, and squirrel pigments that are not shared by the other mammalian pigments. These amino acids are potentially responsible for the blue-shifted, but higher than predicted, $\lambda_{\text{max}}$ values. In this way, we can identify a total of 16 different amino acid substitutions: L244M, A248T, A298S, and L306I for the rabbit; A101V, V133A, and K157R for the deer; I61T, F62I, V68I, Q241H, and A298S for the guinea pig; and L55I, F62I, L135V, V162M, S211Y, and V216M for the squirrel (fig. 2). Among these changes, F62I is common to both the guinea pig and the squirrel, while A298S is common to the rabbit and the guinea pig. With the exception of these two, all amino acid changes are unique to a particular species. Thus, the blue shifts in $\lambda_{\text{max}}$ of the green pigments of the four species appear to have different molecular bases.

As mentioned earlier, the goldfish red (red’) visual pigment with the red-pigment-specific amino acids SHYTA at the five critical sites is known to have a $\lambda_{\text{max}}$ value of 525 nm (Johnson et al. 1993), which is ~35 nm lower than the expected value. The direction of this $\lambda_{\text{max}}$ shift is opposite to those of the green pigments of the rabbit, the deer, the squirrel, and the guinea pig. In this case, we need to search for new amino acid changes that are responsible for the blue shift of the goldfish red’ pigment. At present, the real goldfish red pigment gene has not been found. Both phylogenetic and Southern analyses strongly suggest that the goldfish has an additional green pigment gene (Register, Yokoyama, and Yokoyama 1993). Does this gene encode the real red opsin? Or, as suggested by Johnson et al. (1993), does an allelic form of the red’ pigment gene encode the real red pigment? In either case, once the real goldfish red pigment gene is cloned, comparison of the red and red’ pigments and the mutagenesis analyses using cultured cells will reveal the molecular mechanisms that cause blue shifts in the $\lambda_{\text{max}}$ of the latter pigment. The significant blue shift in the $\lambda_{\text{max}}$ value of the goldfish red’ pigment and the unexpectedly higher $\lambda_{\text{max}}$ value of the green pigment of the rabbit, the deer, the guinea pig, and the squirrel occurred independently of each other. Accordingly, the sites that account for the differential spectral sensitivities of the goldfish red pigment and the green pigment of the four mammalian species can be very different. Thus, the detailed molecular bases for red and green color vision still remain to be clarified.

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LITERATURE CITED


