

The Molecular Genetics of Red and Green Color Vision in Mammals

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ABSTRACT

To elucidate the molecular mechanisms of red-green color vision in mammals, we have cloned and sequenced the red and green opsin cDNAs of cat (*Felis catus*), horse (*Equus caballus*), gray squirrel (*Sciurus carolinensis*), white-tailed deer (*Odocoileus virginianus*), and guinea pig (*Cavia porcellus*). These opsins were expressed in COS1 cells and reconstituted with 11-*cis*-retinal. The purified visual pigments of the cat, horse, squirrel, deer, and guinea pig have λ_{\max} values at 553, 545, 532, 531, and 516 nm, respectively, which are precise to within ± 1 nm. We also regenerated the "true" red pigment of goldfish (*Carassius auratus*), which has a λ_{\max} value at 559 ± 4 nm. Multiple linear regression analyses show that S180A, H197Y, Y277F, T285A, and A308S shift the λ_{\max} values of the red and green pigments in mammals toward blue by 7, 28, 7, 15, and 16 nm, respectively, and the reverse amino acid changes toward red by the same extents. The additive effects of these amino acid changes fully explain the red-green color vision in a wide range of mammalian species, goldfish, American chameleon (*Anolis carolinensis*), and pigeon (*Columba livia*).

MANY long wavelength- (or red-) sensitive and middle wavelength- (or green-) sensitive visual pigments absorb light maximally (λ_{\max}) at ~ 560 nm and 530 nm, respectively. It has been shown that the difference in the color sensitivities of the two types of pigments is due mainly to amino acids AFA (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, although amino acids at sites 277 and 285 have a larger effect than those at 180 (Yokoyama and Yokoyama 1990; Neitz *et al.* 1991; Chan *et al.* 1992; Merbs and Nathans 1993; Asenjo *et al.* 1994). However, some exceptions to this "three-sites" rule have been found. That is, having red pigment-specific amino acids AYT at the three critical sites, the green pigments in mouse, rat, and rabbit have λ_{\max} values at ~ 510 nm. These extreme blue shifts in the λ_{\max} values are fully explained by two amino acid changes, H197Y (H \rightarrow Y at site 197) and A308S (A \rightarrow S at site 308; Sun *et al.* 1997; Radlwimmer and Yokoyama 1998). Thus, red-green color vision appears to be based on amino acids at five sites: 180, 197, 277, 285, and 308.

Using the results from the mutagenesis experiments of the human red pigment (Merbs and Nathans 1993; Asenjo *et al.* 1994; Sun *et al.* 1997) and the mouse green pigment (Sun *et al.* 1997), we have suggested that S180A, H197Y, Y277F, T285A, and A308S shift the λ_{\max} values of the pigments toward blue by ~ 7 , 28, 10, 16, and 18 nm, respectively, in an additive fashion and the reverse

amino acid changes toward red by the same extents (Yokoyama and Radlwimmer 1998). More recent analyses show that the λ_{\max} values of red and green pigments of cat (*Felis catus*), dog (*Canis familiaris*), goat (*Capra hircus*), rabbit (*Oryctolagus cuniculus*), and rat (*Rattus norvegicus*) are accurately predicted by this "five-sites" rule, but the orthologous pigments of white-tailed deer (*Odocoileus virginianus*), gray squirrel (*Sciurus carolinensis*), guinea pig (*Cavia porcellus*), and bottlenose dolphin (*Tursiops truncatus*) differ by ~ 10 nm from the predicted values (Radlwimmer and Yokoyama 1998; Yokoyama and Radlwimmer 1998). A potential problem with this argument is that the λ_{\max} values of many of these red and green pigments are estimated indirectly using the flicker photometric electroretinogram (ERG). An inherent problem with this method is that responses from rods and different types of cones can contribute to the recorded signals and the separation of a specific photoreceptor cell type is sometimes difficult (Neitz and Jacobs 1984).

Fortunately, the λ_{\max} values of virtually any pigment can now be measured by expressing specific opsins in cultured cells, reconstituting them with 11-*cis*-retinal, and measuring the λ_{\max} values of the purified pigments (Yokoyama 1997). Here, using *in vitro* assays, we have measured the λ_{\max} values of the red and green pigments of cat (*F. catus*), horse (*Equus caballus*), gray squirrel (*S. carolinensis*), white-tailed deer (*O. virginianus*), and guinea pig (*C. porcellus*). Using multiple regression analysis based on these and other λ_{\max} values of mammalian pigments, we estimated the magnitudes of the λ_{\max} shifts of the pigments caused by the amino acid changes at sites 180, 197, 277, 285, and 308. The results show that the additive effects of these amino acid

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changes fully explain virtually all observed λ_{max} values of the red and green pigments not only in mammals but also in other vertebrates.

MATERIALS AND METHODS

cDNA cloning and DNA sequencing: Cat (*F. catus*), horse (*E. caballus*), and guinea pig (*C. procellus*) retinas were obtained from Pel-Freez (Rogers, AR), while gray squirrel (*S. carolinensis*) and white-tailed deer (*O. virginianus*) retinas were isolated from road-killed animals. The goldfish (*Carassius auratus*) retinas were isolated from individuals purchased from a local pet store. Total RNAs were prepared from one retina each by acid thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi 1987). On the basis of their partial cDNA sequences (Yokoyama and Radlwimmer 1998) and the 5' and 3' flanking sequences of the orthologous genes of other mammals, the 5'- and 3'-ends of the red and green opsin cDNA fragments of the five mammalian species were cloned using RT-PCR amplification. Using these sequence data, complete cDNA fragments were then cloned.

To obtain the 5'-end subclones, two forward primers, F5A [5'-G(G/T)C(T/C)G(G/A)C(G/A)GG(T/C)(G/A/T)(G/T/C)C(G/A)G(G/A)G-3'] and F5B [5'-GACAGGG(T/C)TTT(G/C)(T/C)A(G/C)AGCCATG-3'], and two reverse primers, R173 [5'-(G/A)(T/C)(G/A)CTGGTGA(G/T/C)GTG(G/A)TA(T/C)ACCC-3'] and R401 [5'-GA(G/C)AC(G/A)GTGTAGCCCTCCA(G/C)(G/A)AC-3'], were used. The 5'-end subclones of horse and cat cDNAs were obtained using primer sets F5A/R173 and F5B/R173, respectively, and those of deer, guinea pig, and squirrel using F5A/R401. Similarly, to obtain the 3'-end subclones, two forward primers, F752 [5'-AGCAGCAGAAAGAATCTGAGTC-3'] and F936 [5'-AAGTGCCACTATCTACAACC-3'], and two reverse primers, R3A [5'-T(G/A)G(G/A)(T/C)G(G/C)(G/A)(G/A)(T/C)(G/A)GGT(A/T/C)GGAGGC-3'] and R3B [5'-TTT(T/C)ACAGGATGGAGAAG-3'], were used. The 3'-end subclones of horse, cat, and deer cDNAs were obtained using primers F936/R3B and those of guinea pig and squirrel using F752/R3A. Using the nucleotide information of the 5'- and 3'-end subclones, we then constructed five sets of the species-specific forward and reverse primers (Figure 1). The forward and reverse primers for goldfish were constructed

using the sequence information obtained by Johnson *et al.* (1993; Figure 1).

For each set of primers, cDNA was reverse transcribed at 42° for 1 hr and at 95° for 5 min, and then PCR was carried out for 30 cycles at 94° for 45 sec, 55° for 1.5 min, and 72° for 2 min. PCR products were gel isolated and subcloned into the T-tailed *EcoRV*-digested Bluescript plasmid vector with T-overhang attached to 3'-ends (Hadjeb and Berkowitz 1996). Nucleotide sequences of the entire region of the cDNA clones were determined by cycle sequencing reactions using the Sequitherm Excell II Long-Read kits (Epicentre Technologies, Madison, WI) with dye-labeled M13 forward and reverse primers. Reactions were run on a LI-COR 4200LD automated DNA sequencer (LI-COR, Lincoln, NE).

Expression and spectral analyses of pigments: The PCR-amplified cDNAs were subcloned into the *EcoRI* and *SalI* restriction sites of the expression vector pMT5 (Khorana *et al.* 1988). These plasmids were expressed in COS1 cells by transient transfection. The pigments were generated by incubation with 11-*cis*-retinal and purified in buffer W1 [50 mM *N*-(2-hydroxyethyl) piperazine-*N*-2-ethanesulfonic acid (HEPES), pH 6.6, 140 mM NaCl, 3 mM MgCl₂, 20% (w/v) glycerol, and 0.1% dodecyl maltoside], as previously described (Kawamura and Yokoyama 1998; Yokoyama *et al.* 1998). UV-visible spectra were recorded at 20° using a Hitachi (Mountain View, CA) U-3000 dual beam spectrophotometer. Visual pigments were bleached for 3 min using a 60-W standard light bulb equipped with a Kodak Wratten no. 3 filter at a distance of 20 cm. Data were analyzed using Sigmaplot software (Jandel Scientific, San Rafael, CA).

Sequence data analyses: The sources of the DNA sequences of the red and green opsin genes of different mammalian species are given in Table 1. Topologies and branch lengths of the phylogenetic trees were inferred by applying the NJ method (Saitou and Nei 1987) to the nucleotide and amino acid sequences. The tree topologies were tested by the bootstrap method with 1000 replications (Felsenstein 1985). The ancestral sequences of the opsins were inferred by using a computer program, PAML, based on a likelihood-based Bayesian method (Yang 1997).

RESULTS

Phylogenetic relationships of mammalian red and green pigments:

The amino acid sequences of the visual pigments deduced from the red and green opsin cDNA sequences of cat (deposited in GenBank with accession no. AF132040), horse (AF132043), deer (AF132041), guinea pig (AF132042), and squirrel (AF132044) consist of 364 amino acids and can be easily aligned with those of the orthologous pigments from other mammals (Figure 2). Note that human (P552) pigment is excluded from Figure 2 because it differs from human (P560) pigment only by one amino acid, having A180 instead of S180. Applying the NJ method to both nucleotide and amino acid sequences of these pigments, the unrooted phylogenetic trees for the 12 mammalian pigments were constructed (Figure 3). The comparison of the two NJ trees reveals three common groupings of the pigments with bootstrap supports at 90–100%: (1) a group consisting of the goat, deer, dolphin, horse, and cat pigments; (2) two human pigments; and (3) two murine pigments (Figure 3). However, neither the evolutionary

FORWARD	
	Eco RI Kozak
Horse	5'-AGGGCTGAATTCACCATGGCCAGCGGTGGG-3'
Cat	5'-AGTGATGAATTCACCATGACCCAGCGGTG-3'
Deer	5'-ACACCTGAATTCACCATGGCCACGAGTG-3'
Guinea pig	5'-CAGGGCGAATTCACCATGGCCCAACGCTGG-3'
Squirrel	5'-AAAGCTGAATTCACCATGGCCAGCGGTGGGAC-3'
Goldfish	5'-ACAACAGAATTCACCATGGCAGAGCAGTGGGGAGA-3'
REVERSE	
	Sal I
Horse	5'-GGAGAGGTCGACGCAGGTGACACTGAAGAGAC-3'
Cat	5'-GGAAGGTCGACGCAGGTGACACCGAAGACAC-3'
Deer	5'-GCAGAGGTCGACGCAGGTGACACCGAAGAGAC-3'
Guinea pig	5'-GGCAGGTCGACGCAGGGGACACCGAAGAGAC-3'
Squirrel	5'-GGCTGTCGACGCAGGAGACTGAAGAG-3'
Goldfish	5'-TGTGCACTCGACGCAGGAGCCACAGAGGACAC-3'

Figure 1.—Oligonucleotide primers for RT-PCR amplification of red and green opsin mRNAs. The *EcoRI* and *SalI* sites are boxed in the forward and reverse primers, respectively, and were used for cloning into the expression vector pMT5. A Kozak sequence (CCACC) was inserted between the *EcoRI* site and the initiation codon to promote translation.

TABLE 1
Mammalian red and green pigments

Pigment	GenBank accession no.	Absorption spectra		
		λ_{\max} (nm)	Method	Reference
Cat (P553)	AF132040	553	<i>In vitro</i>	This study
		555	ERG ^a	Guenther and Zrenner (1993)
Horse (P545)	AF132043	545	<i>In vitro</i>	This study
Deer (P531)	AF132041	531	<i>In vitro</i>	This study
		537	ERG ^a	Jacobs <i>et al.</i> (1994)
Guinea pig (P516)	AF132042	516	<i>In vitro</i>	This study
		529	ERG ^a	Jacobs and Deegan (1994)
Squirrel (P532)	AF132044	532	<i>In vitro</i>	This study
		543	ERG ^a	Blakeslee <i>et al.</i> (1988)
Goat (P553)	U67999	553	<i>In vitro</i>	Radlwimmer and Yokoyama (1997)
		553	ERG ^a	Jacobs <i>et al.</i> (1998)
Rabbit (P509)	AF054235	509	<i>In vitro</i>	Radlwimmer and Yokoyama (1998)
		523	ERG ^a	Nuboer <i>et al.</i> (1983)
Mouse (P508)	AF011389	508	<i>In vitro</i>	Sun <i>et al.</i> (1997)
		510	ERG ^a	Jacobs <i>et al.</i> (1991)
Rat (P509)	AF054241	509	<i>In vitro</i>	Radlwimmer and Yokoyama (1998)
		510	ERG ^a	Jacobs <i>et al.</i> (1991)
Dolphin (P524)	AF055457	524	<i>In vitro</i>	Fasick <i>et al.</i> (1998)
Human (P530)	K03490	530	<i>In vitro</i>	Oprian <i>et al.</i> (1991)
		531	MSP ^b	Bowmaker (1990)
Human (P552)	M13300 ^c	552	<i>In vitro</i>	Merbs and Nathans (1992)
Human (P560)	M13300	560	<i>In vitro</i>	Oprian <i>et al.</i> (1991)
		564	MSP ^b	Bowmaker (1990)

^a Flicker photometric electroretinogram.

^b Microspectrophotometry.

^c See also Winderickx *et al.* (1992).

relationship among the three groups of pigments nor the phylogenetic positions of the rabbit, guinea pig, and squirrel pigments can be established.

Recent molecular phylogenetic analyses of mammals based on much more extensive data sets strongly suggest that (1) cat, goat, and deer are closely related with each other; (2) rabbit appears to be closely related to primates; (3) guinea pig clusters with cat, goat, deer, and rabbit; and (4) mouse, rat, and squirrel are most distantly related (*e.g.*, see Cao *et al.* 1997; Kumar and Hedges 1998; Yang *et al.* 1998). The tree topology in Figure 3A is consistent with the first and third points. The results at the organismal level suggest that the evolutionary relationship of the mammalian pigments is best represented by (((human, rabbit) (((deer, goat), dolphin), horse) cat)), guinea pig), ((mouse, rat), squirrel); see also Yokoyama and Radlwimmer 1998. As we see later in this article, amino acids at sites 180, 197, 277, 285, and 308 are important in determining the λ_{\max} values of the red and green pigments and the NJ tree constructed by excluding these five sites is identical to that in Figure 3B.

It should be pointed out that, when the number of amino acid substitutions is considered, the branch length for dolphin (P524) pigment is much longer than those for the corresponding goat (P553) and deer

(P531) pigments (Figure 3B). For example, taking cat (P553) pigment as a reference, dolphin (P524) pigment- and deer (P531) pigment-specific branch lengths are given by 0.045 ± 0.0114 and 0.018 ± 0.0071 , respectively. The difference is statistically significant at the 5% level. However, when the number of nucleotide substitutions is considered, the difference disappears (Figure 3A). As we argue later, the validity and biological significance of the accelerated amino acid substitution of dolphin (P524) pigment remains to be seen.

Light absorption profiles: When measured in the dark, the visual pigments of guinea pig, cat, deer, squirrel, and horse have λ_{\max} values at 516 ± 1 nm, 553 ± 1 nm, 531 ± 1 nm, 532 ± 1 nm, and 545 ± 1 nm, respectively (Figure 4). The regenerated pigments show very similar patterns of absorption spectra and their functions are characterized by the λ_{\max} values. The respective dark-light difference spectra are given by 518, 552, 531, 534, and 544 nm, all of which are precise to within ± 1 nm (Figure 4, insets) and are very close to the corresponding dark spectra. The λ_{\max} value of cat (P553) pigment obtained from the *in vitro* assay is very close to the ERG estimate, whereas those of deer (P531), guinea pig (P516), and squirrel (P532) pigments are >6 nm lower than the ERG estimates (Table 1). Because responses of rods and different types of cones may con-

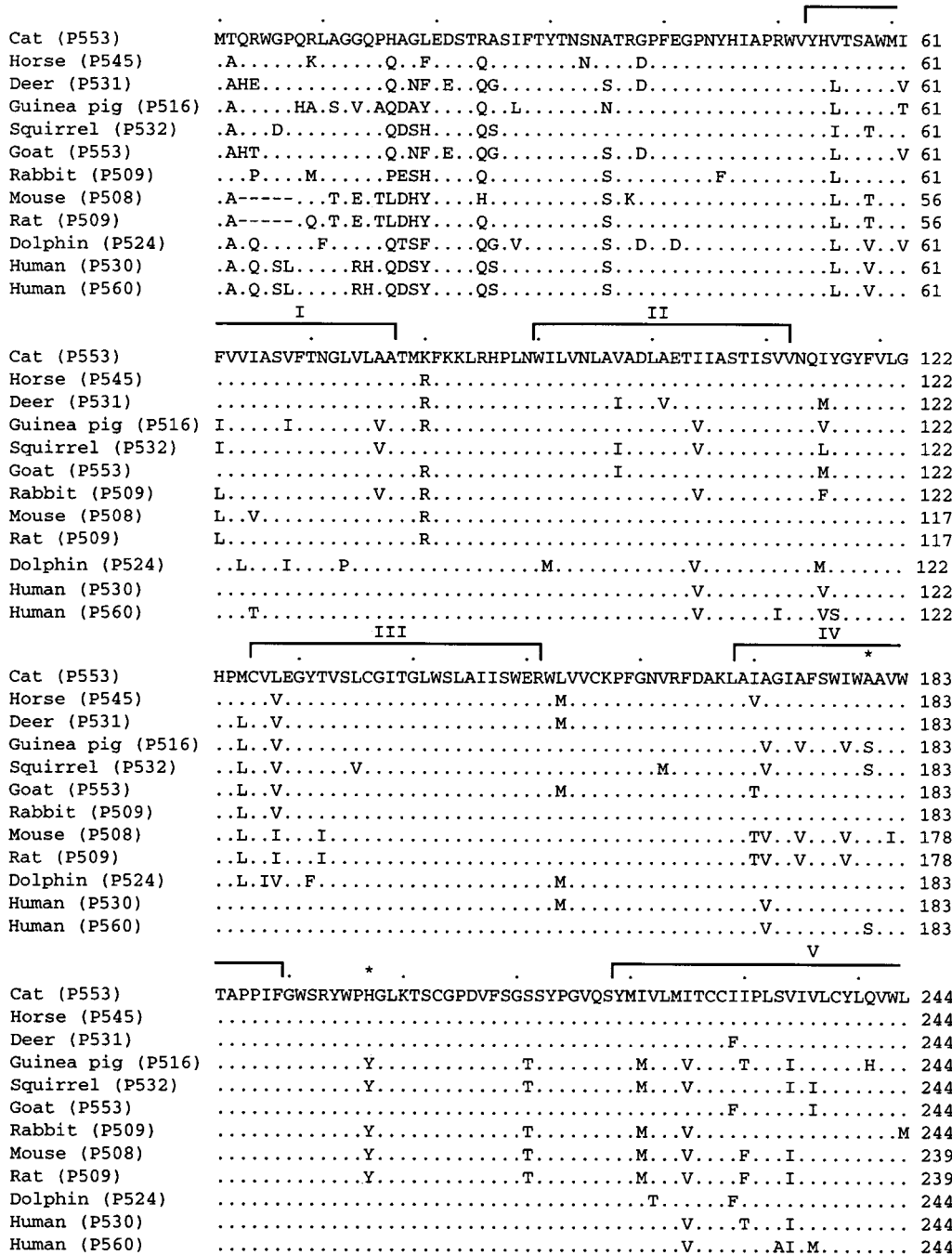


Figure 2.—Alignment of the amino acid sequences of the red and green pigments in mammals. The numbers after P refer to λ_{max} values obtained from the *in vitro* assays. Dots indicate the identity of the amino acids with those of the cat pigment. The seven transmembrane domains (Hargrave *et al.* 1983) are indicated. The positions of five critical sites, 180, 197, 277, 285, and 308 are marked by asterisks.

tribute to the recorded signals, the noninvasive ERG results must be interpreted with caution. Compared to ERG, the visual pigments regenerated using the *in vitro* assay are identical and are expected to provide more reliable λ_{max} values. Thus, the λ_{max} values of deer (P531), guinea pig (P516), and squirrel (P532) pigments should be reexamined using ERG and other physiological methods such as microspectrophotometry (MSP; *e.g.*, see Bowmaker 1991). Note that the λ_{max} value of horse (P545) pigment using the *in vitro* assay is the only estimate available today.

Mechanism of red-green color vision: We previously proposed the “five-sites” rule using information only

from the mutagenesis experiments of Merbs and Nathans (1993), Asenjo *et al.* (1994), and Sun *et al.* (1997). Using λ_{max} values estimated from the *in vitro* assay, we now evaluate the effects of amino acid changes at sites 180, 197, 277, 285, and 308 on the spectral tuning of the mammalian red and green pigments.

Let us assume that $x_1, x_2, x_3, x_4, x_5,$ and x_6 represent the presence or absence of amino acids S180, H197, Y277, T285, A308, and those at the remaining sites in a pigment, respectively. Similarly, let $y_1, y_2, y_3, \dots, y_n$ be the λ_{max} values of n pigments. Furthermore, let $\theta_1, \theta_2, \theta_3, \theta_4, \theta_5,$ and Z be the magnitudes of the λ_{max} shifts caused by S180A, H197Y, Y277F, T285A, A308S, and the

	VI	
Cat (P553)	AIRAVAKQOQKESESTQKAEKEVTRMVMVMIFAYCVCWGPYTFACFAAAHPGYAFHPLVAA	305
Horse (P545)V..F.L.....	305
Deer (P531)F.L.....A.....	305
Guinea pig (P516)V..VL..L.....A.....T.N..S.....	305
Squirrel (P532)V..V..L.....T.N.....	305
Goat (P553)L.....	305
Rabbit (P509)	...T.....V..V..L.....T...S.....	305
Mouse (P508)V..V..L.....T.....S	300
Rat (P509)V..V..L.....T.....S	300
Dolphin (P524)R.....L.....	305
Human (P530)V..VL.F.F.....A.....N..P...M..	305
Human (P560)V.....N.....M..	305
	VII	
Cat (P553)	LPAYFAKSATIYNPIIYVFMNRQFRNCIMQLFGKKVDDGSELSSASRTEASSVSSVSPA	364
Horse (P545)L.....V.K.....	364
Deer (P531)L.....S.....V.K.....	364
Guinea pig (P516)L.....E.S.....T.....	364
Squirrel (P532)L.....T.....K.....	364
Goat (P553)L.....S.....V.K.....	364
Rabbit (P509)	I.S.....L.....E.S.....	364
Mouse (P508)	..S.....LH.....S.....T.K..V.....	359
Rat (P509)	..S.....L.....S.....T.K..V.....	359
Dolphin (P524)	..S.....L.....S.....V.K.....	364
Human (P530)	..F.....V.....L.....K..V.....	364
Human (P560)V.....L.....K..V.....	364

Figure 2.—(Continued)

amino acids at the other sites as a whole in a pigment, respectively. Then, considering the amino acid compositions of the 13 pigments in Table 2, the following relationships hold:

$$\begin{aligned}
 \theta_1 + Z + e_1 &= 553 \\
 \theta_1 + \theta_3 + \theta_4 + Z + e_2 &= 531 \\
 \theta_2 + \theta_4 + Z + e_3 &= 516 \\
 \theta_1 + \theta_3 + Z + e_4 &= 545 \\
 \theta_2 + Z + e_5 &= 532 \\
 \theta_1 + Z + e_6 &= 553 \\
 \theta_1 + \theta_2 + \theta_5 + Z + e_7 &= 509 \\
 \theta_1 + \theta_5 + Z + e_8 &= 524 \\
 \theta_1 + \theta_2 + \theta_5 + Z + e_9 &= 508 \\
 \theta_1 + \theta_2 + \theta_5 + Z + e_{10} &= 509 \\
 Z + e_{11} &= 560 \\
 \theta_1 + Z + e_{12} &= 552 \\
 \theta_1 + \theta_3 + \theta_4 + Z + e_{13} &= 530, \tag{1}
 \end{aligned}$$

where e_i 's ($i = 1, 2, \dots, 13$) denote random errors.

This is represented in matrix form as

$$\mathbf{y} = \mathbf{X}\boldsymbol{\theta} + \mathbf{e}, \tag{2}$$

where

$$\mathbf{y}' = [553 \ 531 \ 516 \ 545 \ 532 \ 553 \ 509 \ 524 \ 508 \ 509 \ 560 \ 552 \ 530],$$

$$\mathbf{X}' = \begin{bmatrix} 1 & 1 & 0 & 1 & 0 & 1 & 1 & 1 & 1 & 1 & 0 & 1 & 1 \\ 0 & 0 & 1 & 0 & 1 & 0 & 1 & 0 & 1 & 1 & 0 & 0 & 0 \\ 0 & 1 & 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 \\ 0 & 1 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 \\ 0 & 0 & 0 & 0 & 0 & 0 & 1 & 1 & 1 & 1 & 0 & 0 & 0 \\ 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 \end{bmatrix},$$

and

$$\mathbf{e}' = [e_1 \ e_2 \ e_3 \ \dots \ e_{13}].$$

If we assume that the random term, \mathbf{e} , has a normal distribution with mean $\mathbf{0}$ and $\sigma^2\mathbf{I}$, then the mean ($\hat{\theta}$) and standard error (\hat{s}) of $\theta' = [\theta_1 \ \theta_2 \ \theta_3 \ \theta_4 \ \theta_5 \ Z]$ are estimated from

$$\hat{\boldsymbol{\theta}} = (\mathbf{X}'\mathbf{X})^{-1} \mathbf{X}'\mathbf{y}, \tag{3}$$

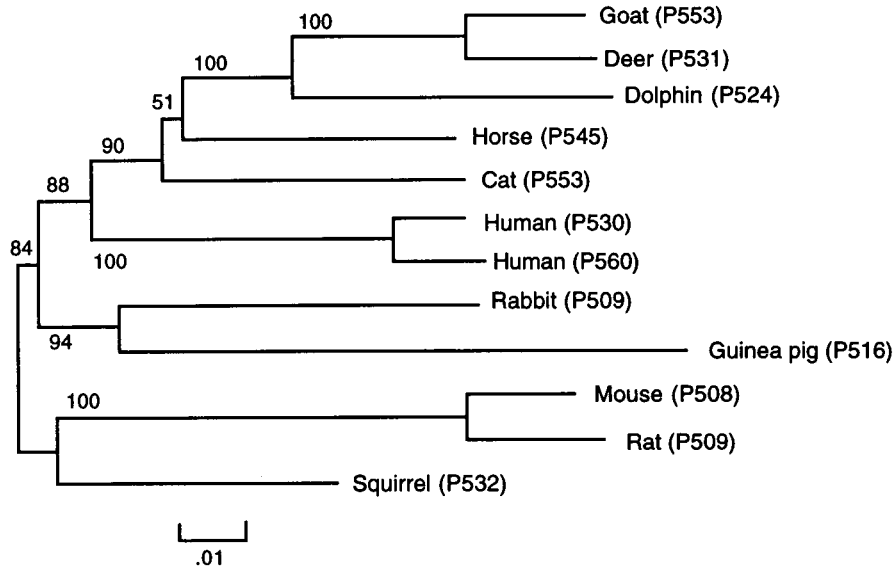
$$\hat{\mathbf{s}} = [(\mathbf{X}'\mathbf{X})^{-1} \text{SSE}/(n - p)]^{1/2}, \tag{4}$$

where

$$\text{SSE} = (\mathbf{y} - \mathbf{X}\hat{\boldsymbol{\theta}})' (\mathbf{y} - \mathbf{X}\hat{\boldsymbol{\theta}}) \tag{5}$$

(Searle 1971). Note that the estimation of $\boldsymbol{\theta}$ does not require the normality assumption of \mathbf{e} under the least-squares estimation procedure. In these formulas, SSE denotes the sum of squares of the deviations of the observed y_i 's from their estimated expected values, while n and p denote the number of samples and that of parameters, respectively. From (3)–(5), $\hat{\theta}_1 = -3 \pm 3$ nm, $\hat{\theta}_2 = -21 \pm 3$ nm, $\hat{\theta}_3 = -6 \pm 3$ nm, $\hat{\theta}_4 = -17 \pm 3$ nm, and $\hat{\theta}_5 = -24 \pm 3$ nm (Table 3). Thus, these estimates have large standard errors and are not always consistent with the corresponding values $-7, -28, -10, -16$, and

A



B

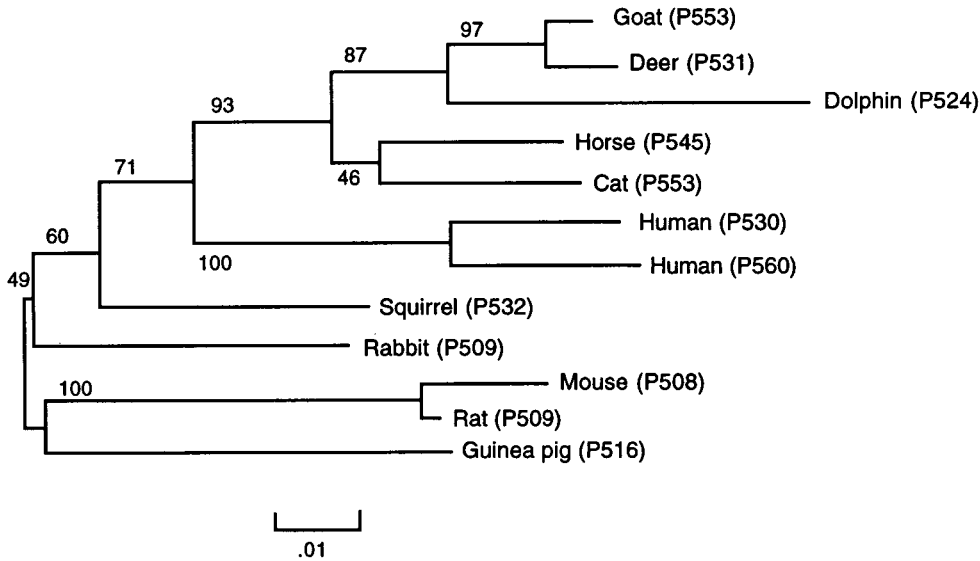


Figure 3.—The unrooted phylogenetic tree for the 12 red and green pigments reconstructed by applying the NJ method to the nucleotide sequences (A) and amino acid sequences (B). The numbers next to the different branches are clustering percentage support generated by 1000 bootstrap replicates. The bars at the bottom indicate evolutionary distance measured as the proportion of nucleotide (A) and amino acid (B) differences per site.

–18 nm observed in the mutagenesis experiments using human (P560) pigment (Asenjo *et al.* 1994; Sun *et al.* 1997).

If we exclude dolphin (P524) pigment in the estimation of θ , we obtain $\hat{\theta}_1 = -7 \pm 1$ nm, $\hat{\theta}_2 = -28 \pm 1$ nm, $\hat{\theta}_3 = -7 \pm 1$ nm, $\hat{\theta}_4 = -15 \pm 1$ nm, and $\hat{\theta}_5 = -16 \pm 1$ nm, which show much smaller standard errors (Table 3). These estimates are much closer to the corresponding observed values in the mutagenesis experiments. The improvement in the estimation procedures with and without dolphin (P524) pigment can be tested by

$$F_{7,6} = (SSE_1/7)/(SSE_2/6), \quad (6)$$

where SSE_1 and SSE_2 indicate the SSE values for the models with and without dolphin (P524) pigment, respectively. For the present case, $F_{7,6} = 20.5$ ($P < 0.01$).

This clearly shows that the estimate $\hat{\theta}$ is superior when dolphin (P524) pigment is excluded from the estimation. When dolphin (P524) pigment is included in the estimation, SSE_1 is 8.49 and dolphin (P524) pigment alone explains 31%, 2.64, of the total SSE_1 value.

Next, let us take human (P530) pigment with AHFAA as a reference. Then θ_1 , θ_2 , θ_3 , θ_4 , and θ_5 denote the λ max-shifts caused by A180S, H197Y, F277Y, A285T, and A308S, respectively. Excluding dolphin (P524) pigment from the estimation, $\hat{\theta}_1$, $\hat{\theta}_3$, and $\hat{\theta}_4$ are given by 7 ± 1 nm, 7 ± 1 nm, and 15 ± 1 nm, respectively (Table 3). The estimate $\hat{\theta}_3$ is close to 6 nm of the red shift generated by the amino acid change F277Y in the mutagenesis experiment using human (P530) pigment (Asenjo *et al.* 1994). However, $\hat{\theta}_1$ and $\hat{\theta}_4$ are much higher than the corresponding red shifts caused by single mutations

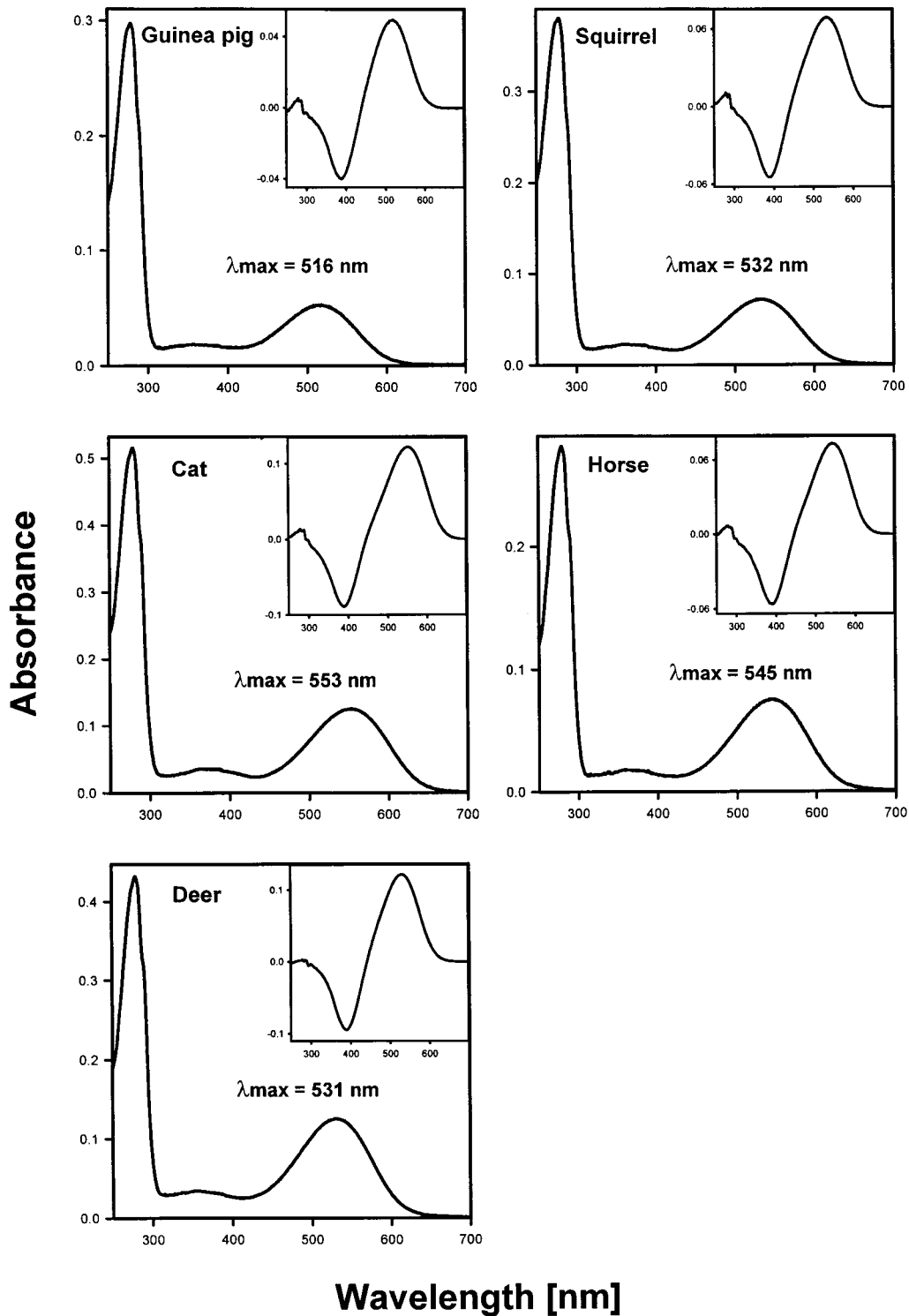


Figure 4.—Absorption spectra of the guinea pig, cat, deer, squirrel, and horse pigments in the dark, and the dark-light difference spectra (inset).

A180S (2 nm) and A285T (10 nm). At present, the cause of this discrepancy is not clear. It is also not clear why the extents of the λ_{\max} shifts generated by amino acid changes at sites 180 and 285 are much smaller in human (P530) pigment than in human (P560) pigment. Similarly, when mouse (P508) pigment with AYYTS is taken as a reference, θ_1 , θ_2 , θ_3 , θ_4 , and θ_5 denote the λ_{\max} shifts generated by A180S, Y197H, Y277F, T285A, and

S308A, respectively. The $\hat{\theta}_5$ value, $16 \pm 1 \text{ nm}$ (Table 3), is close to the 18-nm red shift caused by S308A in a mutagenesis experiment using mouse (P508) pigment (Sun *et al.* 1997).

When dolphin (P524) pigment is excluded in the estimation, $\hat{\theta}_i$'s have reasonably small standard errors (Table 3). This strongly suggests that the red and green color vision in mammals is controlled mainly by the five

TABLE 2
Amino acid compositions at five critical sites and λ_{\max} values of the mammalian red and green pigments

Pigment	180	197	277	285	308	λ_{\max} (nm)	
						Expected	Expected – Observed
Cat (P553)	A	H	Y	T	A	553 ^a (553) ^b	0 ^a (0) ^b
Deer (P531)	A	H	F	A	A	531 (530)	0 (–1)
Guinea pig (P516)	S	Y	Y	A	A	517 (518)	1 (2)
Horse (P545)	A	H	F	T	A	546 (547)	1 (2)
Squirrel (P532)	S	Y	Y	T	A	532 (535)	0 (3)
Goat (P553)	A	H	Y	T	A	553 (553)	0 (0)
Rabbit (P509)	A	Y	Y	T	S	509 (508)	0 (–1)
Dolphin (P524)	A	H	Y	T	S	537 (529)	13 (5)
Mouse (P508)	A	Y	Y	T	S	509 (508)	1 (0)
Rat (P509)	A	Y	Y	T	S	509 (508)	0 (–1)
Human (P560)	S	H	Y	T	A	560 (556)	0 (–4)
Human (P552)	A	H	Y	T	A	553 (553)	1 (1)
Human (P530)	A	H	F	A	A	531 (530)	1 (0)

^a Dolphin (P524) pigment is excluded in the estimation.

^b Dolphin (P524) pigment is included in the estimation.

sites. Namely, S180A, H197Y, Y277F, T285A, and A308S shift the λ_{\max} values of a pigment toward blue by 7, 28, 7, 15, and 16 nm, respectively, in an additive fashion and the reverse changes toward red by the same extents. Note that these estimates are very similar to the previously suggested values 7, 28, 10, 16, and 18 nm in the formulation of the five-sites rule (Yokoyama and Radlwimmer 1998).

With the exception of dolphin (P524), this five-sites rule explains the observed λ_{\max} values of the mammalian red and green pigments extremely well (Table 2). When the five-sites rule is applied to dolphin (P524) pigment, the predicted λ_{\max} value is 13 nm higher than the observed value (Table 2). Fasick *et al.* (1998) obtained the λ_{\max} value of dolphin (P524) pigment using the dark-light difference spectrum in their *in vitro* assay. Because the values of the dark and dark-light difference can disagree (Kawamura and Yokoyama 1998), it is of interest to evaluate the absorption spectrum in the dark and see how well the two spectra coincide. As we see in the goldfish red pigment (discussion), there is also some possibility that unwanted amino acid changes might have been introduced during the cloning of the opsin cDNA, leading to an erroneous λ_{\max} value. If this occurred, the five-sites rule should not apply to the mutant pigment. As we already saw, dolphin (P524) pigment has a higher rate of amino acid substitution compared to other pigments. The cause of this accelerated evolutionary rate is not understood. This high rate may reflect an adaptive evolution of this pigment to a unique marine environment. Or, some of the amino acid changes might have been introduced by spurious mutations. Mutations involved in either of these cases may include E41D, L73P, I91M, and Q260R.

E41 and L73 are completely conserved among the red and green pigments in other vertebrates. I91 is completely conserved in RH1, RH2, SWS2, and LWS/MWS pigment groups, all of which have diverged prior to the evolution of vertebrates (Yokoyama 1997). Q260 is also completely conserved among all RH1, RH2, SWS1, SWS2, and LWS/MWS pigment groups in vertebrates. Thus, it is most important to evaluate whether these and other amino acids of dolphin (P524) actually exist in nature. If these amino acids are validated, then dolphin (P524) pigment provides an exciting opportunity to study not only the molecular mechanism of adaptation of the pigment to a marine environment but also a new genetic mechanism of red-green color vision.

Evolution of the mammalian red-green color vision:

Our analyses show that the five-sites rule explains the λ_{\max} values of virtually all extant red and green pigments in mammals. This implies that it also applies to the ancestral red and green pigments. Thus, it is of interest to study the evolution of red-green color vision of the mammalian ancestors.

To infer the amino acid sequences of visual pigments of ancestral organisms, we consider a composite tree topology of the mammalian red and green pigments inferred by tree topologies in Figure 3 and the organismal tree in Figure 5. Given this tree topology, the amino acid sequences for all ancestral pigments were inferred by using the Dayhoff model of amino acid substitution (Dayhoff *et al.* 1978; Figure 5). When the empirical substitution model (Jones *et al.* 1992) and equal input model are used, virtually identical ancestral amino acid sequences are obtained (results not shown). According to Figure 5, the mammalian ancestral pigment had a λ_{\max} value at 531 nm. Interestingly, this ancestral phe-

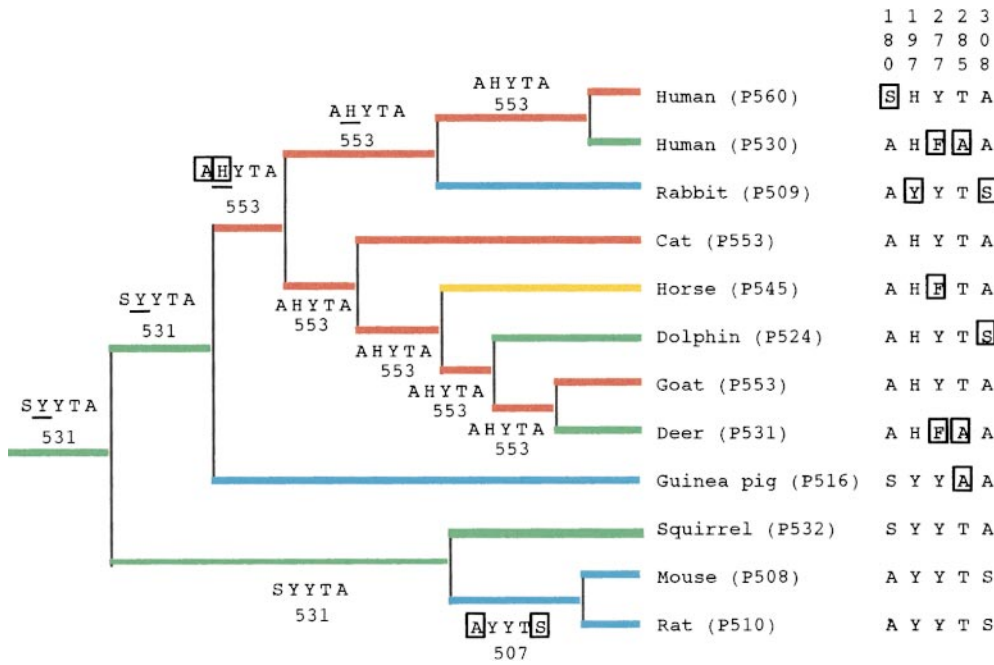


Figure 5.—A composite tree topology of the mammalian red and green pigments and ancestral amino acids at sites 180, 197, 277, 285, and 308. The numbers after P refer to λmax values obtained from the *in vitro* assays, whereas the numbers beside branches are predicted values from the five-sites rule. The amino acids that have a probability of 90% or less are underlined. The rectangles indicate amino acid substitutions. In the estimation, the red pigments of American chameleon (U08-131) and chicken (M62903) were also used as the outgroup.

notype can still be seen in the extant squirrel (P532) pigment. The red color vision at a λmax at 553 nm appears to have been achieved initially in the pigment in the common ancestor of primates (human), Lagomorpha (rabbit), Carnivora (cat), Perissodactyla (horse), Cetacea (dolphin), and Artiodactyla (goat and deer) by two amino acid substitutions S180A and Y197H (Figure 5). Today, this red color vision can be seen in cat (P553) and goat (P553) pigments. Human (P560) pigment achieved further red shift in the λmax by an additional amino acid substitution A180S. The green color sensitivities of human (P530) and deer (P531) pigments were achieved by Y277F and T285A (see also Nei *et al.* 1997) and dolphin (P524) pigment by A308S. Horse (P545) pigment achieved its present blue-shifted λmax from the ancestral red pigment by a single amino acid substitution Y277F.

Guinea pig (P516) appears to have achieved its present green color sensitivity from the original mammalian ancestral green pigment by a single amino acid substitution T285A. The extreme blue shift in a λmax value of rabbit (P509) pigment evolved from the red pigment with a λmax at 553 nm by H197Y and A308S, whereas those of the two murine pigments evolved from the ancestral green pigment by S180A and A308S (Figure 5). Thus, the evolution of red-green color vision in mammals indicates that the extant color vision has been achieved often by independent amino acid substitutions at only a few sites.

DISCUSSION

Red-green color vision in primates: Hominoids and Old World monkeys have two X-linked genes encoding

TABLE 3
The effects of amino acid changes at sites 180, 197, 277, 285, and 308 on the λmax-shifts

Amino acids	Estimator (nm)					
	Z	θ ₁	θ ₂	θ ₃	θ ₄	θ ₅
SHYTA						
Mammalian pigments (n = 13)	556 ± 2	-3.0 ± 2.9	-21.1 ± 2.5	-6.0 ± 2.9	-17.0 ± 2.6	-24.4 ± 2.9
Mammalian pigments (n = 12) ^a	560 ± 1	-7.3 ± 0.7	-28.4 ± 0.8	-7.2 ± 0.6	-15.1 ± 0.6	-15.6 ± 1.0
Vertebrate pigments (n = 18) ^a	560 ± 0.4	-7.2 ± 0.6	-28.0 ± 0.8	-6.8 ± 0.7	-15.9 ± 0.6	-16.0 ± 1.1
AHFAA (n = 12) ^a	530 ± 0.4	7.3 ± 0.7	-28.4 ± 0.8	7.2 ± 0.6	15.1 ± 0.6	-15.6 ± 1.0
AYYTS (n = 12) ^a	509 ± 0.4	7.3 ± 0.7	28.4 ± 0.8	-7.2 ± 0.6	-15.1 ± 0.6	15.6 ± 1.0

^a Dolphin (P524) is excluded from the estimation.

the red and green opsins. With the exception of New World (NW) monkeys, it appears that all mammalian species have only one locus that encodes either red or green opsins (Radlwimmer and Yokoyama 1997, 1998). Most NW monkeys also have one red-green opsin locus (however, see Jacobs *et al.* 1996), but this locus is polymorphic and contains three different alleles (Mollon *et al.* 1984; Neitz *et al.* 1991; Hunt *et al.* 1998). In these species, all males are red-green color blind, but females are either color blind or trichromatic depending on the allelic compositions. Using ERG and MSP, three different allelic pigments have been identified in capuchin monkey (*Cebus nigrivittatus*; P537, P550, and P562; Jacobs and Neitz 1987a), in marmoset monkey (*Callithrix jacchus jacchus*; P543, P556, and P563; Travis *et al.* 1988; Tovee *et al.* 1992), in squirrel monkey (*Saimiri sciureus*; P533–P538, P544–P551, and P559–P565; Mollon *et al.* 1984; Jacobs and Neitz 1987b; and Jacobs *et al.* 1993), and in tamarin monkey (*Saguinus mystax*; P545, P557, and P562; Jacobs *et al.* 1987). All 12 alleles have been sequenced at the nucleotide level. Unfortunately, the λ_{\max} values of these pigments have not been determined directly using the *in vitro* assay. Thus, the relevance of the five-sites rule cannot be discussed for these data yet.

To obtain direct information on the λ_{\max} values of the red and green pigments in NW monkeys, we isolated the three allelic opsin cDNAs from the marmoset retina by RT-PCR using two primers: 5'-AGGGCTGAATTCCA CCATGGCCCAGCAGTGGAG-3' (forward) and 5'-GGC AGAGTCGACGCAGGTGACACCGAGGACA-3' (reverse; see Shyue *et al.* 1998). Using these opsin cDNAs, we regenerated the three allelic pigments using the *in vitro* assay (S. Kawamura, F. B. Radlwimmer and S. Yokoyama, unpublished data). Our analyses show that marmoset pigments with AHYAA, AHYTA, and SHYTA have the λ_{\max} values at 540, 553, and 562 nm, respectively. These λ_{\max} values agree well with the MSP estimates. Furthermore, the three λ_{\max} values are very close to the corresponding predicted values 538, 553, and 560 nm from the five-sites rule.

In human (P530) and human (P560) pigments, amino acids S and Y at site 116, I and T at 230, A and S at 233, and Y and F at 309 have minor effects on the fine tuning of their color sensitivities (Asenjo *et al.* 1994). Although the compositions of amino acids are not the same, the triallelic pigments of NW monkey are also polymorphic at 116, 230, and 233. However, such polymorphic amino acids at 116, 233, and 309 are found only among the primate red and green pigments (Figure 2). Thus, the effects of these polymorphic amino acids on red-green color vision are irrelevant in many other species. One interesting feature of human (P560) pigment is that the population survey shows that 62% of the red pigment consists of SHYTA, a typical human (P560) pigment, but 38% of the allelic red pigment consists of AHYTA (Winderickx *et al.* 1992). The latter

pigment has a λ_{\max} value at 552 nm (Merbs and Nathans 1992), which is virtually identical to the predicted value, 553 nm, from the five-sites rule (Table 2).

Color vision in nonmammalian species: To date, the *in vitro* estimates for the λ_{\max} values of the orthologous pigments in nonmammalian species are available only for goldfish (*C. auratus*) and American chameleon (*Anolis carolinensis*). Although they have the same amino acid SHYTA at the five critical sites, the goldfish and American chameleon red pigments have λ_{\max} values at 525 nm (Johnson *et al.* 1993) and 561 nm (Kawamura and Yokoyama 1998), respectively. Thus, the American chameleon red pigment is consistent with the five-sites rule, but the goldfish red pigment is not.

Many freshwater fishes and amphibians utilize either 11-*cis*-retinal (vitamin A1 aldehyde) or 11-*cis*-3, 4-dehydroretinal (vitamin A2 aldehyde) as a chromophore (*e.g.*, see Dartnall and Lythgoe 1965). In general, visual pigments with 11-*cis*-3, 4 dehydroretinal (A2-pigments) absorb longer wavelengths than those with 11-*cis*-retinal (A1-pigments; Dartnall and Lythgoe 1965; Whitmore and Bowmaker 1989). The relationship between the λ_{\max} value of the A1-pigment (L1) and that of the A2-pigment (L2) is given roughly by empirical formulas $L2_{WB} = (L1/52.5)^{2.5} + 250$ (Whitmore and Bowmaker 1989) and $L2_H = 10^4 / [(10^4/L1) - 0.367 - 0.05054\{(10^4/L1) - 23.347\}^2]$ (Harosi 1994; see also Kawamura and Yokoyama 1998). Almost all the goldfish pigments are A2-types, with A1-pigments representing only 4% of the entire pigment population in the retina (Palacios *et al.* 1998).

Using the *in vitro* assay, Johnson *et al.* (1993) regenerated two green and one red A1-pigments with λ_{\max} values at 505 nm [goldfish (P505)], 511 nm [goldfish (P511)], and 525 nm [goldfish (P525)]. These pigments represent two evolutionarily distinct groups. The first two pigments belong to the RH2 pigment group, whereas the third pigment is orthologous to the mammalian red and green pigments and belongs to the LWS/MWS pigment group (Yokoyama 1997). Palacios *et al.* (1998) measured the spectral sensitivities of cone photoreceptor cells of goldfish by recording membrane photocurrents with suction pipette electrodes. They found three major groups of photoreceptor cells with λ_{\max} values at 623 ± 7 nm, 537 ± 5 nm, and 447 ± 8 nm and two rare types with λ_{\max} values at 356 and 574 nm (see also Table 4). Goldfish (P505) and goldfish (P511) A1-pigments are expected to operate as A2-pigments with λ_{\max} values at 530–540 nm, which correspond to the A2-pigments with λ_{\max} values at 537 nm found by Palacios *et al.* (1998; Table 4). Thus, under normal circumstances, goldfish (P505) and goldfish (P511) pigments have green sensitivities. Goldfish (P525) pigment can have a λ_{\max} value at ~ 565 nm as an A2-pigment, which may correspond to a rare type of A2-pigment with a λ_{\max} value at 574 nm (Palacios *et al.* 1998; Table 4). However, as we see next, the existence

TABLE 4
Absorption spectra of the goldfish red and green pigments

A1-pigment (nm)	Reference	A2-pigment		
		L _{2WB} (nm)	L _{2H} (nm)	Observed ^a (nm)
505	Johnson <i>et al.</i> (1993)	537	532	537
511	Johnson <i>et al.</i> (1993)	546	541	537
525 ^b	Johnson <i>et al.</i> (1993)	566	563	574
559	This study	620	624	623

$L_{2WB} = (L1/52.5)^{2.5} + 250$ (Whitmore and Bowmaker 1989) and $L_{2H} = 10^4 / [(10^4/L1) - 0.367 - 0.05054\{(10^4/L1) - 23.347\}^2]$ (Harosi 1994), where L1 is the λ_{max} value of the pigment with 11-*cis*-retinal (see also Kawamura and Yokoyama 1998).

^aPalacios *et al.* (1998).

^bThis pigment could not be found in this study.

of goldfish (P525) pigment in nature is questionable and needs to be reexamined.

The “true” goldfish red pigment: To date, no one has cloned the “true” goldfish red pigment. To clone the goldfish red opsin cDNA, we constructed forward and reverse primers using sequence information from the goldfish (P525) cDNA (Johnson *et al.* 1993; Figure 1). Using these primers, we cloned an opsin cDNA from a goldfish retina by RT-PCR amplification. The pigment regenerated using the *in vitro* assay has SHYTA at the five critical sites, just like the goldfish (P525) pigment, but it differs from goldfish (P525) pigment by one amino acid. That is, compared to C287 in goldfish (P525) pigment, this pigment has F287. Note that, because of the difference in the pigment lengths, the sites 287 in the human red and green pigments actually cor-

respond to 284 in the goldfish red pigment. When it is measured in the dark, this goldfish pigment has a λ_{max} value at 559 ± 4 nm, while its dark-light difference spectrum is given by 561 ± 2 nm (Figure 6). When goldfish (P559) pigment is reconstituted with 11-*cis*-3, 4 dehydroretinal, the corresponding A2-pigment is expected to have a λ_{max} value at ~ 620 nm (Table 4), which corresponds to the goldfish red A2-pigment with a λ_{max} at 623 ± 7 nm found by Palacios *et al.* (1998). Thus, we have cloned the true goldfish red pigment. The λ_{max} value of goldfish (P559) pigment is again explained nicely by the five-sites rule.

It should be noted that C287 has not been found in any other red and green pigments in a wide variety of vertebrates, including marine lamprey (*Petromyzon marinus*; S. Yokoyama and H. Zhang, unpublished result), Mexican cavefish (*Astyanax fasciatus*), killifish (*Oryzias latipes*), African clawed frog (*Xenopus laevis*), gecko (*Gekko gekko*), American chameleon (*A. carolinensis*), chicken (*Gallus gallus*), and pigeon (*C. livia*; S. Kawamura, N. S. Blow and S. Yokoyama, unpublished results), and mammals. Furthermore, we sequenced the entire coding regions of one red and two green pigments of five river dwelling, six Micos cave, and five Pachon cave fishes of *Astyanax fasciatus* (Yokoyama *et al.* 1995) and could not find C287. The two cave fish populations were derived from the river fish population during the last 1 million years (Avisé and Selander 1972; Chakraborty and Nei 1974; Wilkens 1988). Thus, these cave fish populations are much older than different goldfish varieties.

These observations strongly suggest that C287 may not actually exist and might have been introduced during the cloning process of the red opsin cDNA. To check this possibility, we cloned the red opsin cDNAs from six additional morphologically different breeds of goldfish by RT-PCR using the primers given in Figure 1. This survey reveals only synonymous nucleotide polymorphisms at a small number of sites (Table 5). The critical

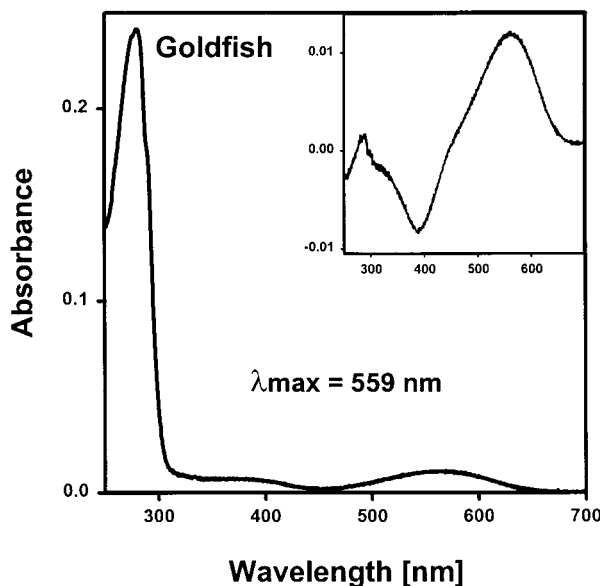


Figure 6.—Absorption spectrum of the goldfish red pigment in the dark and the dark-light difference spectrum (inset).

TABLE 5

DNA polymorphism among the goldfish red cDNA opsins

Individual	Site			
	135	276	600	851 ^a
1 ^b	T	C	T	G
2	C	T	C	T
3	C	T	C	T
4	C	C	C	T
5	C	C	C	T
6	T	C	T	T
7	T	C	T	T
8	C	C	C	T

^a This site corresponds to the second position of the codon 284, where TGT and TTT encode cysteine and phenylalanine, respectively.

^b Johnson *et al.* (1993).

nucleotide G at site 851 found in a red opsin cDNA identified by Johnson *et al.* (1993) cannot be found in the present polymorphism survey. This may mean that the frequency of nucleotides G at this site in a goldfish population is very low. However, it is more likely that the nucleotide G at site 851 was introduced during the process of cloning of the goldfish red opsin cDNA, possibly due to the error-prone reverse transcriptase activity at the time of cDNA library construction.

If goldfish (P525) pigment does not exist, how can we explain the rare goldfish photoreceptor cells with a λ_{\max} value at 574 nm? Three possibilities can be considered. First, because the goldfish retina contains a small population of A1-pigments, the rare photoreceptor cells may arise because goldfish (P559) pigments contain 11-*cis*-retinal rather than 11-*cis*-3, 4-dehydroretinal. Second, some goldfish pigments may be encoded by a polymorphic allele of goldfish (P559) opsin gene, as implicated by Johnson *et al.* (1993). Third, goldfish may have green pigments that belong to the LWS/MWS group in addition to those in the RH2 group, just like Mexican cavefish (Register *et al.* 1994).

The spectral sensitivities of the two rare photoreceptor cells are explained much better by A2-pigments than by A1-pigments (Palacios *et al.* 1998). Thus, 11-*cis*-retinal does not appear to be the cause of the rareness of the photoreceptor cells. The genetic polymorphism hypothesis for the rare photoreceptor cells is also problematic. It turns out that the rare photoreceptor cells are isolated from two retinas of a single fish, each of which contains the red-sensitive photoreceptor cells as well (Palacios *et al.* 1998). Now, suppose that these rare cells contain variant visual pigments, allelic forms of goldfish (P559) pigments, such as goldfish (P525) pigments. Then, this specific goldfish has to be heterozygous at the red opsin gene locus and the wild-type and variant types of red photoreceptor cells should be de-

tected in equal frequencies. However, as already indicated, the frequency of the variant types is 2/20 (Palacios *et al.* 1998) and is significantly <0.5 . Thus, it is unlikely that any allelic forms of goldfish (P559) pigments are contained in the rare photoreceptor cells. The third possibility that a MWS pigment may exist in goldfish has not yet been explored. In the LWS/MWS group, gene duplication of the ancestral LWS and MWS opsin genes predates the speciation between Mexican cavefish and goldfish, suggesting that goldfish can possess at least one MWS gene (Register *et al.* 1994). Having all other necessary retinal pigments in place, it is not unreasonable to assume that such extra pigments may be expressed less abundantly. Thus, MWS pigments appear to be viable candidates for the pigments in the rare photoreceptor cells with a λ_{\max} value at 574 nm. To study the existence of such pigments, more detailed analyses of the opsin genes in the goldfish genome are required.

The five-sites rule in vertebrates: Recently, we also studied the λ_{\max} value of the visual pigments in pigeon (*Columba livia*; S. Kawamura, N. S. Blow and S. Yokoyama, unpublished results). Our analyses show that the pigeon red pigment with SHYTA has a λ_{\max} value at 559 nm that is virtually identical to the predicted value of 560 nm from the five-sites rule. Thus, the red pigments of goldfish, American chameleon, pigeon, and marmoset all with SHYTA at the five critical sites show the λ_{\max} values at 559–562 nm, which are virtually identical to that of human (P560) pigment. The λ_{\max} values of marmoset (P554) and human (P552) pigments with AHYTA are very close to those of cat (P553) and goat (P553) pigments with the identical amino acids at the five sites (Table 2). The λ_{\max} value of the third allelic pigment with AHYAA in marmoset (540 nm) is also close to the predicted value, 538 nm, by the five-sites rule. Thus, when marmoset (P540), marmoset (P553), and marmoset (P562), goldfish (P559), chameleon (P561), and pigeon (P559) pigments are added in the estimation, the θ_i values inferred (vertebrate pigments, Table 3) are virtually identical to those obtained previously.

These observations show that the spectral sensitivities of virtually all red and green pigments in vertebrates known today are fully compatible with the five-sites rule. However, it should be cautioned that only a small number of the λ_{\max} values of the red and green pigments in nonmammalian species have been measured using the *in vitro* assays. Thus, the generality of the five-sites rule for the red-green color vision in vertebrates remains to be seen. The five-sites rule for red-green color vision in mammals may require further modification in its detail, but its validity is strongly supported by the existing data.

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