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Molecular bases of color vision in vertebrates

Shozo Yokoyama

Biological Research Laboratories, Department of Biology, Syracuse University, 130 College Place, Syracuse, NY 13244,

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Visual pigments initiate vision and are characterized by their wavelengths of maximal absorption (λmax). Modifications of the λmax values of visual pigments have allowed organisms to adapt to diverse light environments. The availability of the functional assays of these visual pigments using cultured cells makes vision an ideal genetic system to study the molecular bases of adaptation and genetics of color vision in vertebrates. The visual pigments in vertebrate retinas are distinguished into five evolutionarily distinct groups RH1 (λmax = 490−500 nm), RH2 (470–510 nm), SWS1 (360−420 nm), SWS2 (440−455), and LWS/MWS (510−570 nm). Here, we review amino acid replacements that are associated with the shifts in the λmax values of these visual pigments.

The λmax-shifts in several RH1 pigments seem to reflect adaptive changes to blue environments of the organisms and are explained mostly by amino acid replacements D83N (D → N at residue 83), E122Q, and A292S. Similarly, the blue-shifts in the λmax values of the RH2 pigments can be explained by D83N, E122Q, A164S, and M207L. For the SWS1 pigments of birds, only one amino acid replacement S84C seems to be responsible for the transformation of ultraviolet pigments from the violet pigment. For the LWS/MWS pigments, the additive effects of amino acid differences at 180, 197, 277, 285, and 308 fully explain the red-green color vision in a wide range of vertebrates. All of these observations suggest that the evolution of the extant visual pigments can be explained by amino acid replacements at only a small number of sites.

Visual pigments, a group of G-protein-coupled receptors, initiate visual excitation (Wald 1968). Each visual pigment consists of a transmembrane protein, opsin, and the chromophore, 11-*cis*-retinal, and can be characterized by its wavelength of maximal absorption (λmax). Human color vision is mediated by 'blue', 'green', and 'red' visual pigments. The 'blue' pigments absorb wavelengths ranging from about 370 nm to 570 nm with a λmax at 420 nm, while both 'green' and 'red' pigments are sensitive to wavelength about 450−620 nm with λmax values at 530 nm and 560 nm, respectively (Nathans 1989). The molecular bases of the spectral tuning of these and other visual pigments in vertebrates are still not well understood. Recently, however, some significant progress has been made on this subject.

To evaluate the mechanisms of the functional properties of visual pigments, a large number of amino acid changes have been introduced into the bovine rod-specific visual pigment (rhodopsin) by several groups of vision scientists (for a review, see Yokoyama 1997). In most of these analyses, charged amino acids have been considered. For example, an amino acid change from glutamic acid at residue 113 (E113) to glutamine (E113Q) shifts the λmax of the pigment from 500 nm to 380 nm (Sakmar et al. 1989; Zhukovsky and Oprian 1989; Nathans 1990a, b). E113 is the negatively charged counterion to the positively charged protonated Schiff base and the modification of this opsin structure causes a drastic shift in the λmax value of the pigment. Unfortunately, most of these amino acid changes, including E113Q,

have not been found in nature. Thus, it is not immediately clear how these mutagenesis results are helpful in elucidating the molecular basis for the divergence of λmax values of visual pigments in nature (Yokoyama 1995, 1997).

Fortunately, molecular evolutionary methods often alleviate this problem (Yokoyama and Yokoyama 1990; Yokoyama 1995, 1997; Yokoyama et al. 1999). By analyzing visual pigment sequence data, we can identify potentially important amino acid replacements that may shift the λmax values. Importantly, these hypotheses can be tested rigorously by conducting mutagenesis experiments. Since we study the processes of functional adaptation of visual pigments to various photic environments, the analyses will also elucidate natural selection at the molecular level. In the following, I shall review such analyses by considering dim vision, ultraviolet (UV) vision, and red-green color vision.

BACKGROUND INFORMATION

Phylogenetic relationships of visual pigments. Visual pigments in the vertebrate retinas are classified into five major groups: 1) the RH1 cluster (mostly consisting of rod-specific pigments with λmax values at ~500 nm); 2) the RH2 cluster (a mixture of pigments with λmax values at 470–510 nm); 3) the SWS1 cluster (consisting of short wavelength-sensitive pigments with λmax values at 360–420 nm); 4) the SWS2 cluster (consisting of SWS pigments with λmax values at 440–455 nm); and 5) the LWS/MWS cluster (consisting of long or middle wavelength-sensitive pigments with λmax values of 510–570 nm) (Yokoyama 1994, 1995, 1997; Yokoyama and Yokoyama 1996; see also Okano et al. 1992; Hisatomi et al. 1994).

The phylogenetic relationships of these retinal pigments are often given by ((((RH1, RH2) SWS2) SWS1) LWS/MWS) (e.g. see Yokoyama 1997). Since each of the five groups of visual pigments includes pigments from a wide range of vertebrates, the ancestors of all vertebrates must have possessed all five types of these visual pigments (Yokoyama and Yokoyama 1996).

The evolutionary approach toward understanding of the spectral tuning of visual pigments. The molecular mechanisms of color vision may be elucidated in four steps: 1) cloning and molecular characterization of opsin genes; 2) determination of the λmax values of the visual pigments; 3) identification of potentially important amino acid changes that may shift the λmax values of the visual pigments; and 4) determination of the actual effects of these mutations identified in step three.

In step three, we first construct or utilize an available evolutionary tree for the visual pigments and then infer the amino acid replacements and λmax-shifts in the evolutionary tree. By associating certain amino acid replacements with λmax-shifts, we can identify potentially important amino acid replacements. In this procedure, we consider mostly highly conserved sites because the evolutionary conservation often implies functional importance (Yokoyama 1994, 1995). For the functional assays in steps two and four, we can regenerate wild type and mutant pigments by expressing appropriate opsins in cultured cells, reconstituting them with 11-*cis*-retinal, purifying them using an antibody, and determining the λmax values of the resulting visual pigments (for details, see Yokoyama 2000a).

MUTAGENESIS RESULTS

So far, a majority of single and multiple amino acid changes has been introduced at more than 130 residues of the bovine rhodopsin and other visual pigments (Fig 1). Among these, only a small number of mutants is based on actual polymorphisms detected in natural populations. These mutations have been incorporated into visual pigments from mammals (Nathans 1990a, b; Chan et al. 1992; Merbs and Nathans 1993; Asenjo et al. 1994; Sun et al. 1997; Fasick and Robinson 1998) and fishes (Yokoyama et al. 1995, 1999). So far, these mutagenesis experiments show that amino acid changes at only 10 sites cause more than 5 nm-shifts in the λmax of visual pigments (Fig 1). Nine of the 10 residues are located in the transmembrane regions. This makes sense because the chromophore is embedded inside the transmembrane regions, where the chromophore and an opsin can interact (Hargrave et al. 1983; Baldwin 1993, 1994; Schertler et al. 1993; Unger and Schertler 1995). The mutant 4 is located outside of the transmembrane regions, but still causes a 28 nm blue-shift in the human red pigment, due to the loss of the chloride binding site (Sun et al. 1997).

These mutagenesis experiments show that the magnitudes of the λmax-shifts caused by identical or reverse amino acid changes can differ significantly depending on the background composition of amino acids of visual pigments. For example, in mutants 10 in Fig 1, the magnitudes of the λmax-shifts of pigments range from 8 to 28 nm depending on the direction of mutations (A → S or S → A) and the types of pigments used.

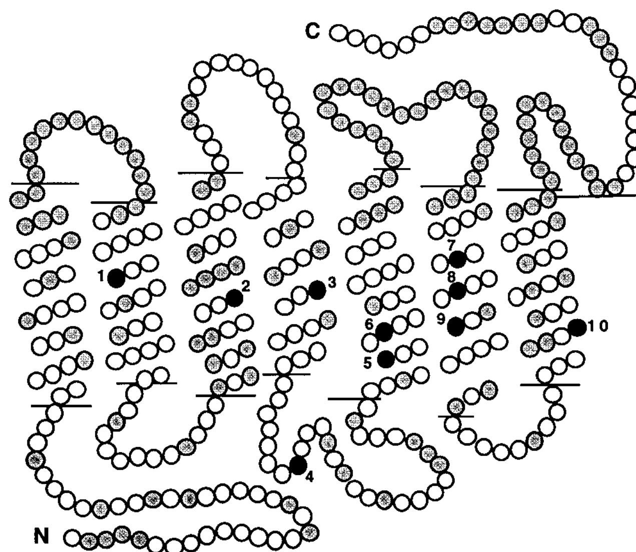

Fig. 1. A compilation of site-directed mutagenesis results. Amino acid changes (1–10) shifting the λmax value more than 5 nm are shown in black circles. Those that do not change or change the λmax value less than 5 nm are shown in stippled circles. The latter group of mutants are constructed using bovine rhodopsin by various authors. Data source: mutant 1 (D83N in bovine rhodopsin (–6 nm), Nathans 1990a, b); 2 (E122Q in bovine rhodopsin (–19 nm), Nathans 1990a, b; Q122E in coelacanth RH1 (10 nm) and RH2 (13 nm) pigments, Yokoyama et al. 1999); 3 (S180A in human LWS pigment (–7 nm), Asenjo et al. 1994); 4 (H197Y in human LWS pigment (–28 nm), Sun et al. 1997); 5 (L207M in coelacanth RH2 pigment (6 nm), Yokoyama et al. 1999); 6 (H211C in bovine rhodopsin (–5 nm), Nathans 1990a); 7 (F261Y in bovine rhodopsin (10 nm), Chan et al. 1992; Y277F in human LWS pigment (–10 nm), Asenjo et al. 1994; Y261F in cavefish RH1 pigment (–8 nm), Yokoyama et al. 1995); 8 (W265Y in bovine rhodopsin (–15 nm), Nakayama and Khorana 1991); 9 (A269T in bovine rhodopsin (14 nm), Chan et al. 1992; T285A in human LWS pigment (–16 nm), Asenjo et al. 1994); and 10 (A292S in bovine rhodopsin (–10 nm), Sun et al. 1997; S303A in mouse MWS pigment (18 nm), Sun et al. 1997; S292A in coelacanth RH1 pigment (8 nm), Yokoyama et al. 1999; S292A in dolphin MWS pigment (28 nm), Fasick and Robinson 1998).

RH1 pigments. At present, we can identify 30 RH1 pigments with known λmax values (Table 1). Given a phylogenetic tree of the RH1 pigments (Fig 2), we can identify three potentially important amino acid replacements D83N, E122Q, and A292S at various branches. In bovine rhodopsin, these changes shift the λmax value 6 nm (mutant 1 in Fig 1), 19 nm (mutant 2), and 10 nm (mutant 10) toward blue, respectively. Sites 83, 122, and 292 are all located in the transmembrane regions (Fig 3). As we can see in Fig 2, the λmax values of most RH1 pigments are at about 500 nm. However, the λmax values of the visual pigment from Conger eel, marine eel, goldfish, John Dory, coelacanth, chameleon, and dolphin are shifted about 10 –20 nm toward blue. Note that, with the exception of lamprey pigments, the three amino acid replacements are highly associated with these blue-shifts. Thus, it is strongly suspected that these amino acid replacements have caused the blue-shift in the λmax values of the seven visual pigments. In the lamprey pigments, the effects of D83N on the λmax-shift might have been reverted by other amino acid replacements.

Conger eel, marine eel, John Dory, coelacanth, and dolphin all live in aquatic environments and their habitats are dominated by dim blue light. Thus, it is conceivable that the RH1 pigments from these animals have achieved the blue-shifted λmax values because of their photic environments. The cause for the blue-shift in the λmax value of the chameleon pigment may be very different from those of the marine organisms' pigments. That is, it has 11-*cis*-3, 4-dehydroretinal instead of 11-*cis*-retinal as the chromophore (Provencio et al. 1992) and, consequently, absorbs longer wavelength (Whitmore and Bowmaker 1989; Harosi 1994). Thus, it appears that the chameleon pigment has achieved a blue-shifted λmax value to attain the λmax at about 500 nm. However, the blue-shift in the λmax of the goldfish pigment is not immediately clear.

So far, the actual effects of two out of the three amino acid replacements on the λmax-shift have been tested using only the coelacanth pigment. When single mutations Q122E and S292A and double mutations Q122E/S292A are introduced into the coelacanth pigment, the mutants have λmax values at 495, 493, and 511 nm, respectively (Yokoyama et al. 1999). This implies that E122Q and A292S together can shift the λmax value 25 nm toward blue and fully explain the observed blue-shifted λmax value of the coelacanth pigment.

RH2 pigments. The RH2 pigments are evolutionarily most closely related to the RH1 pigments. We can iden-

Table 1. The source of the amino acid sequences and λmax values of visual pigments in vertebrates

Group	Pigments	GenBank	Reference for λmax values
RH1	Marine lamprey (P500)	U67123	Harosi and Kleinschmidt (1993)
	River lamprey (P500)	M63632	Hisatomi et al. (1991)
	Skate (P500)	U81514	Cornwall et al. (1989)
	River eel (P502)	L78007	Hope et al. (1997)
	Marine eel (P482)	L78008	Hope et al. (1997)
	Conger eel (P487)	S82619	Archer and Hirano (1996)
	Cavefish (P503)	U12328	Yokoyama et al. (1995)
	Goldfish (P492)	L11863	Johnson et al. (1993)
	Carp (P499)	S74449	Crescitelli and Dartnall (1954)
	John Dory (P492)	Y14484	Dartnall and Lythgoe (1965)
	Sandgoby (P501)	X62405	Archer et al. (1992)
	Guppy (P500)	Y11147	Schwanzara (1967)
	Mosquito fish (P505)	Y11146	Archer and Hirano (1997)
	Coelacanth (P495)	AF131253	Yokoyama et al. (1999)
	Leopard frog (P502)	S49004	Pittler et al. (1992)
	Bullfrog (P500)	S79840	Kayada et al. (1995)
	Clawed frog (P502)	L07770	Batni et al. (1996)
	Salamander (P506)	U36574	Chen et al. (1996)
	Alligator (P499)	U23802	Wald et al. (1957)
	Chameleon (P491)	L31503	Kawamura and Yokoyama (1998)
	Chicken (P503)	D00702	Okano et al. (1992)
	Pigeon (P502)	AF149230	Kawamura et al. (1999)
	Mouse (P498)	M55171	Bridges (1959)
	Rat (P498)	Z46957	Bridges (1959)
	Dog (P508)	X71380	Jacobs (1993)
	Bovine (P500)	M21606	Oprian et al. (1987)
	Dolphin (P488)	AF055456	Fasick et al. (1998)
	Rabbit (P502)	U21688	Bridges (1959)
	Macaque (P500)	S76579	Bowmaker et al. (1980)
	Human (P497)	U49742	Crescitelli and Dartnall (1953)
RH2	Goldfish (P511)	L11865	Johnson et al. (1993)
	Goldfish (P506)	L11866	Johnson et al. (1993)
	Coelacanth (P478)	AF131258	Yokoyama et al. (1999)
	Chicken (P508)	M92038	Okano et al. (1992)
	Pigeon (P503)	AF149232	Kawamura et al. (1999)
	Chameleon (P495)	AF134189	Kawamura and Yokoyama (1998)
	Gecko (P467)	M92035	Kojima et al. (1992)
SWS1	Zebra finch (358)	-	Yokoyama et al. (unpublished data)
	Canary (P366)	-	Das et al. (1999)
	Parakeet (P371)	Y11787	Wilkie et al. (1998)
	Pigeon (P393)	AF149234	Yokoyama et al. (1998)
	Chicken (P415)	M92039	Okano et al. (1989)
LWS/MWS	Cat (P553)	AF132040	Yokoyama and Radlwimmer (1999)
	Horse (P545)	AF132043	Yokoyama and Radlwimmer (1999)
	Deer (P531)	AF132041	Yokoyama and Radlwimmer (1999)
	Guinea pig (P516)	AF132042	Yokoyama and Radlwimmer (1999)
	Squirrel (P532)	AF132044	Yokoyama and Radlwimmer (1999)
	Goat (P553)	U67999	Radlwimmer and Yokoyama (1997)
	Rabbit (P509)	AF054235	Radlwimmer and Yokoyama (1998)
	Mouse (P508)	AF011389	Sun et al. (1997)
	Rat (P509)	AF054241	Radlwimmer and Yokoyama (1998)
	Dolphin (P524)	AF055457	Fasick et al. (1998)
	Human (P530)	K03490	Oprian et al. (1991)
	Human (P552)	M13300[a]	Merbs and Nathans (1992)
	Human (P560)	M13300	Oprian et al. (1991)

[a] see also Winderickx et al. (1992).

The numbers after P refer to λmax values. Alligator, *Alligator mississippinensis*; Bovine, *Bos taurus*; Bullfrog, *Rana catesbeiana*; Carp, *Cyprinus carpio*; Canary, *Serinus canaria*; Cat, *Felis catus*; Cavefish, *Astyanax fasciatus*; Chameleon, *Anolis carolinensis*; Chicken, *Gallus gallus*; Clawed frog, *Xenopus laevis*; Coelacanth, *Latimeria chalumnae*; Conger eel, *Conger conger*; Deer, *Odocoileus virginianus*; Dog, *Canis familiaris*; Dolphin,*Tursiops truncatus*; Gecko, *Gekko gekko*; Goat, *Capra hircus*; Goldfish, *Carassius auratus*; Guinea pig, *Cavia porcellus*; Guppy, *Poecilia reticulata*; Horse, *Equus caballus*; Human, *Homo sapiens*; John Dory, *Zeus faber*; Leopard frog, *Rana pipiens*; Macaque, *Macaca fascicularis*; Marine eel, *Anguilla anguilla*; Marine lamprey, *Lamptera marinus*; Mosquito fish, *Gambusia affinis*; Mouse, *Mus musculus*; Parakeet, *Melopsittacus undulatus*; Pigeon, *Columba livia*; Rabbit, *Oryctolagus cuniculus*; Rat, *Rattus norvegicus*; River eel, *Anguilla anguilla*; River lamprey, *Lamptera japonica*; Salamander, *Ambystoma tigrinum*; Sandgoby, *Pomatoschistus minutus*; Skate, *Raja erinacea*; and Squirrel, *Sciurus carolinensis*; and Zebra finch, *Taeniopygia guttata*.

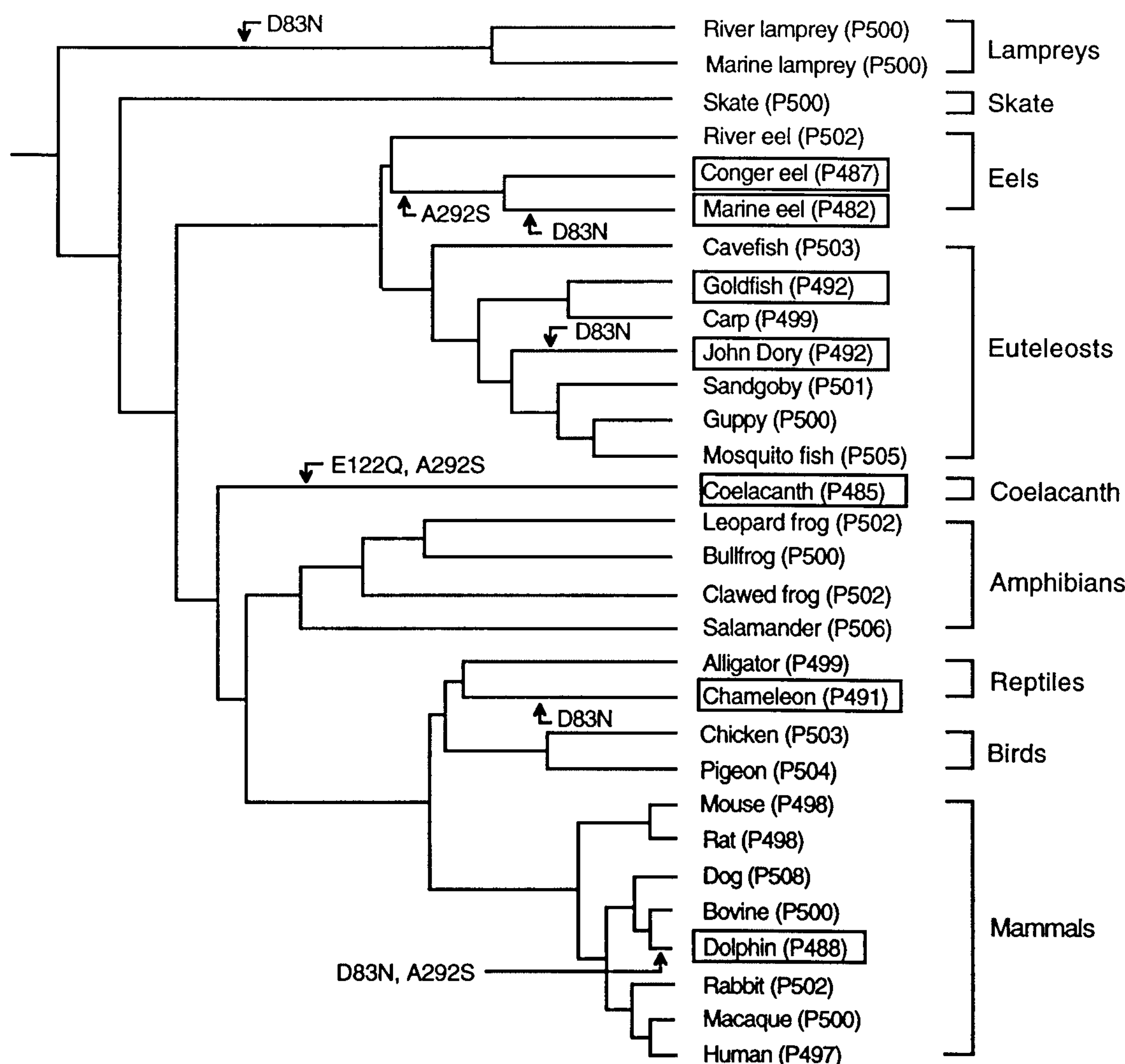

Fig. 2. Amino acid replacements at highly conserved residues indicated by arrows in ancestral RH1 pigments (Yokoyama 2000b). The pigments with blue-shifted λmax values are indicated by rectangles. The tree topology is based primarily on the organismal tree and the detailed phylogenetic relationships within eels, euteleosts, amphibians, and mammals have been obtained by applying NJ method (Saitou and Nei 1987) to the aligned amino acid sequence data.

tify four amino acid replacements D83N, E122Q, M207L, and A164S that are associated with λmax-shifts (Fig 4). As we already saw, D83N and E122Q are known to cause the blue-shifts in the λmax (Fig 1). It is also known that A164S causes 2 nm red-shifts in the λmax values of both bovine rhodopsin (Chan et al. 1992) and human MWS pigment (Asenjo et al. 1994). As we will see below, M207L causes the blue-shift in the λmax of the coelacanth pigment. These four mutations are also located close to the chromophore (Fig 3).

Fig 4 shows that E122Q occurred in the pigment of the tetrapod ancestor, followed by independent amino acid replacements M207L, A164S, and D83N in the coelacanth, chameleon, and gecko pigments, respectively. Among these, the effects of amino acid replacements are tested only for E122Q and M207L using the coelacanth pigment. When single mutations Q122E and L207M and double mutations Q122E/L207M are introduced into the coelacanth pigment, the mutants have λmax values at 491, 484, and 499 nm, respectively (Yokoyama et al. 1999). Thus, the two amino acid replacements again fully explain the blue-shifted λmax value of the coelacanth RH2 pigment. It should be noted that E122Q occurred in the RH1 and RH2 groups independently and their effects on the λmax-shifts in RH1 pigment (10 nm) and RH2 pigment (13 nm) in coelacanth are slightly different (Fig 1).

Thus, we can infer the molecular bases of the blue-shifts in the λmax values of some RH2 pigments. However, the evolutionary analyses also generate a new problem. Since E122Q occurred in the ancestral RH2 pigment

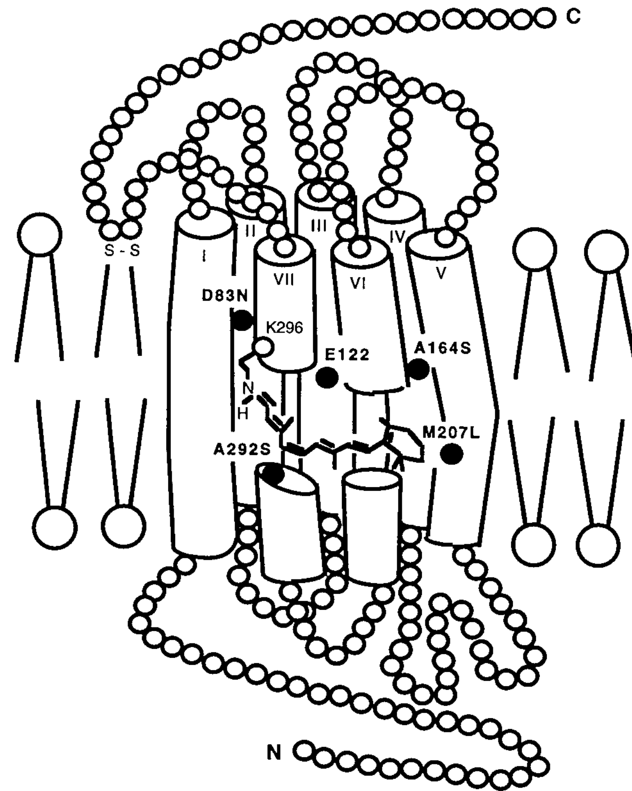

Fig. 3. The locations of amino acid replacements D83N, Q122E, and A292S in the RH1 pigment group and D83N, Q122E, A164S, and M207L in the RH2 pigment group. The model of bovine rhodopsin is taken from Applebury (1990), where the seven transmembrane helices together with the intradiscal amino-terminal and II-III, IV-V, and VI-VII loops, as well as the cytoplasmic carboxy-terminal and I-II, III-IV, and V-VI loops are also indicated. The 11-*cis*-retinal is linked to K296.

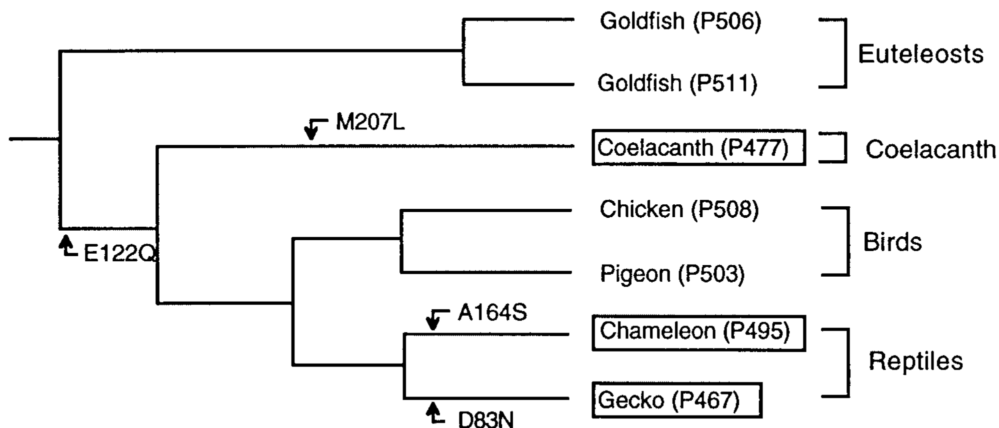

Fig. 4. Amino acid replacements at highly conserved residues in ancestral RH2 pigments (Yokoyama 2000b).

(Fig 4), it is most likely that the λmax value of the ancestral pigment must have been blue-shifted significantly. Then, in the bird RH2 pigments, the λmax values must have been reverted toward red. The validity of this event and the mechanism involved in this process need to be studied.

UV pigments of birds. The λmax values of SWS1 pigments range from 360 nm (UV) to 420 nm (violet). In particular, birds have either UV or violet pigments and appear to be excellent subjects to study the molecular bases of UV vision. At present, we know that the zebra finch, canary, and parakeet SWS1 pigments are UV-sensitive, whereas the orthologous pigeon and chicken pig-

ments are violet-sensitive (Table 1). A phylogenetic tree for these pigments shows that the zebra finch and canary pigments are closely related, but the phylogenetic position of the parakeet pigment cannot be resolved (Fig 5).

By comparing the amino acid sequences of the bird SWS1 pigments, C84 and I85 (following the residue number in the zebra finch pigment) are found to be common only to the zebra finch, canary, and parakeet UV pigments. However, virtually all orthologous SWS1 pigments, including the chicken and pigeon pigments, have S and V at the corresponding residues. Thus, in birds, C84 and I85 are highly correlated to UV-sensitivity. When a single mutation C84S and double mutations C84S/V85I are introduced into the zebra finch pigment, the mutant attained λmax values at 397 nm and 401 nm, respectively, both achieving violet-sensitivity (S. Yokoyama, N. S. Blow and F. B. Radlwimmer, unpublished result). This shows that a single mutation C84S is sufficient to make the ultraviolet pigment violet-sensitive. Since a majority of SWS1 pigments have S84 and I85, it is most likely that S84C and I85V occurred in the ancestral violet pigments, suggesting that the ultraviolet pigments in birds evolved from the violet pigments. Unfortunately, because of the poor resolution of the phylogenetic position of the parakeet pigment, we cannot determine whether the amino acid replacements S84C and V84I occurred once in the common ancestor of the three birds or twice in the zebra finch/canary lineage and parakeet lineage separately (Fig 5). It should be noted that the site 84 is located in the second transmembrane region and are very close to the chromophore.

As already indicated, the other orthologous UV pigments of such species as goldfish (*Carassius auratus*), chameleon, mouse, and rat all have S and V at the corresponding sites. Thus, it seems that UV vision in birds and other vertebrates have been achieved independently by entirely different molecular mechanisms.

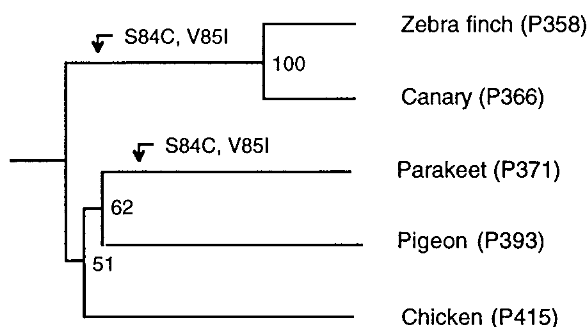

Fig. 5. Amino acid replacements at highly conserved residues of ancestral bird SWS1 pigments. The phylogenetic tree was constructed by applying the NJ method to the aligned amino acid sequence data with the Poisson correction. The numbers next to the different nodes are clustering percent support generated by 1,000 bootstrap replicates (Felsenstein 1985).

Red-green color vision. To study the molecular bases for the red-green color vision, the amino acid sequences of the LWS and MWS pigments from human and the Mexican cavefish, were compared (Yokoyama and Yokoyama 1990). This evolutionary analysis suggested that the LWS and MWS pigments of the two species are derived by independent opsin gene duplications, followed by nucleotide substitutions. It is also suggested that the LWS pigment in both human and fish evolved from the MWS pigment independently by three identical amino acid replacements A180S, F277Y, and A285T (following the residue numbers of the human LWS and MWS pigments) (see also Neitz et al. 1991). The three corresponding amino acid changes A164S, F261Y, and A269T in bovine rhodopsin increased λmax values by 2, 10, and 14 nm, respectively (Chan et al. 1992), explaining the majority of the difference between the λmax values of the LWS and MWS pigments. Essentially the same conclusion has been reached by introducing mutations into the human MWS and LWS pigments, except that the entire 30 nm of λmax-shift requires the minor contributions from amino acid differences at four additional residues (Asenjo et al. 1994). This 'three-sites' rule is applicable to many LWS and MWS pigments (Yokoyama 1997). Recently, however, some exceptions to this rule have been found. That is, having A180, Y277, and T285, the orthologous pigments in mouse, rat, and rabbit have λmax values at about 510 nm (Sun et al. 1997; Radlwimmer and Yokoyama 1998). It turns out that two amino acid changes H197Y and A308S cause the extreme blue-shifts in the λmax values (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 (Fig 6). As already noted, the residue 197 is located outside of the transmembrane regions, but it is known for its important function of chloride binding (Sun et al. 1997).

To test the validity of the 'five-sites' rule of red-green color vision, we recently characterized the LWS and MWS cDNAs of cat, horse, gray squirrel, white tailed deer, guinea pig, and goldfish (Yokoyama and Radlwimmer 1999). The visual pigments regenerated from these mammalian cDNAs and those from other mammals are shown in Table 1. The comparison of amino acids at the five sites and the associated λmax values are subjected to multiple linear regression analysis. The results show that S180A, H197Y, Y277F, T285A, and A308S shift the λmax values of the LWS/MWS pigments toward blue by 7, 28, 7, 15, and 16 nm, respectively, and the reverse amino acid changes toward red by the same extents (Table 2). The additive effects of these amino acid changes fully explain the red-green color vision in mammals and other vertebrates, including goldfish, chameleon, and pigeon. Thus, the spectral sensitivities of virtually all LWS and MWS pigments in vertebrates known today are fully compatible with the 'five-sites' rule. Although the 'five-sites'

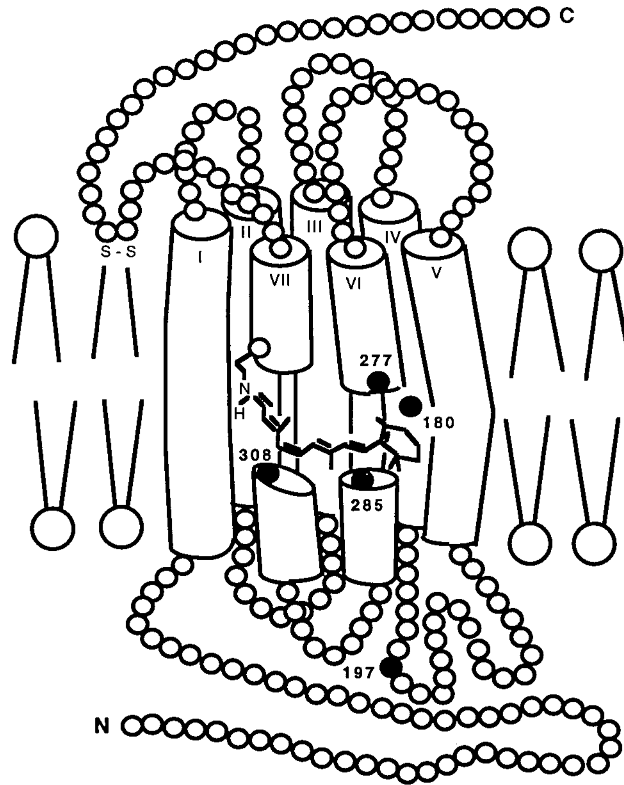

Fig. 6. The five critical amino acid residues that are important in determining variable wavelength-sensitivities of the LWS/MWS pigments.

Table 2. The effects of amino acid changes at sites 180, 197, 277, 285, and 308 on the λmax-shifts.

Amino acids	Estimator (nm)					
	Z	$\hat{\theta}_1$	$\hat{\theta}_2$	$\hat{\theta}_3$	$\hat{\theta}_4$	$\hat{\theta}_5$
SHYTA	560	–7.3	–28.4	–7.2	–15.1	–15.6
AHFAA	530	7.3	–28.4	7.2	15.1	–15.6
AYYTS	509	7.3	28.4	–7.2	–15.1	15.6

Standard errors associated with the estimates are all within ± 1 nm. $\hat{\theta}_1$, $\hat{\theta}_2$, $\hat{\theta}_3$, $\hat{\theta}_4$, $\hat{\theta}_5$, and Z denote the magnitudes of the λmax-shifts caused by S180A, H197Y, Y277F, T285A, A308S, and the amino acids at the other residues as a whole in a pigment, respectively. The negative $\hat{\theta}_i$, values come from amino acid changes to the opposite directions (modified from Yokoyama and Radlwimmer 1999).

rule for the red-green color vision in mammals may require further modification in its detail, its validity is strongly supported by the existing data.

Using the principle of the 'five-sites' rule, we can also infer the evolution of red-green color vision of the mammalian ancestors (Yokoyama and Radlwimmer 1999). The inference on the amino acid composition of the mammalian ancestor suggests that this pigment had S180, Y197, Y277, T285, and A308 with a λmax at 531 nm with green-sensitivity (Fig 7). The first red color vision in mammals appears to have occurred in the pigment in the common ancestor of human, rabbit, cat, horse, dolphin, goat, and deer (λmax = 553 nm). The extent human LWS pigment evolved from this ancestral pigment by A180S. It should be noted that 62% of the LWS pigments consist of S180, H197, Y277, T285, and A308, a typical human LWS pigment, but 38% of the allelic LWS pigments have A180 (Winderickx et al. 1992). The latter pigment is an ancestral type and has a λmax value at 552 nm (Table 1). Interestingly, the human MWS pigment reverted its function and achieved the extant green-sensitivity by Y277F and T285A. Thus, the addition of different mammalian pigments now suggests that the human MWS pigment evolved from the ancestral LWS pigment, not the other way, as originally suggested by Yokoyama and Yokoyama (1990). The green-sensitivities of deer

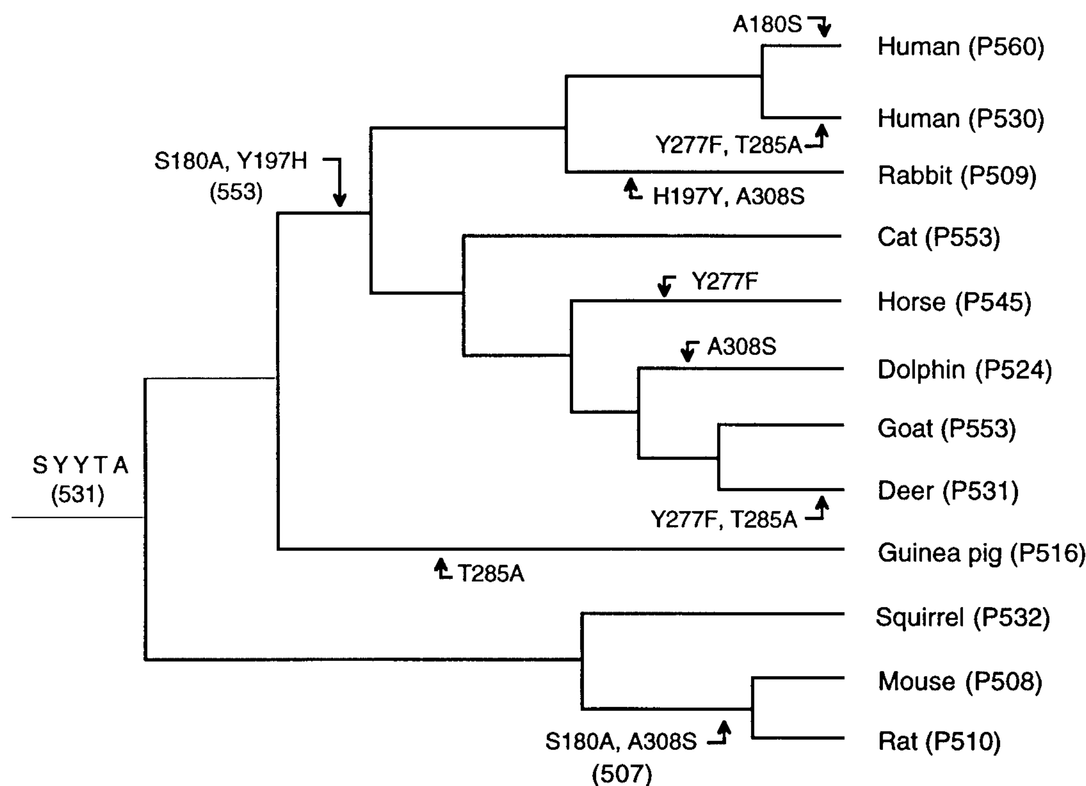

Fig. 7. Amino acid replacements at the five critical residues of the ancestral mammalian pigments. The numbers besides branches are predicted λmax values from the 'five-sites' rule (modified from Yokoyama and Radlwimmer (1999).

and dolphin must have been derived from the ancestral LWS pigment independently by Y277F and T285A and A308S, respectively.

We can see extreme blue-shifts in the λmax values of rabbit, guinea pig, mouse, and rat with λmax values at around 510 nm. The guinea pig pigment appears to have achieved its green-sensitivity from the mammalian ancestral MWS pigment by a single amino acid replacement T285A. The rabbit pigment evolved from the ancestral LWS pigment by H197Y and A308S and the murine pigments by S180A and A308S (Fig 7). These results strongly suggest that the extant red-green color vision in vertebrates has been achieved by independent amino acid replacements at only a few sites.

CONCLUSION

Nathans and his colleagues have cloned and characterized the human RH1, SWS1, and LWS/MWS opsin genes (Nathans and Hogness 1984; Nathans et al. 1986). Using the cDNA clones derived from these studies, the opsin genes from a variety of species have been isolated and characterized. Comparative analyses of these sequence data have proven to be a powerful tool in identifying the potentially important amino acid changes that may be responsible for the λmax-shifts of visual pigments. Based on the amino acid changes identified in this way, site-directed mutagenesis experiments have been conducted and elucidated the genetic bases of dim vision of fish (Yokoyama et al. 1995, 1999) and red-green color vision of mammals (Chan et al. 1992; Asenjo et al. 1994; Sun et al. 1997).

The molecular characterizations of opsin genes from additional species will help to pinpoint the amino acid replacements that are important for the functions of the visual pigments. It is expected that the mutagenesis analyses of visual pigments based on comparative sequence analyses will become common practice in approaching different evolutionary problems. Thus, comparative data analyses can be used as a convenient tool in designing mutagenesis experiments and molecular evolution will have a much more practical use than had been imagined before (Yokoyama 1995, 1997; Yokoyama and Yokoyama 1996). At the same time, these mutagenesis experiments will become an essential tool toward our understanding of the molecular bases of adaptive evolution of visual pigments to various photic environments.

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