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# Primary structures of chicken cone visual pigments: Vertebrate rhodopsins have evolved out of cone visual pigments

(vision/cDNA cloning/molecular evolution)

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**ABSTRACT** **The chicken retina contains rhodopsin (a rod visual pigment) and four kinds of cone visual pigments. The primary structures of chicken red (iodopsin) and rhodopsin have been determined previously. Here we report isolation of three cDNA clones encoding additional pigments from a chicken retinal cDNA library. Based on the partial amino acid sequences of the purified chicken visual pigments together with their biochemical and spectral properties, we have identified these clones as encoding the chicken green, blue, and violet visual pigments. Chicken violet was very similar to human blue not only in absorption maximum (chicken violet, 415 nm; human blue, 419 nm) but also in amino acid sequence (80.6% identical). Interestingly, chicken green was more similar (71–75.1%) than any other known cone pigment (42.0–53.7%) to vertebrate rhodopsins. The fourth additional cone pigment, chicken blue, had relatively low similarity (39.3–54.6%) in amino acid sequence to those of the other vertebrate visual pigments. A phylogenetic tree of vertebrate visual pigments constructed on the basis of amino acid identity indicated that an ancestral visual pigment evolved first into four groups (groups *L*, *S*, $M_1$, and $M_2$), each of which includes one of the chicken cone pigments, and that group *Rh* including vertebrate rhodopsins diverged from group $M_2$ later. Thus, it is suggested that the gene for scotopic vision (rhodopsin) has evolved out of that for photopic vision (cone pigments). The divergence of rhodopsin from cone pigments was accompanied by an increase in negative net charge of the pigment.**

Cone photoreceptor cells of higher vertebrates act under daylight conditions, and their characteristic color sensitivities are attributed to the visual pigments having unique absorption spectra. Humans have three kinds of cone visual pigments (1) with absorption maxima at 558 nm (human red), 531 nm (human green), and 419 nm (human blue) (2), which have been classified into two groups on the basis of amino acid similarity (3): long–middle wavelength-sensitive pigment (group *L*; human red and green) and short wavelength-sensitive pigment (group *S*; human blue). A recent investigation of the primary structure of cone visual pigments in monkeys supports this idea (4).

Microspectrophotometric experiments (5–7) showed that, unlike primates, some vertebrates have a tetrachromatic color vision. In fact, four kinds of cone visual pigments were extracted from chicken retinas (8, 9). We determined absorption maxima of the pigments, which are located at 571 nm (chicken red), 508 nm (chicken green), 455 nm (chicken blue), and 415 nm (chicken violet) (9). It is interesting to examine whether the fourth cone visual pigment, lacking in primate retinas, might belong to one of the known groups or to a previously undisclosed one.

In addition, chicken green-sensitive cone pigment (chicken green) combines characteristics normally found only in rhodopsins with those in cone pigments. Chicken green (508 nm; ref. 9) has an absorption maximum closer to the rhodopsins (around 500 nm) than does any other cone pigment that had been sequenced. Furthermore, it, like rhodopsin, has a high affinity for concanavalin A (8, 9), suggesting that its oligosaccharide chains are similar to those of rhodopsin. On the other hand, unlike rhodopsin, chicken green and the other chicken cone pigments are positively charged at neutral pH as shown by the lack of affinity for DEAE-Sepharose (8, 9). In addition, chicken green and other cone pigments are unstable in the presence of hydroxylamine even in the dark (8, 9). Thus, chicken green possesses properties of both rod and cone pigments. These unique characteristics prompted us to determine the primary structure of chicken green and the other chicken cone pigments.‡

Although our previous work (10) showed that chicken red should be classified into group *L* because of its similarity to human red and green, the present study clearly shows that chicken blue and green should be classified into two different groups. That is, an ancestral cone-like pigment evolved first into four groups, from one of which rhodopsins diverged later. This implies that scotopic vision appeared after animals acquired color vision.

## MATERIALS AND METHODS

**Library and Probes.** A chick retinal cDNA library was constructed in λgt11 phage vector by using 5 μg of poly(A)$^+$ RNA from 1-day-old chick retinas and a cDNA cloning kit (Amersham).

In the primary screening, a 72-nucleotide probe termed RV72 (5′-GATCATCACCACCACCATGCGGGACACCTCCTTCTCCGCCTTCTGCGTCGACTCCGACTCCTTCTGCTGAGC-3′, which is antisense for Ala-Gln-Gln-Lys-Glu-Ser-Glu-Ser-Thr-Gln-Lys-Ala-Glu-Lys-Glu-Val-Ser-Arg-Met-Val-Val-Val-Met-Ile, the loop V–VI region of chicken red; ref. 10) was used. To characterize the cDNA clones isolated in the primary screening, we used three additional probes: LYS30 (5′-AGGATTGTAGATGGCAGAGCTCTTGGCAAA-3′, which is antisense for Phe-Ala-Lys-Ser-Ser-Ala-Ile-Tyr-Asn-Pro, the helix VII region of chicken rhodopsin; ref. 11), CT30 (5′-AGCAGAGCTGTCCTCATCGCCCAGCGGGTT-3′, which is antisense for Asn-Pro-Leu-Gly-Asp-Glu-Asp-Thr-Ser-Ala, the carboxyl-terminal region of chicken rhodopsin; ref. 11), and BL35 [5′-GTCATGGCCTT(G or C)CC(A or G)CA(G or C)ACCATCTTCATGATGCA-3′, which is antisense for Cys-Ile-

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‡The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M92037–M92039).

Met-Lys-Met-Val-Cys-Gly-Lys-Ala-Met-Thr, the carboxyl-terminal region of human blue; ref. 3].

The synthetic probes were radiolabeled with [$\gamma$-$^{32}$P]ATP by the use of Megalabel kit (Takara Shuzo, Kyoto). Inserts of the isolated cDNA clones were also used as probes after being labeled with [$\alpha$-$^{32}$P]dCTP by the use of a random primer labeling kit (Takara Shuzo).

**Cloning and Sequencing.** Plaques of λgt11 phage were transferred to nylon membranes (Hybond-N+; Amersham). A hybridization with the synthetic oligonucleotide probes was carried out in a hybridization buffer composed of 5× SSC (1× SSC = 0.3 M sodium chloride/30 mM sodium citrate, pH 7.0), 10% formamide, 10 mM sodium phosphate, 0.25% sterilized skim milk (Difco), 10% dextran sulfate, and 100 μg of denatured salmon sperm DNA per ml at 42°C. In the case of the hybridization with labeled cDNA inserts, the concentration of formamide in the hybridization buffer was raised to 40%. After the hybridization, the nylon membranes were washed with 2× SSC containing 0.1% SDS at 50°C. Phages comprising DNA hybridized with the probe were identified by autoradiography or a Bio-Image analyzer (FUJIX BA100, Fuji Film).

The cDNA inserts excised from the phage DNAs were subcloned into the pBluescript II KS(+) (Stratagene) plasmid vector. The subclones were subjected to a nested deletion (12). Both strands of all the subclones were sequenced by the dideoxy chain-termination method (13) with the aid of Sequenase version 2.0 in the presence of single-stranded DNA binding protein (United States Biochemical).

**Peptide Analyses of Chicken Green and Blue.** Chicken green was purified as described (9). Chicken blue was purified by subjecting the chicken blue-enriched fraction (9) to carboxymethyl-Sepharose column chromatography (unpublished data). Highly purified chicken green or blue (0.4 mg each) was digested by lysyl-endopeptidase/*Achromobacter lyticus* protease I (8 μg each; Wako Biochemicals, Osaka) in 10 mM Tris·HCl, pH 9.0/4 M urea/2 mM dithiothreitol for 24 hr at 37°C. After the digest was centrifuged (90,000 × *g* for 20 min) to remove insoluble materials, the supernatant was applied to a Cosmosil 5$C_{18}$ P-300 column (4.6 × 150 mm; Nacalai Tesque, Kyoto) equipped with a HPLC system (model 600E; Waters). Proteolytic fragments were eluted with a linear gradient (5–90%) of acetonitrile in 0.05% trifluoroacetic acid at a flow rate of 1 ml/min. Amino acid sequences of the purified peptides thus obtained were determined by a gas-phase automated sequencer (model 477A; Applied Biosystems) and a phenylthiohydantoin amino acid analyzer (model 120A; Applied Biosystems).

**The Phylogenetic Tree.** A phylogenetic tree of visual pigments based on the ratio of amino acid substitution was constructed according to a neighbor-joining method (14). The amino acid identities (% identity) between each pair of the sequences were calculated at every position excluding positions where gaps exist in either of the compared sequences. The evolutionary distance [$k = -\ln(1 - K)$] was estimated by calculating the amino acid difference [$K = 1 - (\%\ \text{identity})/100$]. For estimating the probabilities ($P$) of occurrence of the tree topologies, the bootstrap resampling procedure (15) was repeated 500 times.

**Calculations of a Net Charge and an Isoelectric Point.** The net electric charge (NC) of the opsin moiety at given pH [NC(pH)] was estimated by the following formula:

$$\mathrm{NC(pH)} = \sum_{i}^{R,K,H} n(i)\,\frac{1}{1+10^{\mathrm{pH}-\mathrm{pK_a}(i)}} - \sum_{j}^{D,E,Y} n(j)\,\frac{1}{1+10^{\mathrm{pK_a}(j)-\mathrm{pH}}},$$

where $n(i)$ denotes the number of positively chargeable amino acids arginine (R), lysine (K), and histidine (H); and $n(j)$ denotes the number of negatively chargeable amino acids aspartic acid (D), glutamic acid (E), and tyrosine (Y). Since cysteine residues are often modified posttranslationally (16, 17), the dissociation of sulfhydryl groups was not considered. The isoelectric point (pI) of a pigment was estimated by solving the equation, NC(pI) = 0. The following $pK_a$ values for the amino acid side chains in water at 25°C were used (3.65, 4.25, 6.00, 10.08, 10.53, and 12.48 for D, E, H, Y, K, and R, respectively; ref. 18).

## RESULTS AND DISCUSSION

**Isolation of cDNA Clones.** The DNA sequence encoding the loop V–VI region of chicken red was selected as a screening probe (RV72) because many vertebrate visual pigments have similar sequences in this region. Then, $2.0 \times 10^5$ independent oligo(dT)-primed cDNA clones were screened with the use of radiolabeled RV72, resulting in the isolation of 46 positive clones. From the clones obtained, those encoding chicken red or rhodopsin were eliminated by using plaque hybridization. The clones encoding chicken red or rhodopsin were identified by strong hybridization only with probe λNECO (10) or with both CT30 and LYS30, respectively. Then, we obtained a clone λY2E [761 base pairs (bp)] hybridizing with both probes BL35 and LYS30 but not with CT30 or λNECO. The deduced amino acid sequence of λY2E (217 amino acids) was very similar to that of human blue. The upstream fragment of λY2E (5′ end to the *Pst* I site; 145 bp) was used as a probe for further screening. Briefly, $1.4 \times 10^6$ independent oligo(dT)-primed cDNA clones and $4 \times 10^5$ independent random-primed cDNA clones were screened to isolate more than 20 positive clones from which 2 clones were isolated: λRc2 (1408 bp, a full-length clone of λY2E encoding chicken violet; see below) and λF1 (1711 bp, a clone encoding chicken blue) hybridizing weakly with λY2E. Next, $1.4 \times 10^6$ independent oligo(dT)-primed cDNA clones were again screened by using λF1 as a probe, resulting in isolation of λF7G (2451 bp, a clone encoding chicken green) and λF9Rh (1326 bp, a full-length clone of chicken rhodopsin, whose sequence had already been reported; ref. 11).

**Characteristics of the Deduced Amino Acid Sequences.** The deduced amino acid sequences of the three clones, λRc2, λF1, and λF7G (Fig. 1), had several similarities and differences (Table 1). First, highly conserved amino acids were found in loop III–IV and V–VI regions, which in bovine rhodopsin are known to interact with transducin (26). Thus, these cone pigments would also probably interact with transducin, as was shown for chicken red (27). Second, the amino acid sequences of these three proteins carry a net positive charge at neutral pH (see below). This result is consistent with the chicken cone pigments lacking an affinity for DEAE-Sepharose at pH 6.6 (8, 9). Thus, chicken cone pigments are in contrast with rhodopsin, which is negatively charged as shown by adsorption to the DEAE-Sepharose (8, 9).

**RNA Blot-Hybridization (Northern Blot) Analysis.** A Northern blot analysis of chick retinal poly(A)$^+$ RNA (4 μg per lane) was performed by using the isolated cDNA inserts as hybridization probes (Fig. 2). λF7G and λF1 hybridized with 2.8-kilobase (kb) and 3.1-kb RNA, respectively. λRc2 hybridized with a single faint band (3.7 kb). λF9Rh showed a main band (1.6 kb) and a faint band (2.5 kb), suggesting multiple transcription sites in chicken retina as have been observed in rat, human, and frog opsin genes (28).

**Assignment of the Clones to the Pigments.** The partial amino acid sequence (Thr-Glu-Val-Ser-Ser-Val-Ser-Ser-Ser-Gln-Val-Ser-Pro-Ala) of a carboxyl-terminal fragment obtained by digestion of the highly purified chicken green with *A. lyticus* protease I perfectly coincided with the amino acid sequence

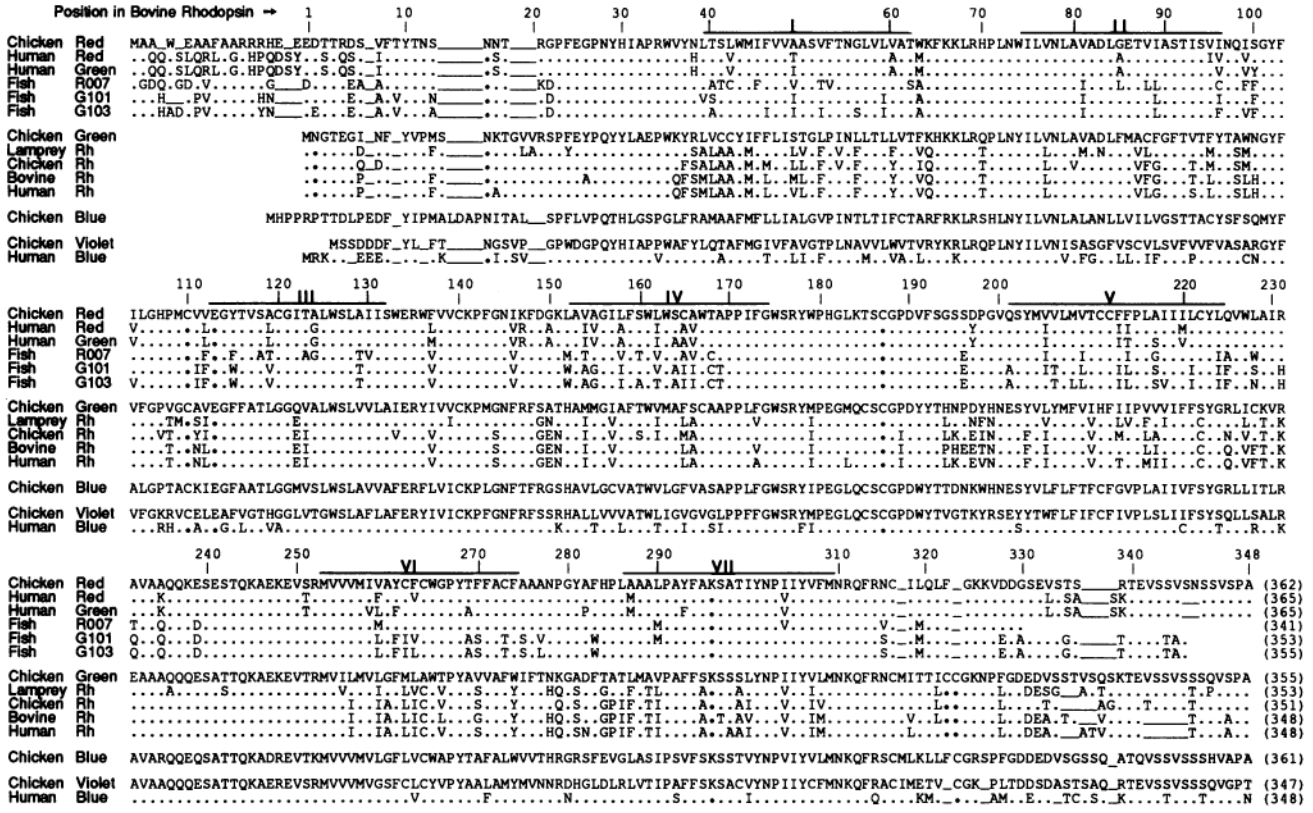


FIG. 1. The alignment of the primary structures of cone and rod visual pigments. The amino acid residues are expressed by the one-letter amino acid code. Deduced amino acid sequences of chicken red (10), human red (3), human green (3), fish putative cone pigments (19, 20), chicken green, lamprey rhodopsin (21), chicken rhodopsin (11), bovine rhodopsin (22, 23), human rhodopsin (24), chicken blue, chicken violet, and human blue (3) are aligned visually to optimize similarity. For simplicity, the sequence positions refer to those of bovine rhodopsin. Breaks in the sequence indicated by lines denote deletions. Dots in human red, human green, and fish pigments indicate the identical amino acids to chicken red. Dots in lamprey, chicken, bovine, and human rhodopsins indicate the identical amino acids to chicken green. Dots in human blue indicate the identical amino acids to chicken violet. Putative transmembrane domains are shown by the lines above the alignment.

deduced from λF7G. Accordingly, the protein (355 amino acids) encoded by λF7G was assigned to be chicken green. Chicken green, which was very similar (73.2% identical) to chicken rhodopsin, displayed two potentially palmitoylated cysteines in the carboxyl-terminal region and two potentially glycosylated asparagines in the amino-terminal region (Table 1). However, the other two clones (λF1 and λRc2) had only one cysteine and one asparagine residue. These characteristics of the deduced sequence of chicken green are consistent with the following biochemical results. (*i*) An antiserum raised against bovine rhodopsin reacted with not only chicken rod cells but also those single cone cells with a deep-yellow oil droplet (29) in which chicken green is present (30, 31). (*ii*) Chicken green and rhodopsin showed a higher affinity for concanavalin A-Sepharose than did chicken red, blue, and violet (8, 9), suggesting chicken green shares with rhodopsin similar amino acid-bound carbohydrates.

On the basis of the partial amino acid sequence of the seventh transmembrane region of chicken blue (Ser-Ser-Thr-Val-Tyr-Asn-Pro-Val-Ile-Tyr-Val-Leu-Met-Asn-Lys), which was determined as described above, the second clone λF1 was assigned to encode chicken blue (absorption maximum,

Table 1. Characteristics of the clones and the deduced amino acid sequences of chicken visual pigments

| Characteristics | Chicken visual pigment | | | | |
|---|---|---|---|---|---|
| | Red | Green | Rhodopsin | Blue | Violet |
| Clone | λNECO | λF7G | λF9Rh | λF1 | λRc2 |
| Size of mRNA, kb | 1.6 | 2.8 | 1.6, 2.5 | 3.1 | 3.7 |
| Pigment | | | | | |
| Absorption maximum, nm | 571 | 508 | 503 | 455 | 415 |
| Deduced amino acids, no. | 362* | 355 | 351† | 361 | 347 |
| Calculated molecular mass, Da | 40,338 | 39,965 | 39,325 | 39,655 | 38,718 |
| Calculated isoelectric point | 9.6 | 9.4 | 5.9 | 9.9 | 9.5 |
| Calculated ε at 280 nm‡ | 100,100 | 62,900 | 51,700 | 52,100 | 66,000 |
| Potential glycosylation site(s)§ | N31 | N2,N15 | N2,N15 | N24 | N12 |
| Chromophore-binding residue§ | K309 | K296 | K296 | K303 | K291 |
| Intramolecular disulfide bond§ | C123-C200 | C110-C187 | C110-C187 | C117-C194 | C105-C182 |
| Possible palmitoylated residue(s)§ | None | C322, C323 | C322, C323 | C330 | C317 |

*From literature (10).
†From literature (11).
‡Calculated by using the molar extinction coefficients (ε) of tryptophan (5559 $M^{-1}\cdot cm^{-1}$) and tyrosine (1197 $M^{-1}\cdot cm^{-1}$) (25).
§Presumed by observations concerned to bovine rhodopsin (single-letter amino acid code is used).

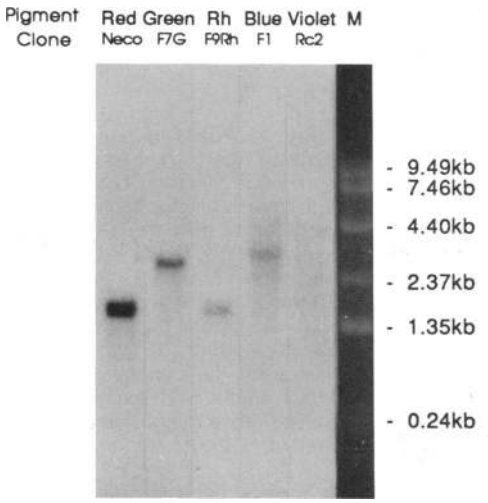


FIG. 2. Northern blot analysis of chicken retinal poly(A)$^+$ RNA with the cDNA clones of chicken visual pigments. Blots of glyoxylated poly(A)$^+$ RNA (4 $\mu$g per lane) from chick retinas were hybridized with the radiolabeled insert DNAs ($1.0 \times 10^6$ dpm) in the hybridization buffer containing 47% formamide, washed with 2× SSC containing 0.1% SDS at 50°C. Lane M shows molecular size markers (0.24- to 9.5-kb RNA ladder) from BRL.

455 nm; ref. 9). The primary structure of chicken blue (361 amino acids) showed relatively low similarity (39.3–54.6%) to any other known vertebrate visual pigment (Fig. 3).

As we have not yet succeeded in purifying chicken violet, no direct sequence data are available at present. However, the assignment that the third clone (λRc2) encodes chicken violet is strongly supported by the following two facts. (*i*) λRc2 mRNA was less abundant than λF1 mRNA, as shown by the Northern blot analysis (Fig. 2). This is consistent with there being less (≈20%; ref. 9) chicken violet in the retina than chicken blue. (*ii*) The amino acid sequence deduced from λRc2 was very similar (80.6% identical, Fig. 3) to that of human blue, whose absorption maximum (419 nm; ref. 2) is close to that of chicken violet (415 nm; ref. 9).

**Net Charge of the Pigments.** It should be noted that many of the positively charged residues in chicken green (Lys-36, Arg-38, Lys-64, Arg-225, Lys-229, and Lys-279) were replaced with noncharged residues as seen in bovine rhodopsin (Fig. 1). Furthermore, noncharged residues in chicken green (Gln-122, Ala-150, and Pro-196) were replaced with glutamate residues as seen in bovine rhodopsin. These replacements brought a striking shift of the isoelectric point from basic (pI = 9.4) to acidic (pI = 5.9) (Table 1). When the net charges of the cone and rod visual pigments were plotted against environmental pH (Fig. 4), the cone pigments clearly split from rhodopsins except for lamprey rhodopsin, which lay between

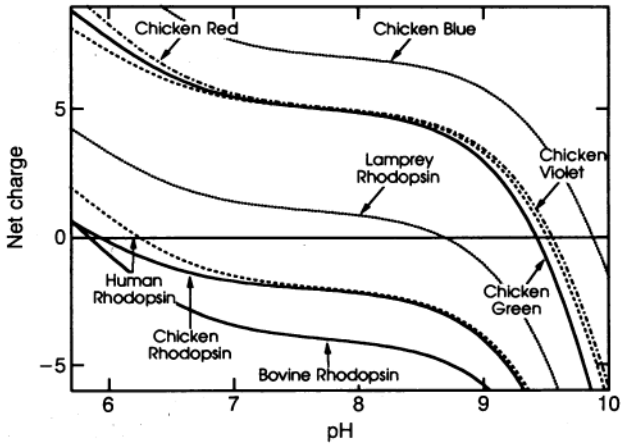


FIG. 4. The net charge of the pigments. The net charge was calculated as a function of pH for every pigment molecule (see *Materials and Methods*).

rod and cone pigments. Thus, in higher vertebrates, rod and cone pigments are negatively and positively charged, respectively, at neutral pH. Although the physiological significance of the molecular charge is not clear yet, some critical differences in the photoresponses between rod and cone cells (pigments) might be attributed to the opposite charges of the pigment molecules.

**Molecular Evolution of Visual Pigments.** The amino acid sequences of chicken visual pigments were compared with other vertebrate pigments (Fig. 1), and the percent identities in amino acid sequence were calculated (Fig. 3). Both figures clearly show that the known vertebrate visual pigments can be classified into four groups (long, short, and two kinds of middle-wavelength pigment groups: *L*, *S*, and $M_1/M_2$, respectively).

A phylogenetic tree of the vertebrate visual pigments (Fig. 5) was constructed by the neighbor-joining method (14) based upon amino acid identity (Fig. 3). The reliability of the tree topology was evaluated by bootstrap resampling (15). All of the tree topologies generated by resamplings 500 times were the same as that shown in Fig. 5 except for two regions. (*i*) Probability of the occurrence of the relationship among pigments in group *L* (chicken red, human red and green, and three fish pigments) illustrated in Fig. 5 is relatively low ($P = 0.81$). (*ii*) Group $M_1$ might be joined directly with group *S* ($P = 0.30$) instead of group $M_2$ ($P = 0.70$) as illustrated in Fig. 5.

The root of the vertebrate pigments (node A in Fig. 5) was determined by a tree including invertebrate rhodopsins (not shown). The bootstrap probabilities of occurrences of the tree topology with the deepest root of vertebrate pigments were 0.91 for the branch between nodes B and E (the present

| | group S | | $M_1$ | group $M_2$ | | | | group L | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Human Blue | Chicken Violet | Chicken Blue | Human Rh | Chicken Rh | Lamprey Rh | Chicken Green | Fish G103 | Fish G101 | Fish R007 | Human Green | Human Red | Chicken Red |
| Chicken Red | 41.9 | 42.3 | 41.8 | 42.0 | 43.1 | 43.1 | 44.6 | ***78.2*** | ***80.4*** | ***82.0*** | ***83.3*** | ***85.0*** | ***100*** |
| Human Red | 42.1 | 42.3 | 42.5 | 44.7 | 45.3 | 45.0 | 45.9 | ***72.0*** | ***73.9*** | ***77.7*** | ***95.9*** | ***100*** | |
| Human Green | 43.3 | 43.8 | 42.5 | 45.9 | 46.2 | 46.2 | 47.4 | ***73.2*** | ***74.7*** | ***76.2*** | ***100*** | | |
| Fish R007 | 40.1 | 40.4 | 42.6 | 41.7 | 40.7 | 40.4 | 42.3 | ***77.7*** | ***80.2*** | ***100*** | | | |
| Fish G101 | 41.0 | 39.6 | 40.5 | 40.8 | 40.8 | 40.5 | 42.0 | ***94.3*** | ***100*** | | | | |
| Fish G103 | 41.6 | 40.5 | 39.3 | 40.2 | 40.8 | 40.5 | 42.6 | ***100*** | | | | | |
| Chicken Green | 47.8 | 49.4 | 53.7 | ***71.0*** | ***73.2*** | ***75.1*** | ***100*** | | | | | | |
| Lamprey Rh | 47.5 | 48.3 | 54.6 | ***81.0*** | ***82.1*** | ***100*** | | | | | | | |
| Chicken Rh | 46.6 | 46.2 | 51.1 | ***87.3*** | ***100*** | | | | | | | | |
| Human Rh | 46.2 | 46.6 | 51.9 | ***100*** | | | | | | | | | |
| Chicken Blue | 49.4 | 49.9 | ***100*** | | | | | | | | | | |
| Chicken Violet | ***80.6*** | ***100*** | | | | | | | | | | | |
| Human Blue | ***100*** | | | | | | | | | | | | |

FIG. 3. The percentage of amino acid identity among several typical pigments. The values were calculated at every position excluding those positions where gaps exist in either of the two sequences. Values greater than 70% are in boldface characters.

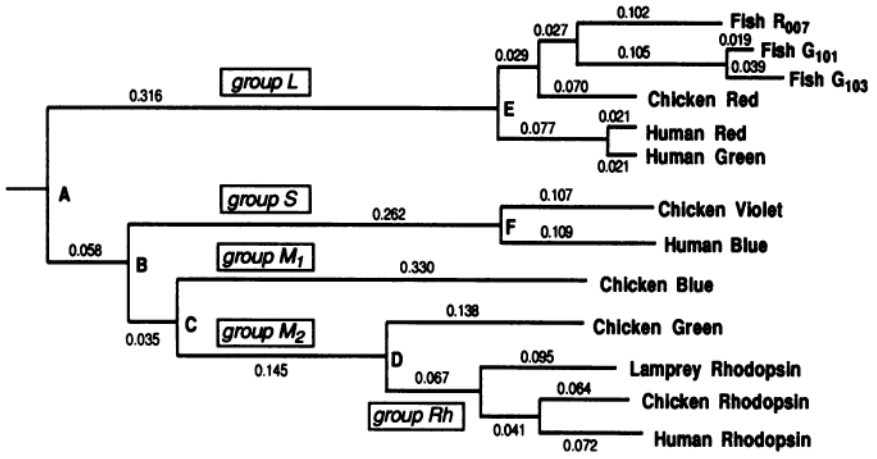


FIG. 5. A phylogenetic tree of visual pigments constructed on the basis of the amino acid identity. The tree is shown with branch lengths calculated from the evolutionary distances ($k$). The deepest root of the tree (node A) and the branch length between nodes A and B were determined by a tree including invertebrate rhodopsins (not shown). For simplicity, some of the mammalian rhodopsins (bovine, sheep, and mouse) whose amino acid sequences are very similar to human rhodopsin were omitted.

topology), 0.08 for a branch between nodes B and F, 0.01 for a branch between nodes C and D, and zero for the other branches.

Chicken red (10), human red (3), human green (3), and three kinds of putative fish visual pigments (19, 20) are clustered in group $L$, while chicken violet and human blue (3) are in group $S$. In contrast, neither chicken blue nor green can be clustered with other known cone pigments. Chicken blue should be classified in a previously unreported group termed "group $M_1$." Most interestingly, chicken green belongs to a large family (group $M_2$) in which many kinds of vertebrate rhodopsins (group $Rh$) are included (see below). The phylogenetic tree demonstrates that an ancestral visual pigment evolved first into four groups (groups $L$, $S$, $M_1$, and $M_2$), each of which includes one of the chicken cone pigments.

All of the sequenced cone visual pigments can be placed in either group $L$ or group $S$ with the exception of chicken blue and chicken green, whose sequences are reported herein. Which diverged earlier, rod and cone pigments or groups $L$ and $S$, has been a moot question (32). The present determination of the primary structure of chicken green, which is closely related to that of vertebrate rhodopsin, should give a definite point of divergence between rod and cone pigments (node D in Fig. 5). Thus, it is evident that vertebrate rhodopsins (group $Rh$) diverged from group $M_2$ after the divergences of the ancestral pigment into the four groups. As already described, lamprey rhodopsin shows an intermediate profile in molecular net charge (Fig. 4). This is consistent with the topology of the phylogenetic tree and suggests that a molecular net charge has varied from basic to acidic in the course of evolution from cone to rod pigments.

It is reasonable to consider that the ancestral visual pigment is similar in character to cone visual pigments rather than vertebrate rhodopsins. Rhodopsin of the lowest vertebrate (lamprey) and that of higher vertebrates diverged much later than the divergence of the cone pigments into the four groups. Taken together, we propose the hypothesis that animals had acquired the ability to distinguish color at least at the stage of the lowest vertebrate and acquired scotopic vision later.

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