Genetic Analyses of Visual Pigments of the Pigeon (*Columba livia*)

Shoji Kawamura,*,† Nathan S. Blow* and Shozo Yokoyama*

**Department of Biology, Syracuse University, Syracuse, New York 13244 and †Department of Biological Sciences, Graduate School of Science, University of Tokyo, Tokyo 113-0033, Japan*

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ABSTRACT

We isolated five classes of retinal opsin genes *rh1*$_{Cl}$, *rh2*$_{Cl}$, *sws1*$_{Cl}$, *sws2*$_{Cl}$, and *lws*$_{Cl}$ from the pigeon; these encode RH1$_{Cl}$, RH2$_{Cl}$, SWS1$_{Cl}$, SWS2$_{Cl}$, and LWS$_{Cl}$ opsins, respectively. Upon binding to 11-*cis*-retinal, these opsins regenerate the corresponding photosensitive molecules, visual pigments. The absorbance spectra of visual pigments have a broad bell shape with the peak, being called λmax. Previously, the SWS1$_{Cl}$ opsin cDNA was isolated from the pigeon retinal RNA, expressed in cultured COS1 cells, reconstituted with 11-*cis*-retinal, and the λmax of the resulting SWS1$_{Cl}$ pigment was shown to be 393 nm. In this article, using the same methods, the λmax values of RH1$_{Cl}$, RH2$_{Cl}$, SWS2$_{Cl}$, and LWS$_{Cl}$ pigments were determined to be 502, 503, 448, and 559 nm, respectively. The pigeon is also known for its UV vision, detecting light at 320–380 nm. Being the only pigments that absorb light below 400 nm, the SWS1$_{Cl}$ pigments must mediate its UV vision. We also determined that a nonretinal P$_{Cl}$ pigment in the pineal gland of the pigeon has a λmax value at 481 nm.

MOST vertebrates have two kinds of photoreceptor cells, rods and cones. Rods function in dim light, while cones function in bright light and are responsible for color vision. Photosensitive molecules, visual pigments, are located in the outer segments of these photoreceptors, each of which consists of a transmembrane protein, an opsin, and a chromophore, either 11-*cis*-retinal or 11-*cis* 3, 4-dehydroretinal (for review see Yokoyama and Yokoyama 1996). These visual pigments are characterized by their wavelengths of maximal absorption (λmax). Since the chromophore is universal to visual pigments, the wide range of λmax values from UV to infrared is generated mainly by the structural differences among various opsins. In many diurnal birds and reptiles, color vision is further modified by colored oil droplets in the inner segments of their cones (Walls 1942; Bowmaker 1991). Although their exact functions have not been fully elucidated, the oil droplets often contain a high concentration of carotenoids and are likely to serve as cut-off filters (Bowmaker 1991).

Because of its easy access and suitability to behavioral and physiological studies, the color vision of the pigeon (*Columba livia*) has been studied extensively. The cone photoreceptor cells in the pigeon retina can be classified into single cones and double cones. The oil droplets in the single cones have been classified into red (R), yellow (Y), clear (C), and transparent (T), according to their cut-off wavelengths (λcut) at ~560–580 nm, 510–540 nm, 440–450 nm, and with no significant absorbance throughout the spectrum, respectively (Bowmaker *et al.* 1997). The principal member of the double cone contains a pale oil droplet with a λcut at 440 nm, whereas the accessory member of the double cone rarely contains an oil droplet (Bowmaker *et al.* 1997). Applying microspectrophotometry (MSP), Bowmaker *et al.* (1997) have identified the rod pigments with a λmax at 506 nm and four different types of cone pigments with λmax at 567 nm (red), 507 nm (green), 453 nm (blue), and 409 nm (violet). Interestingly, there is a strong association between the types of visual pigments and those of photoreceptor cells. That is, the red pigments are found in R-type cones and in both members of double cones (Bowmaker 1991; Bowmaker *et al.* 1997), while the green, blue, and violet pigments are found only in the Y-, C-, and T-type cones, respectively (Bowmaker *et al.* 1997).

UV sensitivity of the pigeon at ~325–385 nm has been detected by both behavioral experiments (Blough 1957; Wright 1972; Kreithen and Eisner 1978; Emmerton and Delius 1980) and electroretinogram (ERG) experiments (Chen *et al.* 1984; Chen and Goldsmith 1986; Vos Hzn *et al.* 1994). It is possible in principle to identify cones containing UV pigments in the retina by MSP, but such analyses have shown no evidence for the existence of the UV receptor (Bowmaker 1977; Bowmaker *et al.* 1997). However, since MSP is based on a random sampling of the photoreceptors in a given retina, the analysis can miss UV pigments entirely if they are present in small numbers (Jacobs 1981; Bowmaker *et al.* 1997).

Another way of identifying visual pigments in the retina is to clone all opsin genes and reconstruct the corresponding visual pigments (*e.g.*, see Yokoyama 1997). At

Corresponding author: Shozo Yokoyama, Department of Biology, Syracuse University, 130 College Pl., Syracuse, NY 13244.
E-mail: syokoyam@mailbox.syr.edu

present, no genetic information on the visual pigments in the pigeon retina is available. Here we report the isolation and molecular characterization of the five classes of retinal opsin genes from the pigeon. The corresponding opsin cDNAs were isolated from the retinal and pineal RNAs, expressed in cultured COS1 cells, and reconstituted with 11-*cis*-retinal, and the λmax values of the resulting visual pigments were determined. The results show that, in addition to the pineal gland-specific pigment (Kawamura and Yokoyama 1996a), the pigeon has one type of rod pigment and four types of cone pigments. No evidence for the "true" UV opsin gene in the pigeon genome was found.

MATERIALS AND METHODS

Background information: Visual pigments in the retinas of vertebrates are classified into five major groups: (1) the RH1 cluster (mostly consisting of rod-specific pigments with λmax values of ~500 nm); (2) the RH2 cluster (a mixture of pigments with λmax values of 470–510 nm); (3) the SWS1 cluster (consisting of *s*hort *w*avelength-sensitive pigments with λmax values of 360–420 nm); (4) the SWS2 cluster (consisting of SWS pigments with λmax values of 440–455 nm); and (5) the LWS/MWS cluster (consisting of *l*ong *w*avelength-sensitive or *m*iddle *w*avelength-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.* 1996). The RH1, RH2, SWS1, SWS2, and LWS or MWS (LWS/MWS) opsins are encoded by *rh1*, *rh2*, *sws1*, *sws2*, and *lws* or *mws* (*lws*/*mws*) opsin genes, respectively.

Some vertebrates such as marine lamprey (Yokoyama and Zhang 1997), lizards (Kawamura and Yokoyama 1997), and birds (Okano *et al.* 1994; Max *et al.* 1995; Kawamura and Yokoyama 1996a) are also known to have an additional group of pineal gland-specific P pigments that are encoded by *p* opsin genes. The pigeon p_{Cl} gene of this type has been determined by Kawamura and Yokoyama (1996a). The recently found VA (vertebrate ancient) pigment of Atlantic salmon, *Salmo salaar* (Soni and Foster 1997), and parapinopsin of channel catfish, *Ictalurus punctatus* (Blackshaw and Snyder 1997), also seem to belong to this P cluster (S. Yokoyama and R. Yokoyama, unpublished results).

Genomic library screening: A λEMBL3 genomic library was constructed using the high-molecular-weight DNA extracted from the blood of one pigeon (*C. livia*; Kawamura and Yokoyama 1996a). The average size of the insert DNA in the genomic library was ~16 kb. Since the nuclear DNA content of the pigeon is ~50% of the human genome (Manfredi Romanini 1973), it requires at least 4.6×10^5 plaques of recombinant λ phages to clone a single-copy gene of ~16 kb in length (Sambrook *et al.* 1989). Thus, we screened a total of 6.3×10^5 recombinant plaques. As hybridization probes, we used bovine RH1 opsin cDNA (Nathans and Hogness 1983), human LWS and SWS1 opsin cDNAs (Nathans *et al.* 1986a), and a mixture of the exons from *sws2* genes of Mexican cavefish, *Astyanax fasciatus* (Yokoyama and Yokoyama 1993), and American chameleon, *Anolis carolinensis* (Kawamura and Yokoyama 1996b). Probe labeling and plaque hybridization were performed as described previously (Kawamura and Yokoyama 1993). Hybridized membranes were washed four times (30 min each) in 1× SSC (0.15 m NaCl/0.015 m Na_3, citrate)/0.1% SDS at 55°, which allows ~30% mismatch (Sambrook *et al.* 1989). The four probes were used sequentially by recycling the membranes. Old probes were removed from the membranes by washing them in 0.4 m NaOH at 45° for 30 min and then in 0.1× SSC/0.1% SDS/0.2 m Tris (pH 7.5) at 45° for 30 min.

A total of 62 phage clones were isolated and subjected to restriction enzyme mapping. Among these, clones λCL89 (representing $rh1_{Cl}$), λCL5 ($rh2_{Cl}$), λCL37 ($sws1_{Cl}$-*S*), λCL36 ($sws1_{Cl}$-*L*), λCL25 ($sws2_{Cl}$-*S*), λCL34 ($sws2_{Cl}$-*L*), and λCL102 (lws_{Cl}) contained entire coding regions of the opsin genes and were subcloned into pBluescript SK(−) vectors. The nucleotide sequences of these clones were determined in both strands by standard dideoxynucleotide-chain-termination method (Sambrook *et al.* 1989). The remaining 55 clones overlap with one of these 7 clones.

Southern hybridization: About 5 μg each of pigeon genomic DNA was digested with restriction enzymes, separated by size on a 0.5% agarose gel, and transferred onto a Hybond-N^+ nylon membrane (Amersham, Piscataway, NJ). Using the random priming method, exon 4 of bovine *rh1*, those of *rh2*, *sws1*, and *sws2* of American chameleon, and a portion of exon 5 of *lws* of American chameleon were labeled with [α-^{32}P]dATP. Different exons of $sws1_{Cl}$ were also used as hybridization probe. Hybridization was carried out at 65°, using the commercial protocol for Hybrid-N^+ membrane. The hybridization membrane was washed at 65° in 1× SSC/0.1% SDS.

cDNA cloning: Using the acid-guanidinium extraction method (Chomczynski and Sacchi 1987), ~50 and 4 μg of total RNAs were prepared from the retina and pineal gland of the second pigeon, respectively. $RH1_{Cl}$, $RH2_{Cl}$, $SWS2_{Cl}$, and LWS_{Cl} opsin cDNAs and P opsin cDNA were amplified from 200 ng of total retinal RNA and 70 ng total RNA from the pineal gland, respectively, by the reverse transcriptase-PCR amplification (RT-PCR) method.

Figure 1 shows RT-PCR primers used to amplify the five types of full-length opsin cDNAs. Forward and reverse primers contain *Eco*RI and *Sal*I linkers, respectively, to permit cloning of the PCR products into the pMT5 expression vector (Kawamura and Yokoyama 1998; Yokoyama *et al.* 1998). To facilitate translation, each forward primer contains the Kozak consensus sequence, CCACC (Kozak 1984), followed by the initiation codon. The *Sal*I linkers in the reverse primers are followed by 21–24 nucleotides of the reverse complement sequence starting from the second nucleotide position of the last codon. The extra 6 nucleotides 5′ to the linker sites are to facilitate restriction digestion and correspond to the genomic noncoding sequences in each respective location.

The first-strand cDNA synthesis was carried out at 42° for 1 hr in a total volume of 20 μl containing reaction buffer (10 mm Tris-HCl, pH 9.0, 1 mm $MgCl_2$, 50 mm KCl, 0.1% Triton

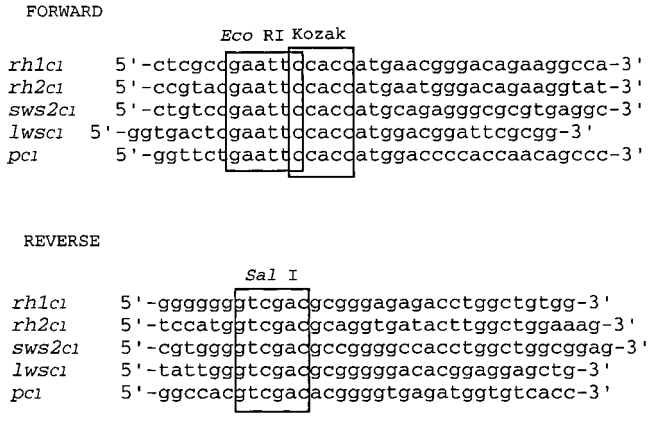

Figure 1.—Oligonucleotide primers for RT-PCR amplification of pigeon opsin mRNAs.

X-100), 1 mm dNTPs, 5 μm reverse primers, 20 units of RNasin (Promega, Madison, WI), and 200 units of SuperScript II reverse transcriptase (Gibco BRL, Gaithersburg, MD). The resulting cDNA was combined with the same reaction buffer containing 200 mm dNTPs, 1 μm each forward and reverse primers, and 5 units of *Taq* polymerase (Promega) in a total volume of 100 μl. PCR amplification was performed by 30 cycles at 92° for 45 sec, 55° for 60 sec, and 72° for 90 sec. At each cycle, the duration of the extension reaction was progressively extended by 3 sec. After the final extension step at 72° for 10 min, the PCR products were resolved in 1.5% agarose gel electrophoresis. The opsin cDNA band of ~1.1 kb was extracted and cloned into the *Eco*RV-digested pBluescript 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 Excel 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). With the exception of p_{Cl}, we selected clones that encode identical amino acid sequences to those of the corresponding sequences deduced from the genomic clones for spectral analyses of the visual pigments (see results).

Regeneration of visual pigments and spectral analysis: The fragment of the *Eco*RI/*Sal*I-digested pMT5 expression vector, ~5 kb in length, contains the sequences necessary for expression in cultured COS1 cells and the last 15 codons of the bovine rhodopsin, encoding Ser-Thr-Thr-Val-Ser-Lys-Thr-Glu-Thr-Ser-Gln-Val-Ser-Pro-Ala that are necessary for immunoaffinity purification (Molday and Mackenzie 1983). This *Eco*RI/*Sal*I fragment was ligated with the *Eco*RI/*Sal*I opsin cDNA fragments. The resulting plasmids were transiently expressed in COS1 cells and the transfected cells were incubated with 11-*cis*-retinal (Storm Eye Institute, Medical University of South Carolina) in the dark. The pigments were purified by binding to the monoclonal anitbody 1D4 Sepharose in buffer W1 (50 mm HEPES, pH 6.6, 140 mm NaCl, 3 mm $MgCl_2$, 20% [w/v] glycerol, and 0.1% dodecyl maltoside; Kawamura and Yokoyama 1998; Yokoyama *et al.* 1998).

UV visible absorption spectra of visual pigments were recorded at 20°, using a Hitachi U-3000 dual beam spectrophotometer. Visual pigments were bleached by a 60-W room lamp with 440-nm cut-off filter. Recorded spectra were analyzed using SigmaPlot software (Jandel Scientific, San Rafael, CA).

Qualitative RT-PCR assay: Total RNA was mixed with the PCR reaction mix (10 mm Tris-HCl, pH 9.0, 1.5 mm $MgCl_2$, 50 mm KCl, 0.1% Triton X-100, 200 μm dNTPs, and primers at 1 μm each), MMLV reverse transcriptase, and *Taq* polymerase in total volume of 25 μl. The primers given in Figure 1 were used for the amplification of $rh2_{Cl}$ and p_{Cl} cDNAs. The primers used for others were: 5′-AGCCCTGGAAGTTCTCG GCT-3′ (forward [F]: starting position at 128) and 5′-TTCATT GTTGATCTCCGGC-3′ (reverse [R]; starting position at 633) for $rh1_{Cl}$; 5′-ACTTCCGCTTCAACTCCAAACACG-3′ (F: position at 419) and 5′-GGCCGCCCGCACACCAG-3′ (R: position at 959) for $sws1_{Cl}$; 5′-AGCCCCGGCGTGTTCCGC-3′ (F: position at 121) and 5′-GAGGGCCAGGGGGACCCC-3′ (R: posit ion at 682) for $sws2_{Cl}$; and 5′-GTGGTGGTGGCGTCGGT GTT-3′ (F: position at 170) and 5′-TGGCCAGCCAGACTTG CAG-3′ (R: position at 720) for lws_{Cl}. The samples were placed in a thermal cycler at 50° for 8 min, followed by 35 cycles of 92° for 45 sec, 55° for 60 sec, and 72° for 90 sec. A total of 5 μl each of PCR products was electrophoresed on 2.5% agarose gel. PCR was also carried out without reverse transcriptase for each opsin gene, resulting in no amplification in both tisses.

Phylogenetic analysis: The six types of visual pigments of the pigeon were compared to those of chicken, *Gallus gallus* (RH1 pigment, GenBank accession no. D00702; RH2 pigment, M92038; SWS1 pigment, M92039; SWS2 pigment, M92037; LWS pigment, M62903; and P pigment, U15762) and American chameleon (RH1 pigment, L31503; RH2 pigment, AF134189–AF134191; SWS1 pigment, AF134192–AF134194; SWS2 pigment, AF133907; LWS pigment, U08131; and P pigment, AF134767–AF134771). To construct a rooted phylogenetic tree of these pigments, we used four different pigments of *Drosophila melanogaster* (Rh1 pigment, K02315; Rh2 pigment, M12896; Rh3 pigment, M17718; and Rh4 pigment, X65880) as the outgroup. The deduced amino acid sequences were aligned by CLUSTAL W program (Thompson *et al.* 1994) and adjusted visually. The number (K) of amino acid substitutions per site for two sequences was estimated from $K = -\ln(1 - p)$, where p is the proportion of different amino acids for a pair of sequences. The phylogenetic tree was reconstructed by applying the NJ method to the K values (Saitou and Nei 1987). The reliability of the phylogenetic tree was evaluated by the bootstrap analysis with 1000 replications (Felsenstein 1985).

RESULTS

Retinal opsin genes and visual pigments: We cloned all five major groups of the retinal opsin genes $rh1_{Cl}$, $rh2_{Cl}$, $sws1_{Cl}$, $sws2_{Cl}$, and lws_{Cl} from the pigeon, where both $sws1_{Cl}$ and $sws2_{Cl}$ have two forms with different intron sizes (Figure 2). $rh1_{Cl}$, $rh2_{Cl}$, $sws1_{Cl}$, and $sws2_{Cl}$ contain five putative exons and four introns, whereas lws_{Cl} contains one extra exon. The introns 1, 2, 3, and 4 of $rh1_{Cl}$, $rh2_{Cl}$, $sws1_{Cl}$, and $sws2_{Cl}$ and the corresponding introns 2, 3, 4, and 5 of lws_{Cl} interrupt the coding sequence at exactly the same positions. These exon-intron structures have been well conserved among the retinal opsin genes in vertebrates (Yokoyama and Yokoyama 1996). Splice junction signals (GT/AG) are conserved in all introns and there are no nonsense mutations in the coding regions.

By comparing the five opsin genes of the pigeon to those of chicken and American chameleon, we evaluated the proportions of identical nucleotides per site ($p_{I,\,nuc}$) for all pairs. The amino acid sequences of $RH1_{Cl}$, $RH2_{Cl}$, $SWS1_{Cl}$, $SWS2_{Cl}$, LWS_{Cl}, and P_{Cl} pigments deduced from the nucleotide sequences of $rh1_{Cl}$, $rh2_{Cl}$, $sws1_{Cl}$ (*swsCl-S* and *sws1Cl-L*), $sws2_{Cl}$ (represented by $sws2_{Cl}$-*S*), lws_{Cl}, and p_{Cl} are shown in Figure 3, where amino acid residue numbers correspond to those of $RH1_{Cl}$ pigment. By comparing these amino acids to those of the chicken and American chameleon, the proportions of identical amino acids per site ($p_{I,\,aa}$) were also evaluated. The comparisons of the $p_{I,\,nuc}$ and $p_{I,\,aa}$ show that $RH1_{Cl}$, $RH2_{Cl}$, $SWS1_{Cl}$, $SWS2_{Cl}$, and LWS_{Cl} pigments belong to RH1, RH2, SWS1, SWS2, and LWS/MWS groups, respectively (see materials and methods). That is, the $p_{I,\,nuc}$ values between orthologous opsin genes range from 0.74 to 0.92, whereas those between paralogous genes range from 0.51 to 0.75. Similarly, the $p_{I,\,aa}$ values between orthologous and paralogous pigments range from 0.82 to 0.98 and from 0.41 to 0.73, respectively.

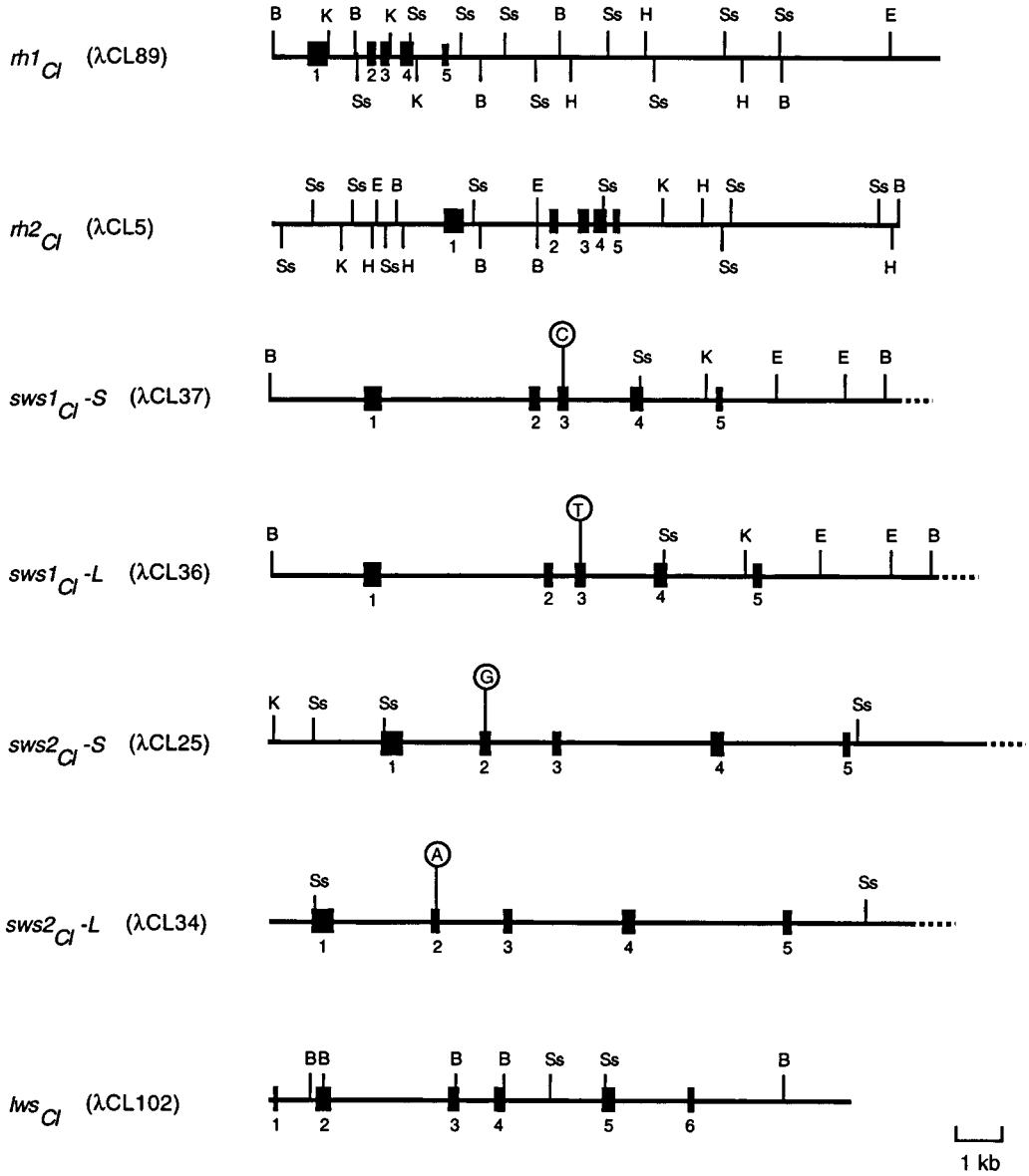

Figure 2.—The genomic structures of the retinal opsin genes of the pigeon. The coding regions are indicated by solid boxes. Introns 1, 2, 3, and 4 of $rh1_{Cl}$ consist of 856 bp, 99 bp, 241 bp, and 0.6 kb, respectively. Introns 1, 2, 3, and 4 of $rh2_{Cl}$ consist of 1.7 kb, 457 bp, 91 bp, and 173 bp, respectively. Introns 1, 2, 3, and 4 of $sws1_{Cl}$-*S* consist of 3 kb, 420 bp, 1.4 kb, and 1.5 kb, respectively, and the corresponding introns of $sws1_{Cl}$-*L* consist of 3.3, 0.5, 1.5, and 1.8 kb. Introns 1, 2, 3, and 4 of $sws2_{Cl}$-*S* consist of 1.6, 1.3, 3, and 2.4 kb, respectively, and those of $sws2_{Cl}$-*L* are given by 2, 1.3, 2.2, and 3 kb, respectively. Introns 1, 2, 3, 4, and 5 of lws_{Cl} consist of 0.8, 2.2, 0.7, 2, and 1.5 kb, respectively. Circled nucleotides indicate polymorphisms between $sws1_{Cl}$-*S* and $sws1_{Cl}$-*L* and between $sws2_{Cl}$-*S* and $sws2_{Cl}$-*L*. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; Sl, *Sal*I; Ss, *Sst*I. The sequences reported in this article have been deposited in the GenBank database (AF149230–AF149231 for $rh1_{Cl}$; AF149232–AF149233 for $rh2_{Cl}$; AF149234–AF149237 for $sws1_{Cl}$; AF149238–AF149242 for $sws2_{Cl}$; AF149243–AF149248 for lws_{Cl}).

From Figure 3, we can also identify functionally important residues that are conserved among these pigments. They include Lys296 for Schiff base linkage to the chromophore (Wang *et al.* 1980), Glu113 for Schiff base counterion (Sakmar *et al.* 1989; Zhukovsky and Oprian 1989; Nathans 1990), Cys110 and Cys187 for disulfide bond (Karnik *et al.* 1988), and multiple Ser and Thr in the C-terminal region for the targets of opsin kinase (Ohguro *et al.* 1994). $RH1_{Cl}$ and $RH2_{Cl}$ pigments have Asn2 and Asn15 for *N*-glycosylation (Okano *et al.* 1992) and Cys322 and Cys323 for palmitoylation sites (Ovchinnikov *et al.* 1988) as those in the orthologous pigments of other vertebrates. Furthermore, Ser164, His181, Tyr261, Thr269, and Ala292 of the LWS_{Cl} pigment exhibit the LWS-specific character that is important for a red-light detection (Yokoyama and Radlwimmer 1998, 1999). Note that sites 164, 181, 261, 269, and 292 correspond to 180, 197, 277, 285, and 308 in human red and green pigments.

$rh1_{Cl}$, represented by λCl89, spans 2.9 kb from the start to stop codons (Figure 2). When 1056 nucleotide sites of the entire coding region of $rh1_{Cl}$, including the stop codon, are compared to those of the orthologous chicken and American chameleon genes, the $p_{I,\,nuc}$ values are given by 0.92 and 0.78, respectively. The $p_{I,\,nuc}$ value for *rh1* genes between chicken and American chameleon is 0.78 and is identical to the corresponding value between pigeon and American chameleon. The $p_{I,\,aa}$ values for RH1 pigments between pigeon and chicken, between pigeon and American chameleon, and between chicken and American chameleon are given by 0.98, 0.85, and 0.86, respectively. These $p_{I,\,nuc}$ and $p_{I,\,aa}$ values between the orthologous molecules of the two bird species are higher than the corresponding values between the bird

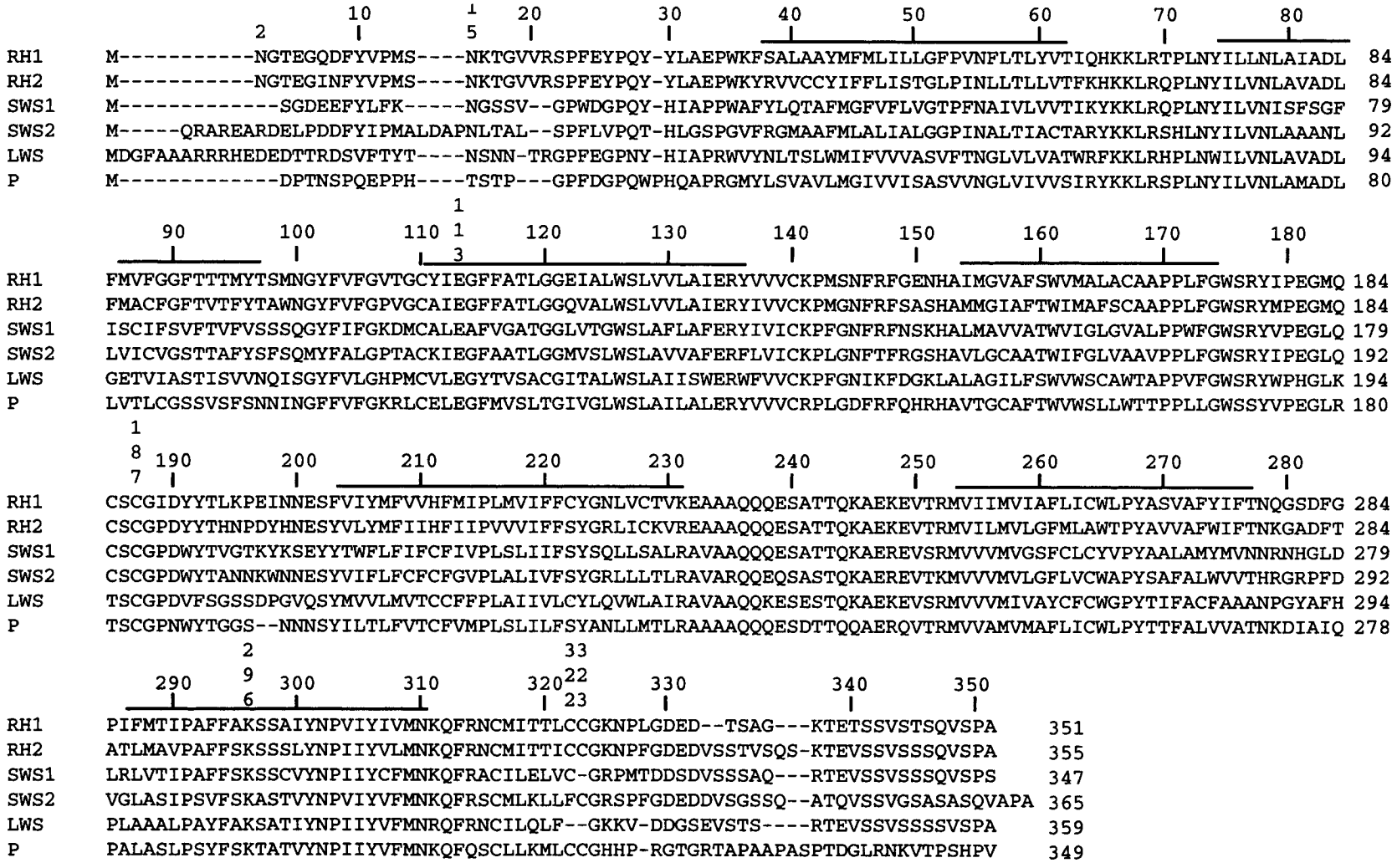

Figure 3.—Alignment of the deduced amino acid sequences of the retinal and nonretinal opsins of the pigeon. Gaps necessary to optimize the alignment are indicated by dashes. Seven transmembrane regions (Hargrave *et al.* 1983) are indicated by horizontal lines above the sequences. RH1, RH2, SWS1, SWS2, LWS, and P denote $RH1_{Cl}$, $RH2_{Cl}$, $SWS1_{Cl}$, $SWS2_{Cl}$, and LWS_{Cl} pigments, respectively. The residue number is according to that of $RH1_{Cl}$.

and reptile, reflecting the phylogenetic relationships of the three species.

The length of *rh2*$_{Cl}$, represented by λCl5, is ~3.5 kb from the start to stop codons (Figure 2). The $p_{I, nuc}$ values for *rh2* genes between pigeon and chicken, between pigeon and American chameleon, and between chicken and American chameleon are given by 0.91, 0.83, 0.84, respectively. The $p_{I, aa}$ values for RH2 pigments between pigeon and chicken, between pigeon and American chameleon, and between chicken and American chameleon are given by 0.98, 0.92, and 0.92, respectively. Again, the two comparisons reflect the phylogenetic relationships of the three species well.

sws1$_{Cl}$*-L*, ~8 kb in length, represented by λCl36, is ~0.8 kb longer than *sws1*$_{Cl}$*-S*, represented by λCl37 (Figure 2). When 1044 nucleotide sites of the entire coding region of these two genes are compared, there is only one silent nucleotide difference, C in *sws1*$_{Cl}$*-S* and T in *sws1*$_{Cl}$*-L*, at nucleotide position 519 in exon 3 (Figure 2). When 898 bp in the noncoding regions of the two opsin genes are compared, there are only 5 different nucleotides (0.56% difference). This small difference and the Southern analysis (see discussion) show that *sws1*$_{Cl}$*-S* and *sws1*$_{Cl}$*-L* are different alleles rather than two genes at different loci. The $p_{I, nuc}$ for *sws1* genes between pigeon and chicken, between pigeon and American chameleon, and between chicken and American chameleon are given by 0.84, 0.87, and 0.83, respectively, while the corresponding $p_{I, aa}$ values are given by 0.87, 0.87, and 0.84. These $p_{I, nuc}$ and $p_{I, aa}$ values are about the same for all pairwise combinations of the pigeon, chicken, and American chameleon pigments and, surprisingly, do not reflect the phylogenetic relationships of the three species. As we see later, this seems to be caused mainly by a slow evolution of *sws1* gene in the common ancestor of the two bird species.

λCl25 and λCl34 represent two types of *sws2*$_{Cl}$ genes, *sws2*$_{Cl}$*-S* and *sws2*$_{Cl}$*-L*, respectively. *sws2*$_{Cl}$*-S* and *sws2*$_{Cl}$*-L* span 9.4 and 9.6 kb from the start to stop codons, respectively (Figure 2). Along the 1098 bp sites of the coding regions, the two genes differ at one nucleotide site at nucleotide position 499 in exon 2, causing one amino acid difference (Ala167 and Thr167 for SWS2$_{Cl}$-S and SWS2$_{Cl}$-L pigments, respectively; Figure 2). When 688 bp sites in the noncoding regions of the two genes are compared, there are 21 different nucleotides (3.1% difference). Interestingly, both *sws2*$_{Cl}$*-S* and *sws2*$_{Cl}$*-L* are physically linked to *lws*$_{Cl}$ (Figure 4). The distance between the stop codon for *sws2*$_{Cl}$*-L* and the initiation codon of *lws*$_{Cl}$ is ~7 kb, while that between *sws2*$_{Cl}$-S and *lws*$_{Cl}$ is ~5 kb (Figure 4). Thus, like *sws1*$_{Cl}$*-S* and *sws1*$_{Cl}$*-L*, it is most likely that *sws2*$_{Cl}$*-S* and *sws2*$_{Cl}$*-L* also represent two alleles rather than two distinct genes. The $p_{I, nuc}$ values of *sws2* genes between pigeon and chicken, between pigeon and American chameleon, and between chicken and American chameleon are given by 0.84, 0.74, and 0.74, respectively, while the corresponding $p_{I, aa}$ values are 0.87, 0.84, and 0.82. Thus, the phylogenetic relationships of the three species are reflected in the $p_{I, nuc}$ and $p_{I, aa}$ values.

lws$_{Cl}$, represented by λCl102, has six exons and five introns spanning 8.3 kb from the start to stop codons

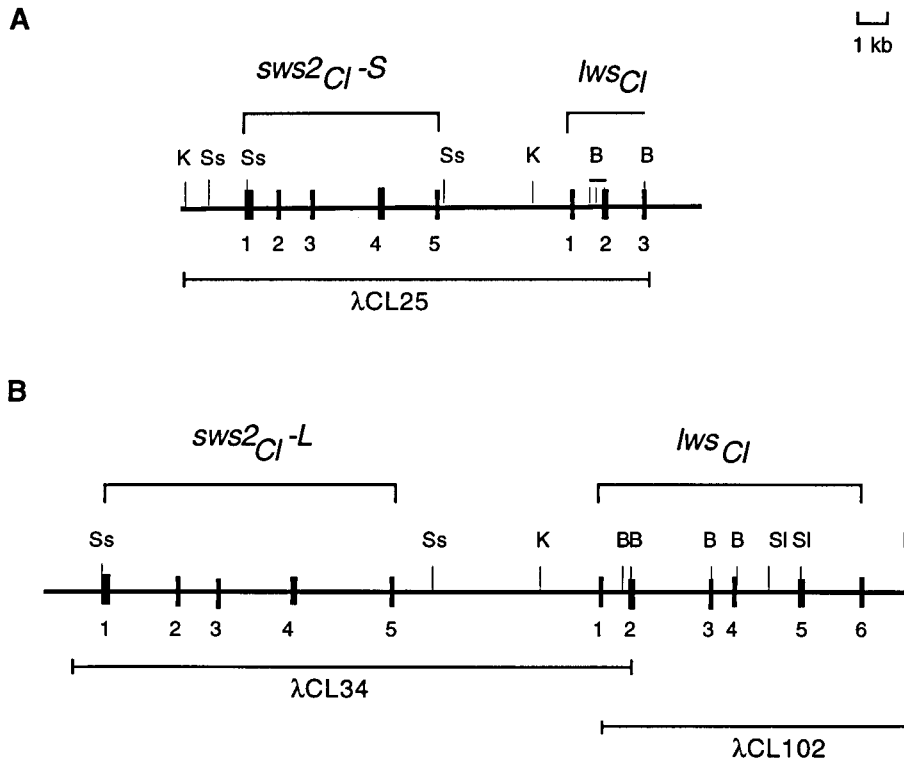

Figure 4.—Genomic structure of the *sws2*$_{Cl}$ and *lws*$_{Cl}$. The coding exons are indicated by solid boxes. B, *Bam*HI; K, *Kpn*I; Sl, *Sal*I; Ss, *Sst*I.

(Figure 2). The $p_{I,\,nuc}$ values for *lws* genes between pigeon and chicken, between pigeon and American chameleon, and between chicken and American chameleon are given by 0.91, 0.78, and 0.76, respectively, while the corresponding $p_{I,\,aa}$ values for LWS opsins are given by 0.96, 0.90, and 0.91. Like RH1, RH2, and SWS2 pigments, these values also reflect the phylogenetic relationships of the three species reasonably well.

Evolution of the pigeon pigments: All RH1, RH2, SWS1, SWS2, LWS, and P pigments have been characterized in chicken (Okano *et al.* 1992, 1994) and in American chameleon (see materials and methods). The phylogenetic relationships of the five different types of retinal pigments and P pigments from pigeon, chicken, and American chameleon are given by (((((RH1, RH2) SWS2) SWS1) P) LWS) (Figure 5). However, since the bootstrap support for the cluster of RH1, RH2, and SWS2 pigments is only 72%, the phylogenetic position of SWS2 pigments is not as clear-cut as the tree topology in Figure 5 may indicate. Similarly, the bootstrap value for the cluster of RH1, RH2, SWS2, SWS1, and P pigments is 80% and the phylogenetic position of P pigments in Figure 5 is also not reliable. Thus, the six groups of pigments may be distinguished roughly into three groups: (i) RH1, RH2, SWS1, and SWS2 clusters; (ii) P cluster; and (iii) LWS/MWS cluster.

With the exception of SWS1 pigments, the bootstrap supports for the sets of the paired orthologous bird pigments are 100% and are highly reliable. Figure 5 shows that the uncertainty of the pigeon and chicken pigment cluster is due to a very slow rate of amino acid replacement in the common bird ancestor (see also discussion).

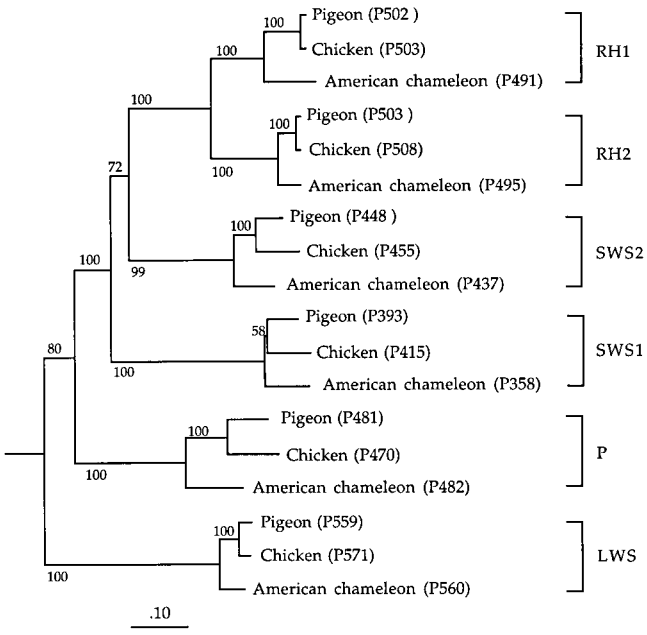

Figure 5.—The phylogenetic tree for retinal (RH1, RH2, SWS1, SWS2, and LWS) and pineal gland-specific (P) pigments of pigeon, chicken, and American chameleon. The bootstrap supports are indicated next to branch nodes. Values after P indicate λmax values.

Light absorption profiles: For spectral analyses, with the exception of p_{Cl}, we used cDNAs that encode identical amino acids to those of the corresponding pigments deduced from genomic clones. At codon position 77, P_{Cl} opsin cDNA clone contains one nonsynonymous nucleotide difference from its genomic DNA sequence that encodes Val instead of Met (Figure 3). Since the P pigments of American chameleon and chicken both have Val at the corresponding sites, the difference between the cDNA sequence from one pigeon and the corresponding genomic DNA sequence obtained from another is interpreted as a naturally occurring DNA polymorphism rather than a cloning artifact.

The λmax values of pigments can be measured directly from the dark spectra (Figure 6) and from the dark-light difference (Figure 6, insets). The former measurements for $RH1_{Cl}$, $RH2_{Cl}$, $SWS2_{Cl}$-S, LWS_{Cl}, and P_{Cl} pigments are given by 502 ± 3, 503 ± 2, 448 ± 2, 559 ± 2, and 481 ± 2 nm, respectively. The respective λmax values estimated from the dark-light difference are given by 505, 506, 459, 559, and 486 nm. With the exception of $SWS2_{Cl}$-S, the corresponding two values for each pigment are close. Using the same method, we previously obtained the λmax value of $SWS1_{Cl}$ pigment, 393 ± 2 nm (Yokoyama *et al.* 1998). Out of these five types of retinal pigments, the λmax values of $RH1_{Cl}$, $RH2_{Cl}$, $SWS2_{Cl}$, and LWS_{Cl} pigments generally agree with the corresponding values estimated either by MSP or by ERG (Table 1). The λmax value of 393 nm for $SWS1_{Cl}$-S pigment is close to the MSP estimate of 409 nm (Bowmaker *et al.* 1997) and the λmax values of about 400 nm observed by Remy and Emmerton (1989), Graf and van Norren (1974), van Norren (1975), and Wortel *et al.* (1984). However, it differs considerably from the corresponding λmax values of 370 nm estimated by ERG (Table 1). The cause of this discrepancy remains to be elucidated (see also discussion).

Expression of retinal and P opsins: Using opsin gene-specific primers, we have examined the expressions of $rh1_{Cl}$, $rh2_{Cl}$, $sws1_{Cl}$, $sws2_{Cl}$, lws_{Cl}, and p_{Cl} in the retina and pineal gland of the pigeon by RT-PCR assay. In the retina, all five visual opsin genes are expressed, but p_{Cl} is not, whereas in the pineal gland, only p_{Cl} is expressed (Figure 7). Similar analyses in the chicken show that the five visual opsins and P opsin are expressed in the retina and in the pineal gland, respectively (Okano *et al.* 1994; Max *et al.* 1995). One study reports a low level of the *lws* gene expression in the pineal gland (Okano *et al.* 1994, 1997). However, this cannot be confirmed in another study (Max *et al.* 1995). Thus, the expression of the *lws* opsin in the pineal gland of the chicken is controversial. To settle the issue in the chicken, additional analyses of the *lws* gene expression are needed.

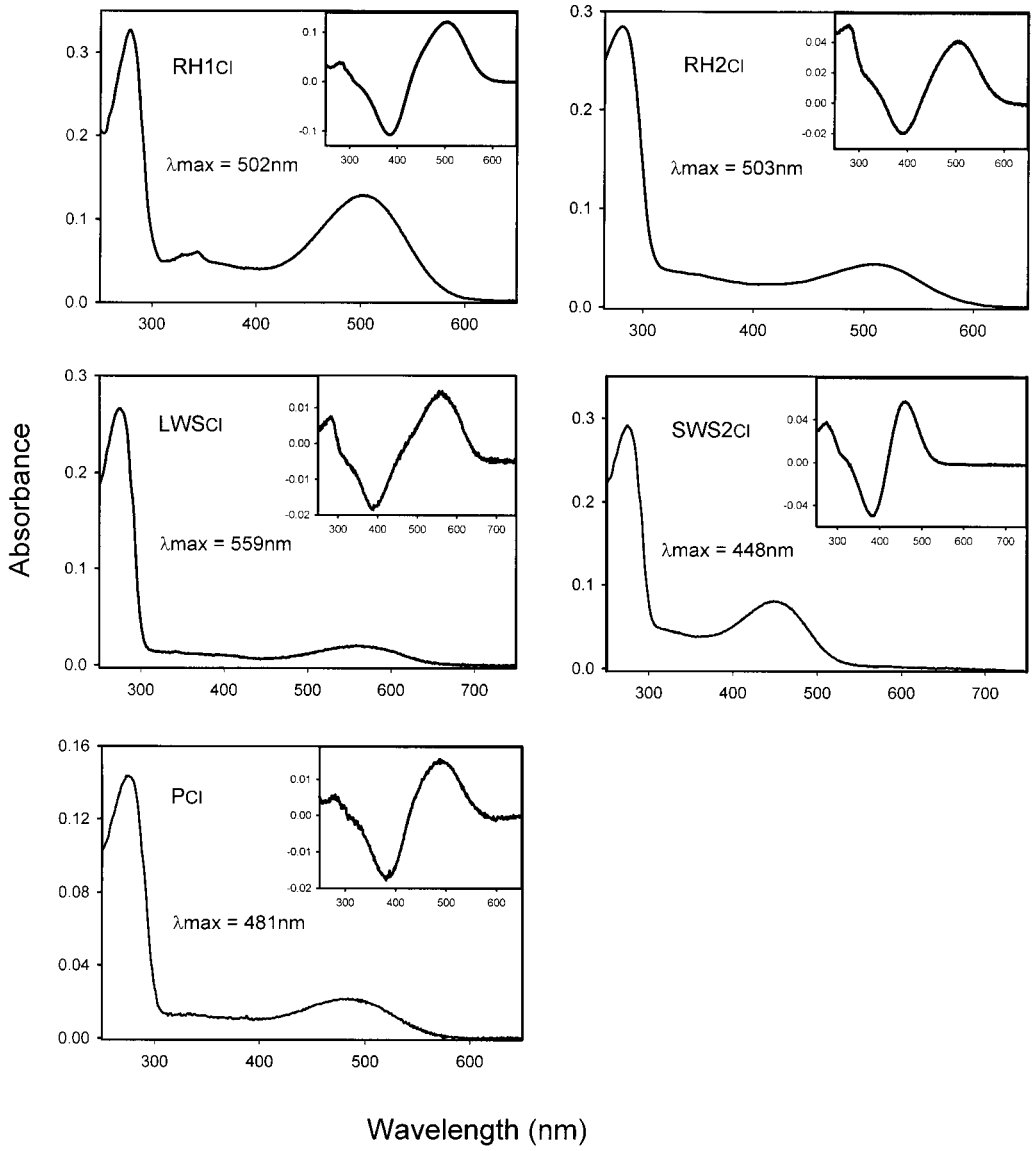

Figure 6.—Absorption spectra of regenerated pigeon pigments measured in the dark and the dark-light difference spectra (insets).

DISCUSSION

Opsin genes and visual pigments of the pigeon: During the molecular characterization of the pigeon opsin genes, we found physical linkage between *sws2*$_{Cl}$ and *lws*$_{Cl}$. Previously, we reported a similar linkage relationship between *sws2* and *lws* genes in Mexican cavefish, *Astyanax fasciatus* (Yokoyama and Yokoyama 1993). The same linkage relationships in fish and bird species suggest that the structure of 5′-*sws2*-*lws*-3′ was established in the vertebrate ancestor, some 450 mya. So far, neither *sws2* gene nor *rh2* gene has been isolated from any mammals and they appear to have been nonfunctionalized in the early stage of mammalian evolution. Incidentally, in human, *sws1* gene is located on chromosome 7 (Nathans *et al.* 1986b), whereas *lws* and relatively recently duplicated *mws* gene(s) are located tandemly on the X chromosome (Nathans *et al.* 1986a, b).

We have seen that the genetic distances of SWS1$_{Cl}$ pigments of the pigeon, chicken, and American chameleon do not necessarily reflect the phylogenetic relationships of the three species. To evaluate the evolutionary patterns of amino acid replacements in the pigments in more detail, we estimated the numbers of amino acid replacements per site (K) for the pigments of pigeon and chicken separately (Table 2). The results show that the K values for the orthologous retinal pigments of the two bird species are similar to each other. However, the branch lengths between the two P pigments are significantly different. In the pigeon, SWS1 pigment has the highest K value, followed by P, SWS2, LWS, RH1, and RH2 pigments, in that order. In the chicken, the K values for P, SWS1, and SWS2 pigments are about one order of magnitude higher than those for RH1 and RH2 pigments. These trends can also be seen in the

TABLE 1

λmax values (nm) of the pigeon visual pigments

Pigment	Opsin + 11-*cis*-retinal	MSP[a]	ERG
$RH1_{Cl}$	502 ± 3	506 ± 1	ND
$RH2_{Cl}$	503 ± 2	507 ± 2	507[b]
$SWS1_{Cl}$	393 ± 2[c]	409 ± 7	370[d]
$SWS2_{Cl}$	448 ± 1	453 ± 5	467[b]
LWS_{Cl}	558 ± 2	567 ± 3	562[b]
P_{Cl}	481 ± 2	ND	ND

ND, not determined.
[a] Bowmaker *et al.* (1997).
[b] Govardovskii and Zueva (1977).
[c] Yokoyama *et al.* (1998).
[d] Chen *et al.* (1984); Chen and Goldsmith (1986).

TABLE 2

Numbers of amino acid replacements per site after the divergence between pigeon and chicken pigments (× 100)[a]

Pigment	Pigeon	Chicken
RH1	1.4 ± 0.67[b]	0.7 ± 0.48
RH2	0.6 ± 0.43	0.9 ± 0.54
SWS1	6.0 ± 1.38	7.9 ± 1.59
SWS2	5.1 ± 1.28	8.7 ± 1.68
LWS	2.4 ± 0.86	1.7 ± 0.73
P	5.7 ± 1.35*	10.7 ± 1.86*

[a] In computing the numbers of the pigeon and chicken pigment-specific amino acid replacements, the orthologous American chameleon pigments were used as the outgroup.
[b] Standard errors were computed from $[p/\{n(1-p)\}]^{1/2}$, where p is the proportion of different amino acids per site, and n (= 324) is the number of amino acid sites compared.
* Difference in branch lengths is significantly different at the 5% level.

phylogenetic tree in Figure 5. A close inspection of Figure 5 reveals that the common ancestor of the bird species experienced an unusually small number of amino acid replacements in its SWS1 pigments, followed by equally accelerated amino acid replacements in the pigeon and chicken pigments.

The "near-UV" pigment of the pigeon: We have determined the λmax values of $RH1_{Cl}$, $RH2_{Cl}$, $SWS2_{Cl}$, LWS_{Cl}, and P_{Cl} pigments. This functional assay has shown no evidence for the existence of a true UV opsin gene in the pigeon genome. The only pigment that absorbs light at around the UV to violet range is a near-UV $SWS1_{Cl}$ pigment with a λmax at 393 nm (Yokoyama *et al.* 1998). Using MSP, Bowmaker *et al.* (1997) also identified only the "violet" pigment that absorbs light at 409 nm. Thus, although there is a small difference in the two λmax values, the near-UV and violet pigments appear to be the same pigment. As already noted, pigment regeneration is the most direct method for evaluating absorption spectrum of the visual pigment. Pigments were synthesized abundantly *in vitro* and highly purified to eliminate background absorbance of other cellular materials. Consequently, the method provides very high signal/noise ratio and small standard errors. Thus, we may conclude that the near-UV or violet pigment has a λmax at 393 nm. It should be noted that, being expressed in the T-type cones (see Introduction), the λmax values of $SWS1_{Cl}$ pigments are not modified further by the oil droplet.

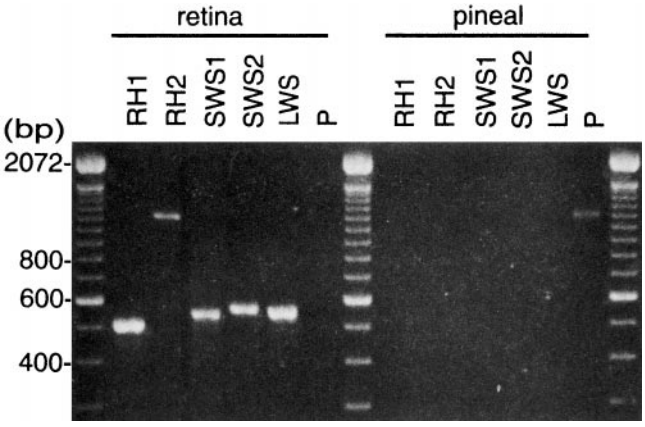

Figure 7.—RT-PCR assay for the visual and P opsin gene expression in the retina and pineal gland of the pigeon. The 100-bp DNA ladder marker is shown at the left margin.

Bowmaker *et al.* (1997) suggested that the true UV opsin gene in the pigeon might have arisen from $sws1_{Cl}$ by gene duplication. This is reasonable because all known UV and violet opsin genes in vertebrates belong to the SWS1 cluster (Yokoyama 1997). To test the possible existence of such duplicated *sws1* genes in the pigeon genome, we have isolated genomic DNA from the second pigeon (see materials and methods) and conducted Southern hybridization analysis. When the exons 3 and 4 of $sws1_{Cl}$-*S* are used as the probe, the double-digested DNAs with *Bam*HI/*Sst*I (Figure 8, lane A) and *Bam*HI/*Kpn*I (lane B) show single hybridizing bands of 7.4 and 8.6 kb in size, respectively. They correspond to $sws1_{Cl}$-*S* (Figure 2), indicating that this particular pigeon is homozygous for the $sws1_{Cl}$-*S* allele. When exons 1–3 of the same gene were used as the probe, the identical hybridizing bands were detected (results not shown). These results strongly suggest that there is not an additional $sws1_{Cl}$ gene in the pigeon genome and that $SWS1_{Cl}$ pigment is the sole pigment that detects light below 400 nm. The existence of a $sws1_{Cl}$-*S*/$sws1_{Cl}$-*S* homozygous individual also demonstrates that $sws1_{Cl}$-*S* and $sws1_{Cl}$-*L* are two alleles rather than two genes at separate loci. We have also tested the possible existence of duplicated loci of $rh1_{Cl}$, $rh2_{Cl}$, $sws1_{Cl}$, $sws2_{Cl}$, and lws_{Cl} in the pigeon genome, but hybridizing bands are consistent with the restriction maps of the genomic clones in Figure 2 (results not shown). It should also be noted that no extra genes were included in the 62 clones isolated (materials and methods). All these results strongly suggest that the pigeon contains only five reti-

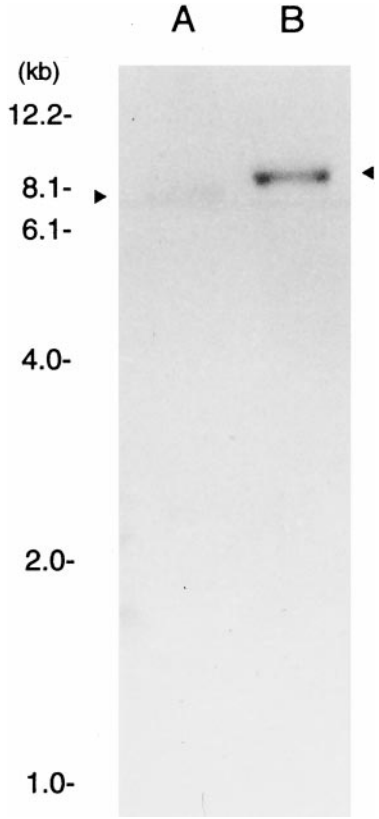

Figure 8.—Southern hybridization of *Bam*HI/*Sst*I- (lane A) and *Bam*HI/*Kpn*I- (B) digested genomic DNA of the pigeon with exons 3 and 4 of $sws1_{Cl}$-*S*. The 1-kb DNA ladder marker is shown at the left margin. The hybridizing bands are indicated by the arrowheads.

nal opsin genes $rh1_{Cl}$, $rh2_{Cl}$, $sws1_{Cl}$, $sws2_{Cl}$, and lws_{Cl} in its genome. Thus, pigeon, chicken, and American chameleon seem to have five different types of retinal visual pigments (Figure 5).

UV vision of the pigeon: As already noted, both behavioral experiments and ERG analyses demonstrated the pigeon's ability to detect UV (see Introduction). However, there exist some conflicting data on the absorption spectra of the pigeon in the range of UV–violet. For example, from behavioral experiments, Remy and Emmerton (1989) and Romeskii and Yager (1976a,b) detected only one absorption maximum at 400–415 nm. Some ERG studies also show single λmax values at 400–413 nm (Graf and van Norren 1974; van Norren 1975; Govardovskii and Zueva 1977; Wortel *et al.* 1984). On the other hand, using behavioral experiments, Emmerton and Remy (1983) detected double peaks of spectral sensitivities at 360 and 400–420 nm. Using ERG, Vos Hzn *et al.* (1994) also proposed that pigeon uses two pigments with λmax at 366 and 415 nm (Table 1).

These conflicting results may mean that pigeon's UV sensitivity varies from one individual to another. If this turns out to be the case, then the structural difference of the pigeon's retinas in different individuals may become an important factor. The dorsal red field of the pigeon retina contains mostly single cones and encompasses much of its binocular field of view, while the remaining ventral yellow field contains a higher proportion of rods and double cones (Jacobs 1981). The λcut values of the oil droplets in the yellow field are often lower than the corresponding droplets in the red field (Bowmaker 1977; Martin and Muntz 1978; Wortel *et al.* 1984; Bowmaker *et al.* 1997). The sensitivities of the red and yellow fields of the pigeon's retina in different individuals differ for both "visible" and UV light (Remy and Emmerton 1989). Due to this variation, different numbers of UV sensitive receptors in the two fields may result in different λmax values among different individuals (Remy and Emmerton 1989).

Where do these UV-sensitive pigments come from? Humans are normally blind to UV light because it is strongly absorbed by the yellow-pigmented lens (Said and Weale 1959). However, if the lens is surgically removed, then we can detect UV light (Wald 1945). The UV vision in humans in this unusual circumstance must be mediated by SWS1 pigments that absorb wavelengths of 370–530 nm with λmax values at 420 nm (Boynton 1979). The pigeon's cornea, lens, and vitrous body transmit both visible and UV light that can reach and excite the retina (Emmerton *et al.* 1980). Thus, it is most likely that the pigeon can detect UV light using $SWS1_{Cl}$ pigments whose λmax values are much lower than those of the orthologous pigments in humans.

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