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Articles

A Visual Pigment from Chicken That Resembles Rhodopsin: Amino Acid Sequence, Gene Structure, and Functional Expression†,‡

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ABSTRACT: The amino acid sequence of a rhodopsin-like visual pigment from chickens has been determined by isolating and sequencing its gene. The predicted sequence is between 70% and 80% identical to bovine, human, and chicken rhodopsins and between 40% and 50% identical to human blue, green, and red cone pigments, the chicken red cone pigment, and cavefish long-wave cone pigments. The encoded pigment, produced by transfection of cDNA into cultured cells, absorbs maximally at 495 nm as determined from photobleaching difference spectra and reacts at 20 °C with 50 mM hydroxylamine with a half-time of 16 min. These properties, together with a high p*I* predicted from the amino acid sequence, suggest that this cloned gene encodes the chicken green pigment previously identified by biochemical and spectroscopic studies. This sequence defines a new branch of the visual pigment gene family.

Visual pigments are retinal-containing proteins that mediate photoreception. In vertebrates, different members of the visual pigment family reside in the rod and cone photoreceptors, where they mediate vision under conditions of dim and bright illumination, respectively. It is generally accepted that most vertebrates have only a single class of rod photoreceptors and, within them, a single form of visual pigment, rhodopsin. By contrast, multiple cone types exist in most vertebrates, and these differ from one another with respect to the absorbance properties of their pigments. Hue discrimination (color vision) is achieved by a comparison of the relative extents of excitation of the different cone pigments.

Cones and rods also differ in several other important respects. For example, they contain different isoforms of phototransduction proteins, such as transducin and phosphodiesterase subunits (Gillespie & Beavo, 1988; Lerea et al., 1989; Charbonneau et al., 1990; Li et al., 1990). They also differ morphologically: in rods the outer segments form uninterrupted cylinders containing stacks of isolated photopigment-rich disks, whereas in cones the outer segments are tapered and the disks remain connected to the plasma membrane (Rodieck, 1973). The 100-fold lower current response per captured photon exhibited by cones, compared to rods, is likely to derive from these differences.

The high degree of sequence homology between rod and cone visual pigments and phototransduction proteins suggests a model in which new photoreceptor classes evolve by sequentially recruiting duplicated genes encoding photoreceptor proteins. A comparison of visual pigment sequences indicates that the earliest duplication in the vertebrate lineage occurred before the divergence of birds, fish, and mammals. At the amino acid level, for example, chicken and human rhodopsins are 87% identical, and the long-wave-sensitive pigments of chickens, cavefish (*Astyanax fasciatus*), and humans are greater than 73% identical. In contrast, none of the long-wavelength pigments so far sequenced are greater than 48% identical to rhodopsin (Nathans & Hogness, 1984; Nathans et al., 1986; Takao et al., 1988; Kuwata et al., 1990; Tokunaga

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et al., 1990; Yokoyama, 1990).

The chicken retina is an attractive system in which to study photoreceptor differentiation, function, and evolution. In the chicken retina, cones are approximately 10 times more abundant than in typical mammalian retinas, and the differentiation of chicken cones from precursor neuroblasts has been achieved in dissociated cell culture (Adler et al., 1984). Five chicken visual pigments have been identified, and these have been extracted, partially purified, and characterized spectroscopically (Wald et al., 1955; Fager & Fager, 1981; Yen & Fager, 1984; Okano et al., 1989). Recently, the amino acid sequences of chicken rhodopsin (Takao et al., 1988) and chicken iodopsin (the red cone pigment) (Kuwata et al., 1990; Tokunaga et al., 1990) have been determined by sequencing of cloned DNA.

We began the present study with the goal of isolating one or more chicken cone pigment genes. Using a PCR-based approach, we have identified genomic and cDNA clones encoding a visual pigment that closely resembles, but is distinct from, chicken rhodopsin. This pigment is likely to correspond to the previously characterized chicken green pigment on the basis of its absorbance maximum at 495 nm, its sensitivity to hydroxylamine, and its predicted basic isoelectric point.

Materials and Methods

PCR Amplification. A pair of PCR primers, 5′GGGGGAATTCCCAGAAGGCAGAGAAGGAAGT and 5′GGGGAAGCTTCTGNCGGTTCATPuAAGACATA, corresponding to codons 259–266 and the reverse complement of codons 322–328 of the human red pigment gene and carrying *Eco*RI and *Hin*dIII sites near their 5′ termini, respectively, were derived from a comparison of human and cavefish long-wavelength pigment gene sequences (Nathans et al., 1986; Yokoyama & Yokoyama, 1990) (Pu, purine; N, all four DNA bases). These primers were used to direct PCR amplification from chicken genomic DNA. After 25 cycles at 45 °C for 30 s and 94 °C for 30 s, the PCR products were digested with *Eco*RI and *Hin*dIII, gel purified, and ligated into pUC119. For PCR amplification of the coding region from first-strand cDNA, total RNA was prepared from newly hatched chick retinas (Chomczynski & Sacchi, 1987), first-strand cDNA was synthesized, and PCR amplification was performed as described (Kawasaki et al., 1988; Rappoleee et al., 1988) using primers corresponding to the first six codons and the reverse complement of the last seven codons of the pRA1 coding region. The PCR product was cloned into the mammalian expression vector pCIS (Gorman et al., 1990) and its sequence verified.

Recombinant DNA Analysis and Sequence Comparisons. A chicken genomic library was constructed from a 15–20-kb *Sau*3A partial digest of chicken genomic DNA in λ EMBL3, and clones were identified by plaque hybridization at high stringency (Frischauf et al., 1983). The DNA sequence was determined on one strand by the dideoxy method using as templates a nested deletion series constructed with DNase I in the presence of manganese (Nathans & Hogness, 1984). The complementary strand of each putative exon was sequenced by priming with oligonucleotide primers synthesized on the basis of the first-strand sequence. The percent amino acid identity between a pair of sequences was calculated by summing the number of amino acid differences and the number of gaps in the aligned sequences, subtracting that sum from the length of the longest sequence, and dividing by the length of the longest sequence.

Production of Visual Pigment in Tissue Culture and Reconstitution with 11-cis-Retinal. A cDNA clone containing the coding region of pRA1 was inserted into the expression vector pCIS (Gorman et al., 1990). Transfection, membrane isolation, and incubation with 11-*cis*-retinal were performed essentially as described in Nathans (1990). In a typical experiment, 20 10-cm-diameter plates of 293S cells (a suspension-adapted variant of a human embryonic kidney cell line; American Type Culture Collection CRL 1573) were cotransfected with 100 μg of expression plasmid together with 10 μg of pRSV TAg (an SV40 T-antigen expression plasmid) by the calcium phosphate method (Gorman, 1985). Forty eight hours after transfection, the cells were collected in cold phosphate-buffered saline and homogenized in a Polytron homogenizer (Brinkmann) in 10 mL of 100 mM sodium phosphate, pH 6.5, 1 mM EDTA, 100 mM sodium chloride, 250 mM sucrose, and 0.2 mM phenylmethanesulfonyl fluoride. The homogenate was layered onto a 1.5 M sucrose cushion in the same buffer and centrifuged in a swinging bucket rotor (SW28) at 105000*g* for 30 min. Membranes were collected from the interface, adjusted to 1 mM DTT, and incubated in the dark with 50 nmol of 11-*cis*-retinal at 23 °C for 2 h. To remove excess 11-*cis*-retinal, the sample, typically 1.5–2 mL, was diluted with 20 volumes of 4% BSA in 100 mM sodium phosphate, pH 6.5, 100 mM sodium chloride, and 1 mM EDTA and centrifuged in a swinging bucket rotor (SW28) at 105000*g* for 30 min. The membrane pellet was resuspended in the dark in 0.4 mL of 100 mM sodium phosphate, 100 mM sodium chloride, 1 mM EDTA, and 5% digitonin (Kodak).

Absorbance Spectra and Hydroxylamine Reactivity. Before the absorbance spectrum was measured, samples were centrifuged at 11000 rpm in an Eppendorf microfuge for 5 min. Absorbance spectra were obtained on the supernatant before and after 20 s of photobleaching from a fiber optic light source filtered through a 530–nm short-wave cutoff filter. Spectra were recorded on a Kontron instruments Uvikon 860 spectrophotometer with the sample maintained at 20 °C in a water-jacketed cuvette holder. For the hydroxylamine bleaching experiment, 0.8 M hydroxylamine, pH 6.0, was added to a final concentration of 50 mM, and spectra were recorded at the time intervals indicated in Figure 6. A control experiment showed that less than 0.5% of the pigment was bleached by the recording beam during each complete spectral determination.

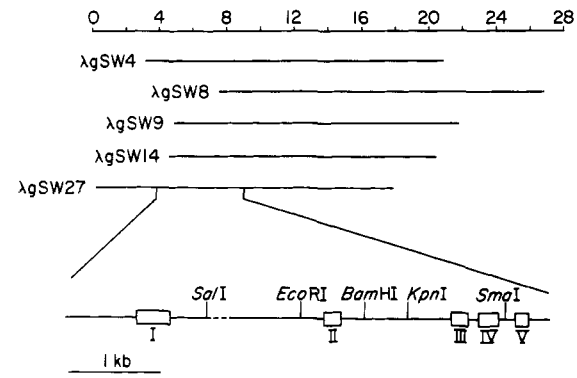

FIGURE 1: Structure of the pRA1 gene. The chromosomal DNA restriction map is shown above the set of overlapping λ clones that define it. The scale in kilobases is shown across the top. At the bottom (enlarged scale) exons are shown as open boxes. Solid lines indicate regions sequenced; the dashed line indicates a region which has not been sequenced.

Results

Isolation of a Novel Visual Pigment Gene from Chicken Genomic DNA. On the basis of a comparison of conserved nucleotide sequences in the human (Nathans et al., 1986) and

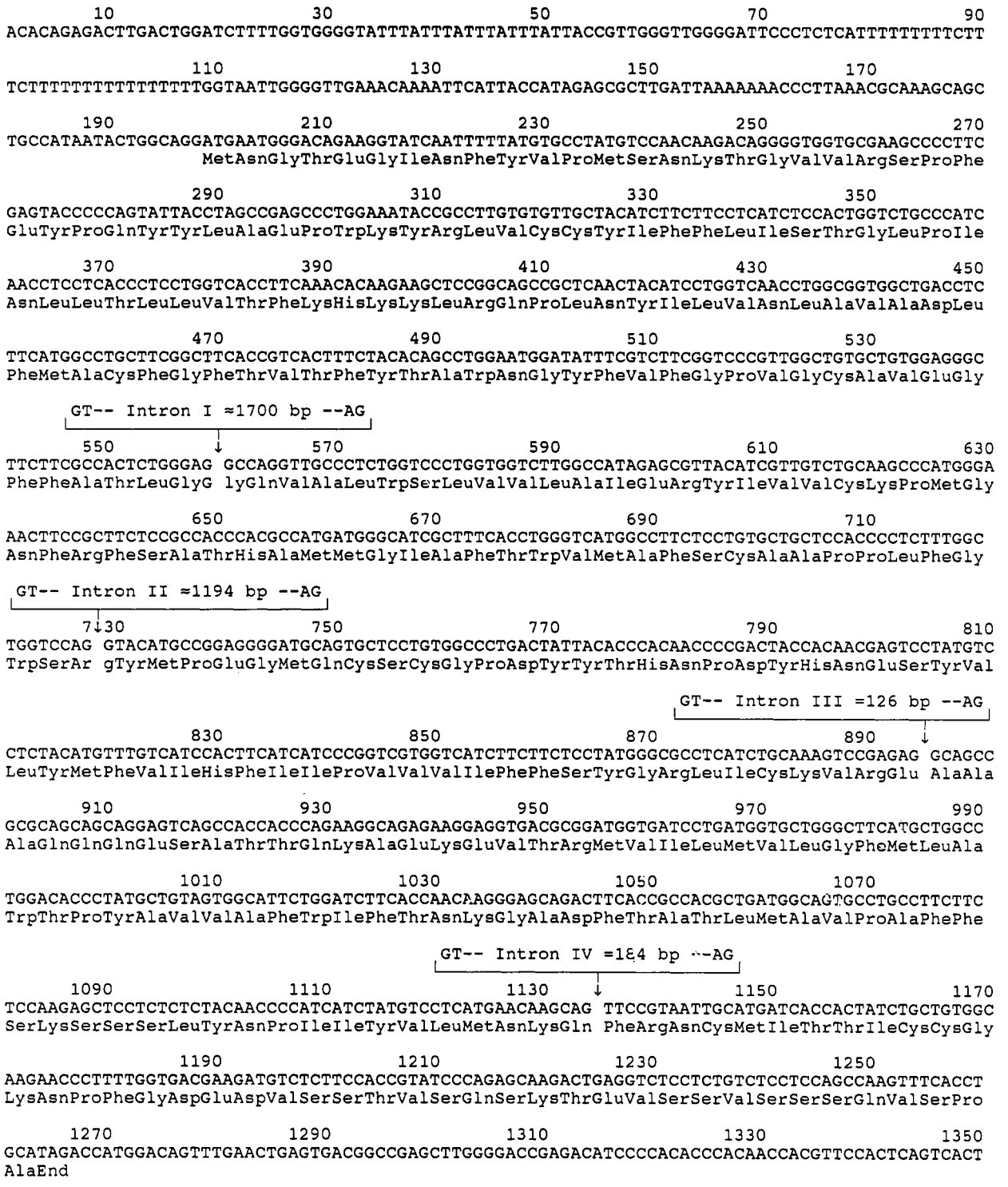

FIGURE 2: Nucleotide and deduced amino acid sequence of the pRA1 gene. The locations of the four introns are indicated above the exon sequence. The sequence presented was determined from genomic DNA; the nucleotide sequence derived from PCR amplification of retina cDNA shows three silent nucleotide substitutions: C^{267} to T, G^{738} to A, and C^{786} to T.

cavefish (Yokoyama & Yokoyama, 1990) long-wavelength cone pigment genes, two PCR primers were designed to amplify a region corresponding to codons 259–328 of the human long-wavelength visual pigment gene. Following low stringency amplification of chicken genomic DNA with this primer pair, agarose gel electrophoresis revealed a variety of product sizes. PCR products with the same electrophoretic mobility as a control PCR product generated from a human red pigment cDNA were cloned and sequenced. One clone, pRA1, showed strong homology to vertebrate rhodopsins but was clearly distinct from chicken rhodopsin.

To determine the complete sequence of the visual pigment encoded by pRA1, we isolated the corresponding gene from a chicken genomic DNA library (Figure 1). Exons were identified by sequencing of one DNA strand followed by comparison of the predicted translation products with known visual pigment sequences. As shown in Figures 2 and 3, the predicted translation products of five separate regions—presumed to be exons of the pRA1 gene—show strong homology to known visual pigment sequences. The assignment of an initiator methionine codon for the pRA1 gene is based upon a comparison with other visual pigments: the assignment

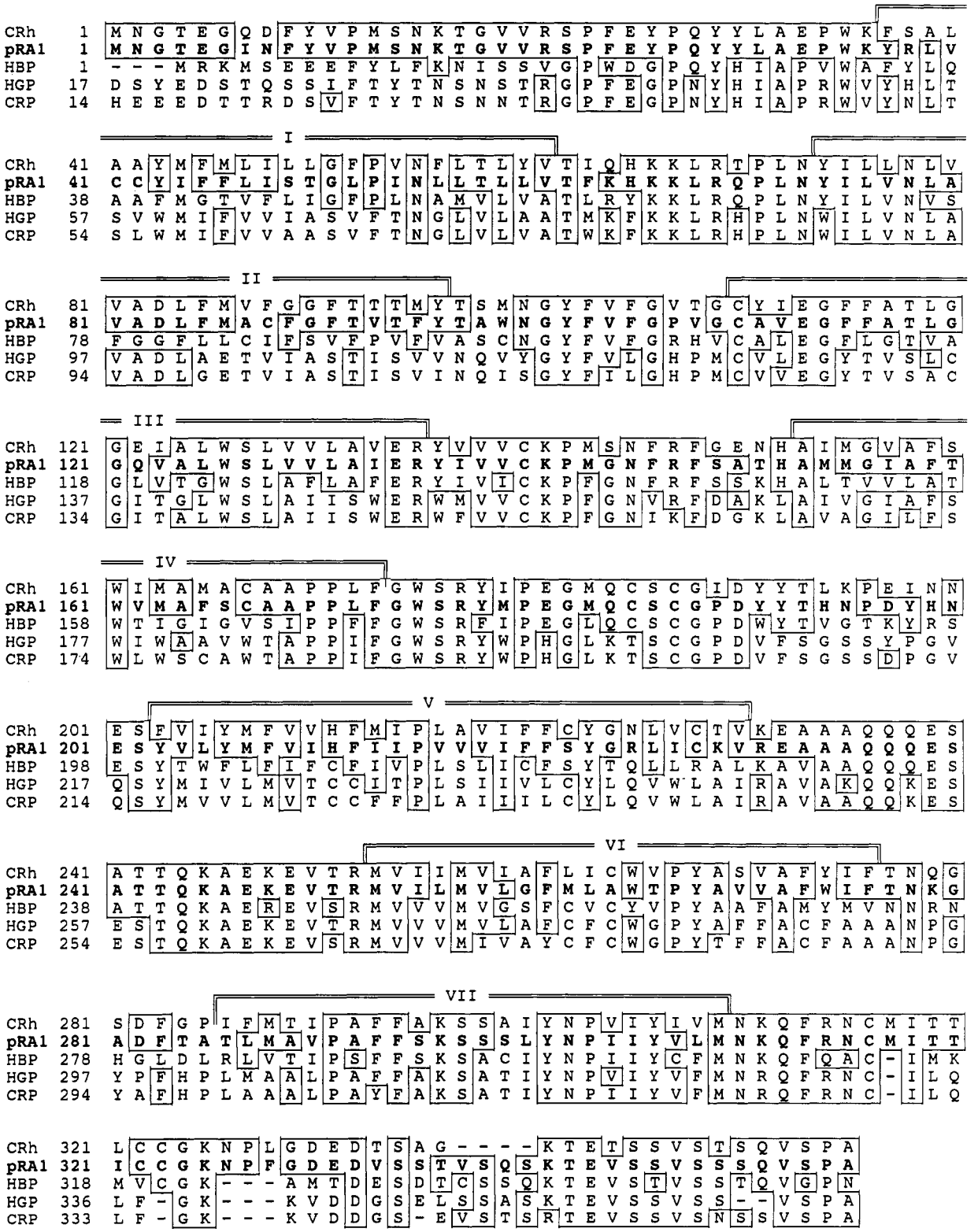

FIGURE 3: Alignment of amino acid sequences between chicken rhodopsin (CRh), the pRA1 pigment (pRA1), human blue (HBP), human green (HGP), and chicken red/iodopsin (CRP). Amino acids shared with pRA1 are boxed. The seven putative transmembrane regions are indicated by roman numerals. Dashes indicate gaps in the sequence introduced to maximize homology, as determined by inspection.

shown in Figure 2 produces a perfect match for the first seven amino acids with bovine, human, and chicken rhodopsins. The internal boundaries of the putative exons correspond precisely to the intron–exon boundaries of vertebrate rod and cone pigments, and the putative intron sequences abutting them conform to consensus splice junction sequences. To test this assignment of intron–exon structure and to determine whether this gene is expressed in the chicken retina, primers corresponding to the first six codons and the reverse complement of the last seven codons were used to direct PCR amplification from a template of first-strand cDNA prepared from newly hatched chicken retina RNA. A 1.1-kb PCR product was obtained. This product was cloned, and the DNA sequence of one representative clone showed the predicted exons precisely joined together. We conclude that the pRA1 gene has an intron–exon structure resembling that of other vertebrate visual pigment genes and that it encodes a protein of 355 amino acids.

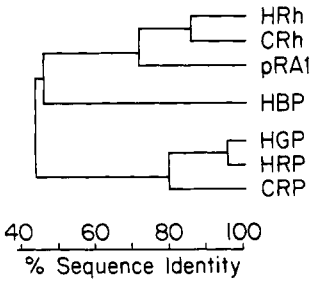

FIGURE 4: Dendrogram showing percent amino acid identity between the pRA1 pigment and other vertebrate visual pigments. The nodal point between each pair of branches indicates the percent amino acid identity. Abbreviations: human rhodopsin (HRh), chicken rhodopsin (CRh), the pRA1 pigment (pRA1), human blue (HBP), human green (HGP), human red (HRP), and chicken red/iodopsin (CRP).

Comparisons with Other Visual Pigment Sequences. Alignment of the pRA1 amino acid sequence with representative members of the vertebrate visual pigment family revealed a greater percent identity to rod pigments [74% vs chicken, 72% vs bovine (Ovchinnikov, 1983; Hargrave, 1983; Nathans & Hogness, 1983), and 73% vs human] than to cone pigments (44% vs iodopsin, 46% vs human red, 46% vs human green, and 47% vs human blue; Figure 3). The percent amino acid identity is summarized in a dendrogram (Figure 4), which serves as a close approximation to the corresponding evolutionary tree.

Amino acids known to be functionally important in bovine rhodopsin are conserved in the pRA1 pigment: lysine[296], the site of retinylidene Schiff's base formation (Bownds, 1967; Wang et al., 1980); cysteine[110] and cysteine[187], which form an essential disulfide bond (Karnik & Khorana, 1990); glutamate[113], the Schiff's base counterion (Zhukovsky & Oprian, 1989; Sakmar et al., 1989; Nathans, 1990); and multiple serines and threonines near the carboxy terminus, the sites of phosphorylation by rhodopsin kinase (Hargrave, 1982). Two potential N-glycosylation sites are located in the amino-terminal domain at positions corresponding to the two known sites of N-linked glycosylation in bovine rhodopsin (Hargrave, 1982), and two cysteines are present at positions 322 and 323, homologous to the pair that is palmitylated in bovine rhodopsin (Ovchinnikov et al., 1988). Histidine[211], which is absent from all cone pigments and present in all vertebrate rhodopsins sequenced to date, is present in the pRA1 pigment. This histidine has recently been shown to regulate the transition of metarhodopsin I to metarhodopsin II (C. J. Weitz and J. Nathans, unpublished). At pH 6.5, the pRA1 pigment is predicted to be eight charge units more positive than chicken rhodopsin.

Absorbance Properties of Recombinant Visual Pigment. To characterize the visual pigment encoded by the pRA1 gene, a cDNA clone containing the entire coding region (synthesized by PCR; see above) was inserted into the mammalian expression vector pCIS (Gorman et al. 1990) and transiently transfected into the human embryonic kidney cell line 293S. The expressed visual pigment was reconstituted in vitro with 11-*cis*-retinal and solubilized in digitonin.

In an initial series of experiments, photobleaching difference spectra were determined using a 420-nm short-wave cutoff filter. A photolabile species was observed with a broad bell-shaped absorbance curve that was maximal near 500 nm. Subsequent photobleaching experiments were therefore carried out with a 530-nm short-wave cutoff filter to eliminate absorbance changes due to isomerization of residual free 11-*cis*-retinal. (The broad visual pigment absorbance band includes wavelengths longer than 530 nm.) Figure 5 shows a

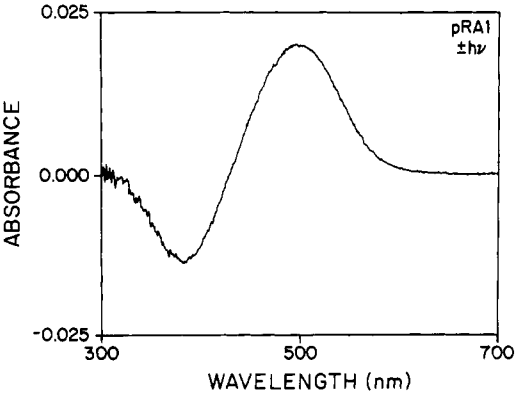

FIGURE 5: Photobleaching difference spectrum of the pRA1 pigment. Spectra were obtained at 20 °C with a 530-nm short-wave cutoff filter. The positive peak at 495 nm derives from the visual pigment; the negative peak at 380 nm derives from released *all-trans*-retinal.

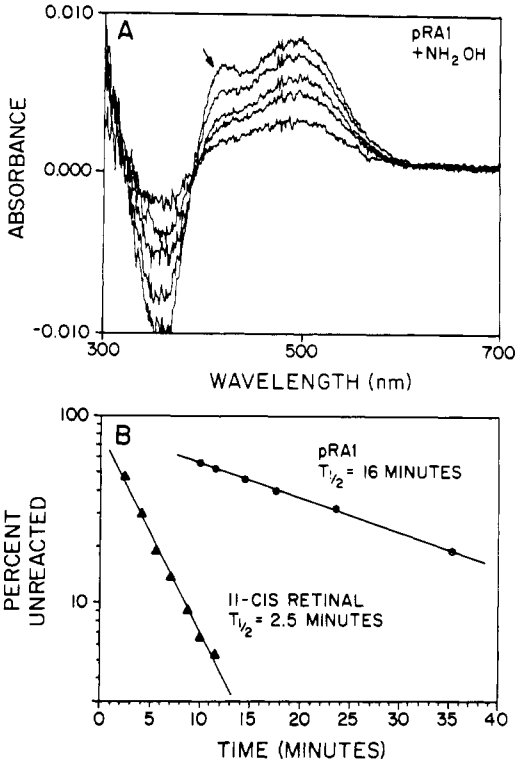

FIGURE 6: Time course of hydroxylamine reactivity with the pRA1 pigment and free 11-*cis*-retinal at 20 °C. The reaction was initiated by addition of hydroxylamine to a final concentration of 50 nm. (A) An absorbance spectrum obtained 35 min after hydroxylamine addition was subtracted from (top to bottom) spectra obtained after 10, 11.5, 14.5, 17.5, and 23.5 min. The reaction of hydroxylamine with residual 11-*cis*-retinal can be seen in the short-wavelength region of the spectrum. The difference spectrum between retinal oxime and free 11-*cis*-retinal generates a large negative peak at approximately 370 nm and two smaller positive excursions at wavelengths less than 330 nm and at approximately 415 nm (arrow). (B) Rates of reaction with hydroxylamine are plotted on a semilogarithmic plot. To minimize baseline distortion due to reaction of free 11-*cis*-retinal, data for the pRA1 pigment were collected after 10 min, after 94% of the free 11-*cis*-retinal had reacted to form retinal oxime.

typical photobleaching difference spectrum obtained at room temperature in the absence of hydroxylamine. The positive component, maximal at 495 nm, is derived from the expressed visual pigment; the negative component, maximal at 380 nm, is derived from released *all-trans*-retinal. In three independent experiments the pRA1 pigment absorbance maximum was observed to be 495 ± 1 nm. The photobleaching difference curve closely matches in shape that of bovine rhodopsin. The

yield of pRA1 pigment following transient transfection and reconstitution with 11-*cis*-retinal is reproducibly 4–5-fold lower than that of bovine rhodopsin.

To further characterize the properties of the pRA1 pigment, its sensitivity to bleaching by 50 mM hydroxylamine at 20 °C was determined (Figure 6). In this experiment, the reaction of residual free 11-*cis*-retinal with hydroxylamine occurs with a half-time of 2.5 min and serves as an internal standard. The pRA1 pigment reacts with a half-time of 16 min. An identical sample monitored in the absence of hydroxylamine showed no pigment loss over the 35-min incubation. Recombinant bovine rhodopsin incubated under these conditions reacted with a half-time of greater than 10 h.

Discussion

Does pRA1 Encode Chicken Green? The experiments reported here demonstrate that the chicken genome encodes a second rhodopsin-like visual pigment, defined by the pRA1 DNA sequence. In the chicken two visual pigments have been reported to absorb maximally near 500 nm. The absorbance maximum of chicken rhodopsin is 503 nm (Okano, 1989), and the absorbance maximum of chicken green has been determined by hydroxylamine bleaching in two studies to be approximately 500 nm (Yen & Fager, 1984) and 508 nm (Okano et al., 1989). Unlike chicken rhodopsin, chicken green does not bind to DEAE-Sepharose in low salt at pH 6.5, and it is susceptible to hydroxylamine attack (Yen & Fager, 1984; Okano et al., 1989). In detergent extracts of chicken photoreceptor outer segments, the chicken green pigment is found at approximately 10% the level of chicken rhodopsin. The pRA1 pigment differs from chicken rhodopsin in its higher predicted isoelectric point and its sensitivity to hydroxylamine, properties that also distinguish green from chicken rhodopsin. The pRA1 pigment absorbance maximum of 495 nm determined by photobleaching is not identical to that determined for chicken green by hydroxylamine bleaching. Unfortunately, the presence of residual 11-*cis*-retinal in the preparation of recombinant pRA1 pigment precluded obtaining an accurate hydroxylamine bleaching spectrum. If the discrepency in absorbance maxima can be ascribed to differences in starting material, extraction conditions, and/or analytical techniques, then the evidence suggests that the pigment encoded by pRA1 is chicken green.

Implications for Structure/Function Studies. The pRA1 pigment joins a growing list of visual pigments, including site-directed mutants of bovine and human rhodopsin, that absorb maximally at or near 498 nm. This data base should be helpful in identifying those amino acids involved in tuning of the chromophore absorbance, by revealing those variable residues that do not affect the absorbance maximum.

Accessibility to attack by hydroxylamine has been a consistently observed characteristic of cone pigments (Wald et al., 1955; Fager & Fager, 1981; Okano et al., 1989). It presumably reflects a greater accessibility in cone than in rod pigments of the retinal binding pocket, an hypothesis consistent with the greater speed of cone pigment regeneration with 11-*cis*-retinal (Wald et al., 1955). The limited number of amino acid differences between pRA1 and vertebrate rhodopsins together with their large difference in hydroxylamine reactivity should facilitate the identification of those amino acids that play a role in determining binding pocket accessibility.

Evolutionary Considerations. It is not yet known whether the pRA1 pigment resides in rod or cone photoreceptors, nor is it known which photoreceptor cell type contains chicken green. The similarity of pRA1 to rhodopsin suggests that the photoreceptors in which it resides may have evolved from rods. Alternatively, these photoreceptors may have evolved from cones by replacing a cone pigment with the pRA1 gene product. Concomitant immunolocalization and in situ hybridization studies of the pRA1 pigment and of rod and cone isoforms of other phototransduction proteins should clarify this issue. In either case, the pRA1 pigment defines a novel evolutionary branch in the visual pigment tree. It will be interesting to determine which other organisms carry representatives from this branch.

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Identification and Functional Importance of Tyrosine Sulfate Residues within Recombinant Factor VIII

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ABSTRACT: Sulfated tyrosine residues within recombinant human factor VIII were identified by [^{35}S]sulfate biosynthetic labeling of Chinese hamster ovary cells which express human recombinant factor VIII. Alkaline hydrolysis of purified [^{35}S]sulfate-labeled factor VIII showed that greater than 95% of the [^{35}S]sulfate was incorporated into tyrosine. [^{3}H]Tyrosine and [^{35}S]sulfate double labeling was used to quantify the presence of 6 mol of tyrosine sulfate per mole of factor VIII. Amino acid sequence analysis of thrombin and tryptic peptides isolated from [^{35}S]sulfate-labeled factor VIII demonstrated tyrosine sulfate at residue 346 in the factor VIII heavy chain and at residues 1664 and 1680 in the factor VIII light chain. In addition, the carboxyl-terminal half of the A2 domain contained three tyrosine sulfate residues, likely at positions 718, 719, and 723. Interestingly, all sites of tyrosine sulfation border thrombin cleavage sites. The functional importance of tyrosine sulfation was examined by treatment of cells expressing factor VIII with sodium chlorate, a potent inhibitor of tyrosine sulfation. Increasing concentrations of sodium chlorate inhibited sulfate incorporation into factor VIII without affecting its synthesis and/or secretion. However, factor VIII secreted in the presence of sodium chlorate exhibited a 5-fold reduction in procoagulant activity, although the protein was susceptible to thrombin cleavage. These results suggest that tyrosine sulfation is required for full factor VIII activity and may affect the interaction of factor VIII with other components of the coagulation cascade.

Hemophilia A is a bleeding disorder caused by a deficiency or abnormality in factor VIII. Factor VIII functions in the intrinsic pathway of coagulation. After proteolytic activation, it serves as the cofactor for the factor IXa dependent proteolytic activation of factor X. The isolation of the factor VIII gene and its expression in mammalian cells have greatly enhanced the understanding of the structure and synthesis of factor VIII (Vehar et al., 1984; Wood et al., 1984; Toole et al., 1984). The deduced amino acid sequence revealed a domain structure of A1-A2-B-A3-C1-C2 (Figure 1). The A-domains occur twice in the heavy chain and once in the light chain and share homology to the coagulation protein factor V and to the plasma copper binding protein ceruloplasmin (Kane & Davie, 1988; Mann et al., 1990). The C-domains are repeated twice in the light chain and are homologous to the C-domains in factor V and other phospholipid binding proteins (Kane & Davie, 1988; Stubbs et al., 1990). The B-domain does not share homology with other known proteins and contains 19 of the 25 potential asparagine (N)-linked glycosylation sites. Previous studies have demonstrated that the B-domain is not required for procoagulant activity (Toole et al., 1986; Eaton et al., 1986b; Kaufman et al., 1987). Two regions containing a high content of acidic amino acids occur between domains A1 and A2 and domains B and A3.

Since there are no known naturally occurring cell lines which produce factor VIII, analysis of the biosynthesis, processing, and secretion of factor VIII has required the heterologous expression of the human factor VIII cDNA in mammalian cells (Kaufman et al., 1988). Factor VIII is synthesized as a 2351 amino acid precursor from which a 19 amino acid signal peptide is cleaved. Upon transit into the Golgi compartment, factor VIII is modified by addition of O-linked oligosaccharides to serine and threonine and addition of complex structures to N-linked oligosaccharides (Kaufman et al., 1988). Also in the Golgi compartment the polypeptide is cleaved after residues 1313 and 1648 to generate the heavy

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