A Human Opsin-Related Gene That Encodes a Retinaldehyde-Binding Protein[†,⊥]

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ABSTRACT: The ligand-binding property of a cytoplasmic membrane-bound protein from bovine retinal pigment epithelium (RPE) has been demonstrated. The putative RPE–retinal G protein coupled receptor (RGR) covalently binds both *all-trans*- and 11-*cis*-retinal after reduction by sodium borohydride. The 32-kDa receptor binds *all-trans*-retinal preferentially, rather than the 11-*cis* isomer. The amino acid sequence of the opsin-related protein in humans is 86% identical to that of bovine RGR, and a lysine residue, analogous to the retinaldehyde attachment site of rhodopsin, is conserved in the seventh transmembrane domain of RGR in both species. The human gene that encodes the novel retinaldehyde receptor spans 14.8 kb and is split into seven exons. The structure of the gene is distinct from that of the visual pigment genes. These findings support the notion that the *rgr* gene represents the earliest independent branch of the vertebrate opsin gene family. A second form of human RGR in retina is predicted by alternative splicing of its precursor mRNA. This RGR variant results from the alternative use of an internal acceptor splice site in the second intron of the human gene, and it contains an insertion of four amino acids in the connecting loop between the second and third transmembrane domains. Since RGR binds *all-trans*-retinal preferentially, one of its functions may be to catalyze isomerization of the chromophore by a retinochrome-like mechanism.

The retinal pigment epithelium (RPE[1]) is a specialized cell monolayer that lies adjacent to the photoreceptors and performs functions that are essential to the visual process. One function of the RPE is to restore the chromophore 11-*cis*-retinal from its *all-trans* configuration and allow synthesis and regeneration of the visual pigments (Saari, 1990; Rando et al., 1991). The regeneration of rhodopsin in the dark is dependent on the production of 11-*cis*-retinal by a pathway that involves isomerization of *all-trans*-retinol to the 11-*cis* conformation (Bernstein & Rando, 1986). The isomerization step has been characterized *in vitro* by the demonstration of cell-free 11-*cis*-retinoid biosynthesis (Bernstein et al., 1987). A lecithin retinol acyl transferase and an isomerohydrolase participate in the endothermic reaction of converting *all-trans*- to 11-*cis*-retinoids at the alcohol, rather than aldehyde, oxidation state (Rando, 1991). The substrate for the isomerohydrolase is an *all-trans*-retinyl ester, which is converted directly into 11-*cis*-retinol (Deigner et al., 1989; Trehan et al., 1990). 11-*cis*-Retinol is then either oxidized to 11-*cis*-retinal for regeneration of the visual pigments or esterified and stored in the RPE as an 11-*cis*-retinyl ester.

The invertebrate visual cycle uses another method to regenerate active visual pigments. In molluscan eyes, there are a pair of photopigment systems: one containing rhodopsin and the other containing retinochrome (Hara & Hara, 1987). Photopigments are regenerated in the rhodopsin–retinochrome conjugate system by exchange of retinal chromophores. Retinochrome binds and photoisomerizes *all-trans*-retinal to 11-*cis*-retinal, which is returned to the rhodopsin system by the retinal-binding protein (RALBP) (Terakita et al., 1989). The arthropod eye may have a similar mechanism for producing 11-*cis*-retinal (Schwemer et al., 1984; Smith & Goldsmith, 1991).

We have recently identified an opsin-related gene that is preferentially expressed at high levels in the RPE and Müller cells of the neural retina (Jiang et al., 1993). The gene encodes a putative RPE–retinal G protein coupled receptor (RGR) with seven transmembrane segments. The putative receptor most closely resembles the subfamily of visual pigments and retinochromes. A lysine residue, analogous to the retinaldehyde attachment site of rhodopsin, is conserved in the seventh transmembrane domain of RGR. The subcellular distribution of the membrane-bound RGR is unique for the seven transmembrane domain receptors. The opsin homologue is localized predominantly in an intracellular cytoplasmic compartment of RPE and Müller cells, rather than in the plasma membrane at the cell surface (Pandey et al., 1994).

In this study, we demonstrate the binding of isomeric retinals to bovine RGR and deduce the primary structure of human RGR by characterization of human *rgr* gene clones and cDNAs. The structure of the human *rgr* gene, the organization of its exons and introns, and the expression of an alternatively spliced mRNA transcript in human retina are described herein. Significant evolutionary differences between the structure of the human *rgr* gene and that of known visual pigment genes are shown.

MATERIALS AND METHODS

Materials. Human genomic DNA clones were isolated from a λFIXII bacteriophage library (Stratagene, Inc., La Jolla, CA), which was constructed from human placental DNA by the insertion of partially digested *Sau*3AI DNA fragments into the *Xho*I cloning site. The human retinal cDNA library was kindly provided by Jeremy Nathans (Johns Hopkins University School of Medicine, Baltimore, MD). Oligonucleotides were synthesized using an Applied Biosystems 380B DNA synthesizer (Applied Biosystems, Foster City,

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[⊥] Sequences have been deposited under GenBank accession numbers U14910 and U14911.

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[1] Abbreviations: RPE, retinal pigment epithelium; RGR, putative RPE–retinal G protein coupled receptor; *all-trans*-[^{3}H]retinal, *all-trans*-[11,12-$^3H_2$]retinal; DTT, dithiothreitol.

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CA). Tritium-labeled vitamin A, [11,12-$^3H_2$]retinol (40–60 Ci/mmol), was purchased from New England Nuclear Research Products (Boston, MA). Purified *all-trans*-retinal was purchased from Sigma (St. Louis, MO), and 11-*cis*-retinal was obtained from the National Eye Institute, courtesy of Dr. Rosalie K. Crouch (Medical University of South Carolina, Charleston, SC).

Isolation of RPE Microsomes. Postmortem bovine eyes were obtained from a local abattoir. The isolation of bovine RPE cells and the preparation of RPE microsomes were carried out under dim yellow or red light within 2 h of enucleation. After excision of the anterior segment and removal of the lens, vitreous, and neural retina, RPE cells were removed by gently scraping the cell monolayer with a spatula. The cells were collected by centrifugation and homogenized in an ice-cold sucrose buffer of 0.25 M sucrose, 30 mM Tris–acetate (pH 7.0), and 1 mM DTT using a Dounce glass homogenizer. The homogenate was centrifuged at 300*g* at 4 °C to remove nuclei and unbroken cells. The pellet was resuspended, and the homogenization and centrifugation steps were repeated four times. The combined supernatants from the homogenization steps were centrifuged in a Sorvall SS-34 rotor at 15000*g* for 20 min at 4 °C. The 15000*g* supernatant contained the RPE microsome fraction from which the membranes were then collected by centrifugation in a Beckman 70 Ti rotor at 150000*g* for 1 h at 4 °C.

Preparation and Analysis of [^3H]Retinal. all-trans-[11,12-$^3H_2$]Retinal was prepared by chemical oxidation of [11,12-$^3H_2$]retinol (40–60 Ci/mmol), as described previously (Ball et al., 1948). The [11,12-$^3H_2$]retinol (250 μCi) was oxidized in the presence of 2.4 mg of MnO_2 in a hexane solution saturated with retinoic acid. The reaction was performed for 2 h in the dark under a nitrogen atmosphere, and the products of the reaction were filtered through a GF/C glass filter. The *all-trans*-[11,12-$^3H_2$]retinal was isolated by HPLC and used to prepare 11-*cis*-[11,12-$^3H_2$]retinal by photoisomerization. The irradiation of purified *all-trans*-[11,12-$^3H_2$]retinal in ethanol by a fiber optic light source for 5 min resulted in a mixture containing 13-*cis*-, 11-*cis*-, 9-*cis*-, and *all-trans*-retinals. The *all-trans*- and 11-*cis*-[11,12-$^3H_2$]retinal isomers were isolated and routinely analyzed for purity by normal phase chromatography using a LiChrosorb RT Si60 silica column (4 × 250 mm, 5 μm) (E. Merck, Darmstadt, Germany) and a Bio-Rad HPLC system. The HPLC column was precalibrated using purified isomers of retinal. The standards were eluted with 2% dioxane in hexane as the mobile phase and detected by UV absorbance at 325 nm. The isolation of the labeled retinoids was based on the elution times of the standards. The crystals of isomeric retinaldehyde standards were stored at –80 °C in a light-protected container before use.

Binding of all-trans-[^3H]- and 11-cis-[^3H]Retinal to RPE Microsomal Protein. RPE microsomes from 10 bovine eyes were prepared as described earlier and resuspended in 1.0 mL of cold 67 mM sodium phosphate (pH 6.5) (protein concentration: 0.85 mg/mL). The membranes were exposed for 1 h to light from a fiber optic light source, and then equal aliquots (0.25 mL) of the membrane suspension were mixed in the dark with 2.5 μL of an ethanolic solution of *all-trans*-[^3H]- or 11-*cis*-[^3H]retinal (each isomer at 1×10^5 or 0.4×10^5 cpm, 50 Ci/mmol). The mixtures were incubated in the dark with gentle agitation for 3 h at room temperature. After incubation, the membranes were collected by centrifugation at 38 500 rpm for 25 min at 4 °C using a Beckman SW60 rotor. The pellet was washed three times and resuspended in 1.0 mL of 67 mM sodium phosphate (pH 6.5). After adjustment of the buffer pH to 8.0 with 1 M NaOH, the membrane suspension was mixed with 38 mg of sodium borohydride (1 M $NaBH_4$, final concentration) and then immediately irradiated for 5 min by a flood lamp light source. The membranes were recovered again by centrifugation and washed three times with phosphate buffer. The preceding experiment was repeated, except that the sodium borohydride reaction and all subsequent washing steps were carried out completely in the dark. Labeled microsomal proteins were analyzed by fluorography after SDS–PAGE. For fluorography, the 12% polyacrylamide gel was saturated with Enlightning reagent (DuPont-NEN Research Products, Boston, MA), dried, and exposed to Kodak X-omat AR film at –80 °C for a period of 5 days.

Immunoprecipitation of [^3H]Retinal-Labeled Bovine RGR. After incubation with *all-trans*-[11,12-$^3H_2$]retinal and treatment with sodium borohydride, as described earlier, the RPE microsomal proteins were solubilized in a solution of 1.2% digitonin and 67 mM sodium phosphate (pH 6.5). For each condition, the digitonin extract (200 μL) was added to 600 μL of binding buffer (500 mM NaCl and 10 mM sodium phosphate (pH 7.2)) and 150 μL of immunoaffinity resin (described below). The mixtures were incubated at 4 °C for 2 h in the presence or absence of excess blocking peptide. Two of the samples included a high concentration of bovine RGR peptide: either 100 μM amino-terminal peptide (AESGTLPTGFGELEVC) or 100 μM carboxyl-terminal peptide (CLSPQRREHSREQ). The immunobeads were washed in binding buffer containing 0.3% digitonin and recovered by centrifugation. Samples of the immunoprecipitates and original extract were analyzed by fluorography after electrophoresis in 12% SDS–polyacrylamide gels. The gel was exposed to Kodak X-omat AR film at –80 °C for a period of 8 weeks.

The immunoaffinity resin was conjugated with anti-bovine RGR monoclonal antibody 2F4, which was produced and purified as will be described later. The antibody-containing fractions from Mono Q chromatography were pooled and dialyzed three times in 0.1 M MOPS (pH 7.5) at 4 °C. Activated Affi-Gel 10 resin (Bio-Rad, Hercules, CA) was added to the antibody solution, and the suspension was agitated gently for 4 h at 4 °C. After the coupling reaction, the gel was incubated for 1 h in 0.1 M ethanolamine to block the remaining reactive sites. The immunoaffinity gel was then washed with water, equilibrated with binding buffer, and stored at 4 °C until use.

Preparation of Antibody. A monoclonal antipeptide antibody was produced against the carboxyl-terminal amino acid sequence (CLSPQRREHSREQ) of bovine RGR. Hybridomas were obtained from Susan K.-H. Ou (Division of Biology, Caltech, Pasadena, CA) and were produced as described previously (Ou et al., 1991). The synthetic peptide was conjugated to keyhole limpet hemocyanin, emulsified with RIBI adjuvant (RIBI Immunochemical Research, Hamilton, MT), and injected intraperitoneally into 5-week-old female BALB/c mice. Spleen cells from the immunized mice were fused with myeloma-653 cells from Ventrex Laboratories, Inc. (Portland, ME). The hybridomas were grown initially in selection medium containing RPMI 1640 supplemented with 1% fetal calf serum (Hyclone, Logan, UT), 4 mM glutamine, 10 units/mL each of penicillin and streptomycin, 1 mM sodium pyruvate, 75 μM adenine, 0.4 μM aminopterin, and 16 μM thymidine. Immunoreactivities of the monoclonal antibodies and antisera were tested by enzyme-linked im-

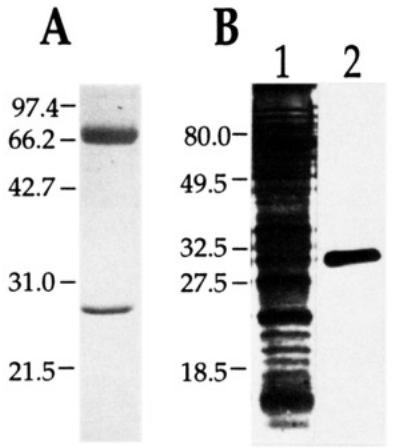

FIGURE 1: Characterization of antipeptide monoclonal antibody 2F4 directed against the carboxyl terminus of bovine RGR. (A) Purified monoclonal antibody 2F4 was electrophoresed on a 12% SDS–polyacrylamide gel and stained with Coomassie Brilliant Blue. (B) The specificity of monoclonal antibody 2F4 was tested by binding to a protein immunoblot of bovine RPE microsomes. Lanes: 1, microsomal proteins were visualized by silver staining; 2, protein immunoblot developed with monoclonal antibody 2F4 as primary antibody and alkaline phosphatase-conjugated anti-mouse IgM and IgG as secondary antibody. Each lane was loaded with 16 μg of protein. The molecular sizes of protein standards are indicated in kilodaltons on the left side.

munosorbent assay (ELISA) and by immunoblot analysis. Anti-RGR positive hybridoma cells were cloned by culturing single cells in HL-1 medium (Ventrex Laboratories, Inc.) containing 1% fetal bovine serum, 200 mM glutamine, and 10 units/mL each of penicillin and streptomycin. The hybridoma clones were grown on a lawn of BALB/c thymus feeder cells at 37 °C in a humidified air atmosphere of 5% CO_2.

The hybridoma clone 2F4 cells were grown on a large scale in HL-1 or UltraDOMA-PF (BioWhittaker, Inc., Walkersville, MD) medium that contained 1% fetal bovine serum, 200 mM glutamine, and 10 units/mL each of penicillin and streptomycin. The cells were removed by centrifugation, and the culture supernatant was mixed slowly with ammonium sulfate until the solution was 50% saturated with the salt. The mixture was stirred overnight at 4 °C, and the resultant protein precipitate was sedimented by centrifugation at 3000*g* for 30 min at 4 °C. The protein pellet was resuspended in phosphate-buffered saline and dialyzed thrice in 20 mM sodium phosphate (pH 7.0) at 4 °C. The dialyzed antibody solution was loaded onto an FPLC Mono Q column, and the proteins were eluted by a gradient of NaCl from 0 to 1.0 M in 20 mM sodium phosphate (pH 7.0). The column fractions were assayed for antibody activity by ELISA. The purity of the antibody was analyzed by SDS–polyacrylamide gel electrophoresis (Figure 1A). The size of the heavy chain of the monoclonal antibody (approximately 72 kDa) corresponded to that of an IgM molecule. On Western blots, the 2F4 monoclonal antibody specifically recognized a 32-kDa protein in a crude extract of bovine RPE microsomes (Figure 1B).

Isolation of DNA Clones and Methods in Molecular Biology. Genomic and cDNA clones were identified by plaque hybridization to a radiolabeled bovine RGR cDNA, RPE12 (Jiang et al., 1993). *Not*I-digested λFIXII DNA clones containing the human *rgr* gene were completely and partially cleaved with *Bam*HI, *Eco*RI, and *Sac*I restriction enzymes. Restriction maps were determined by Southern blot hybridization of the resulting fragments using oligonucleotide probes complementary to the flanking T3 and T7 promoters. DNA sequencing was carried out using single- and double-strand phagemid DNA, sequence-specific primers, and Sequenase (U.S. Biochemical Corp., Cleveland, OH) or Bst DNA polymerase (Bio-Rad, Hercules, CA), according to the manufacturers' protocols.

RNA and DNA blot hybridizations were performed according to established techniques (Sambrook et al., 1989). Human retina poly(A)$^+$RNA from 22 pooled tissue specimens (ages 16–70) was purchased from Clontech Laboratories, Inc. (Palo Alto, CA). Postmortem eyes were obtained from the Lions Doheny Eye Bank (Los Angeles, CA), and retina poly-(A)$^+$RNAs from individuals were prepared using a Mini RiboSep Ultra mRNA Isolation Kit (Becton Dickinson Labware, Bedford, MA), according to instructions from the manufacturer.

Rapid Amplification of the 5′-End of Human rgr mRNA. Amplification of the 5′-end of human *rgr* mRNA was performed with reagents from the 5′-AmpliFinder RACE Kit (Clontech Laboratories, Inc.), according to published procedures (Edwards et al., 1991). Ten picomoles of a human RGR-specific primer, A290 (5′-ATGCGATGGCTGCACTGCTGC), were used in the reverse transcription of 0.5 μg of pooled human retina poly(A)$^+$RNA. Following the ligation of a single-stranded oligonucleotide anchor directly to the 3′-end of the first-strand cDNAs, the 5′-end of the RGR cDNA was amplified using the A290 primer and a primer (5′-CTGGTTCGGCCCACCTCTGAAGGTTCCAGAATCGATAG) that was complementary to the anchor sequence. The amplification by PCR was carried out for 40 cycles, with each cycle performed for 1 min at 94 °C, 1.5 min at 55 °C, and 2 min at 72 °C. The 5′-end PCR fragment was then subcloned and several clones were sequenced.

RESULTS

Binding of all-trans- and 11-cis-Retinals to Bovine RGR. The hypothesis that RGR is a receptor for one or more isomers of retinal was tested by analysis of the covalent binding of ^{3}H-labeled *all-trans-* and 11-*cis*-retinals to bovine RPE microsomal proteins. A preparation of RPE microsomes was first exposed to light for 1 h in an attempt to photobleach RGR that may be bound endogenously to retinal. Equal aliquots of the membrane suspension were then incubated in the dark with either *all-trans*-[^{3}H]- or 11-*cis*-[^{3}H]retinal. After the incubation, sodium borohydride was added to the microsomes, and the membranes were then either kept in the dark or immediately irradiated for 5 min. The labeling of proteins in the microsomes was analyzed by fluorography after SDS–polyacrylamide gel electrophoresis.

Although added to a highly complex mixture of membrane proteins, both *all-trans*-[^{3}H]- and 11-*cis*-[^{3}H]retinals covalently bound and specifically labeled a single protein in the RPE microsomes, both under light (Figure 2A) and in the dark (Figure 2B). The labeled protein was 32 kDa conforming to bovine RGR in size (Jiang et al., 1993). The degree of labeling was consistently greater with *all-trans*-[^{3}H]retinal than with the 11-*cis* isomer. Exposure to light during the reduction step in the presence of sodium borohydride did not significantly affect the intensity of the bands. Although some rhodopsin was present as a contaminant, the labeled protein in RPE microsomes differed in size from rhodopsin, as detected by Western immunoblot (results not shown).

The binding of retinal to RGR was confirmed by specific immunoprecipitation of the protein from RPE microsomes (Figure 3). A portion of RPE microsomes that were labeled covalently with *all-trans*-[^{3}H]retinal was solubilized in digi-

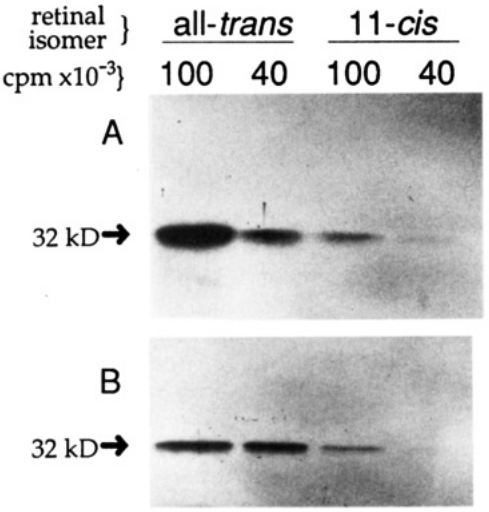

FIGURE 2: Binding of *all-trans*-[^{3}H]- and 11-*cis*-[^{3}H]retinals to protein in RPE microsomes. Bovine RPE microsomes (0.2 mg of protein/0.25 mL) were incubated with either *all-trans*-[^{3}H]- or 11-*cis*-[^{3}H]retinal, with each isomer at 100 000 and 40 000 cpm and specific activity 46 Ci/mmol. The samples were subsequently mixed with 1 M sodium borohydride and either (A) irradiated immediately with a fiber optic light source or (B) kept completely in the dark. The reaction products were analyzed by SDS–polyacrylamide gel electrophoresis and fluorography.

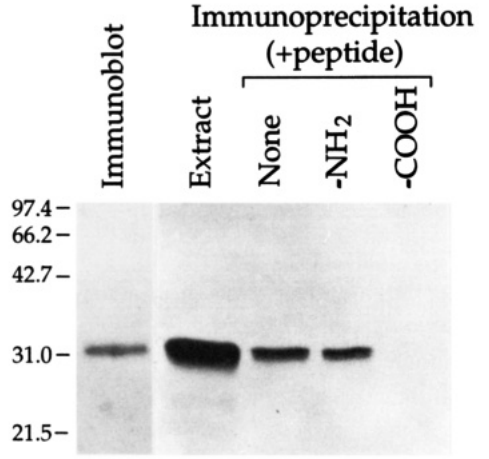

FIGURE 3: Immunoprecipitation of RGR from RPE microsomes labeled with *all-trans*-[^{3}H]retinal. An extract of RPE microsomal proteins that were labeled with *all-trans*-[^{3}H]retinal was prepared by solubilization of the membranes in a solution of 1.2% digitonin. RGR was immunoprecipitated from equal aliquots (0.02 mg of protein) of the extract in the presence or absence of amino-terminal (NH_2) or carboxyl-terminal (COOH) peptide. The immunoprecipitated samples were analyzed by SDS–polyacrylamide gel electrophoresis and fluorography. The immunoprecipitated protein comigrated with RGR, as detected by Western immunoblot.

tonin-containing phosphate buffer, and RGR in the soluble extract was immunoprecipitated using a monoclonal antibody directed against the carboxyl terminus of bovine RGR. The labeled 32-kDa protein was immunoprecipitated in a specific manner by the antibody-conjugated Affi-Gel resin. The immunoprecipitation of the protein was completely blocked when the antibody was incubated in the presence of 100 μM carboxyl-terminal peptide, but was not affected when the antibody was incubated with the same concentration of amino-terminal bovine RGR peptide. The immunoprecipitated protein comigrated with RGR, as detected by Western immunoblot (Figure 3).

Characterization of Human RGR cDNA. Six human RGR cDNA clones were isolated from a λgt10 retina library, following hybridization to the bovine RGR cDNA clone RPE12 (Jiang et al., 1993). One of these clones, HRGR1-2, contained a 1.3-kb incomplete cDNA insert and was used to determine the human RGR cDNA sequence from the nucleotide position 224 to the 3′-terminal poly(A) tract (Figure

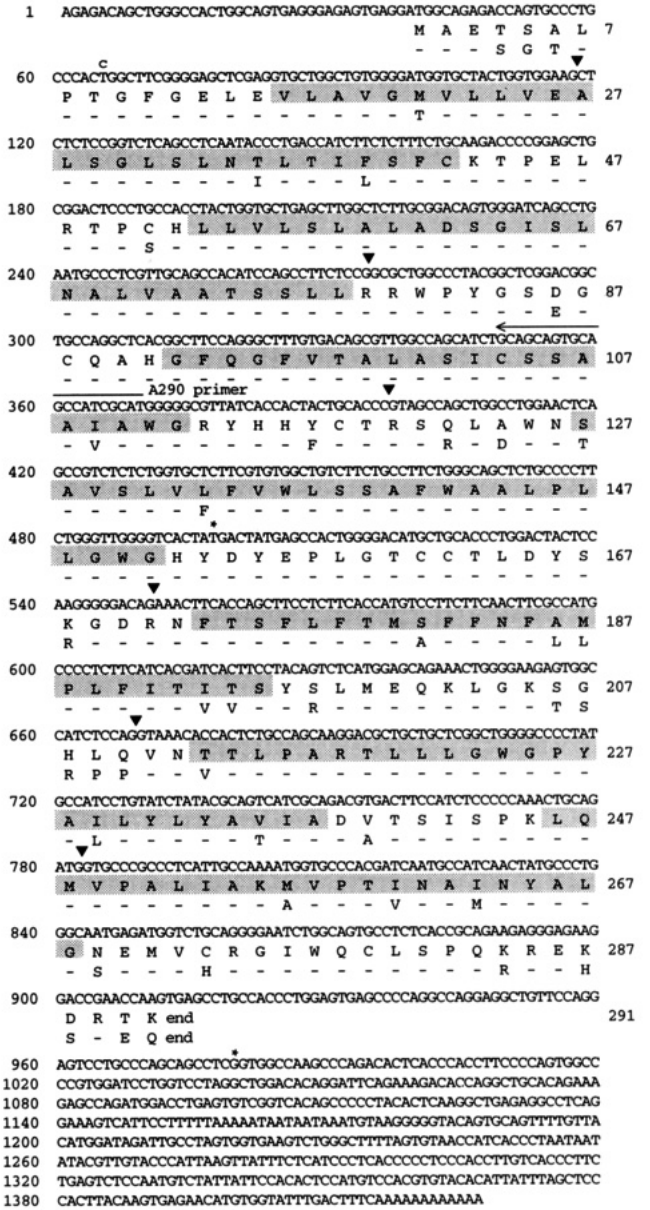

FIGURE 4: Nucleotide and deduced amino acid sequences of human RGR cDNA. The nucleotide and translated amino acid (one-letter code) sequences for human RGR are numbered on the left and right sides, respectively. Seven stretches of hydrophobic amino acids are shaded. Below the amino acid sequence of human RGR is that of the bovine protein; for comparison, only amino acid sequence differences are shown and identities are indicated by dashes. The locations of six intervening sequences in the human *rgr* gene are marked above the cDNA sequence by the inverted triangles. The sequence of the A290 primer is complementary to the cDNA sequence aligned under the arrow. Two sites of nucleotide sequence differences between the cDNA clone HRGR1-2 and human *rgr* gene clones are indicated by the asterisk. Several amplified copies of the 5′-end cDNA fragment were cloned and sequenced, and about half (13/23) of the clones contained a cytidine residue at nucleotide position 65, while the other half (10/23) contained thymidine. The cDNA clone HRGR33 contained an insertion of 12 nucleotides (TGTCTCCCA-CAG) between nucleotide positions 274 and 275. Amino acid residues (three-letter code) in human and bovine RGR that conform to conserved sequence motifs in G protein-coupled receptors include Asn^{34}, Asp^{62}, Cys^{88}, Arg^{113}-Tyr^{114}, Trp^{136}, Cys^{162}, Pro^{188}, Tyr^{196}, Trp^{224}, Pro^{226}-Tyr^{227}, Asn^{261}, and Tyr^{265} (GenBank accession number U14910.).

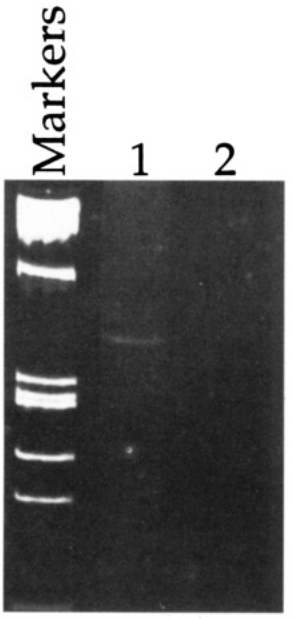

FIGURE 5: Primer extension and amplification of the 5′-end of human RGR cDNA. The antisense primer A290 was annealed to human retina poly(A)$^+$RNA and extended with reverse transcriptase. The 5′-end of the RGR cDNA was then amplified using a modified RACE procedure (see Materials and Methods). Lane 1: The results of the primer extension and PCR were the amplification of a DNA fragment about 380 bp in length. The DNA fragment was cloned and several copies were sequenced. Lane 2: PCR control in which all reagents were included except single-strand cDNA template. The DNA markers were the *Hae*III digest of *φX174* DNA.

4). The first 57 nucleotides of the actual 5′-end of the cDNA clone HRGR1-2 showed poor homology to the sequence of bovine RGR cDNA and appeared to be an artifact of cDNA cloning. This unknown sequence was later shown to be an inversion of the 5′-end of the human RGR cDNA.

The sequence of the cDNA from nucleotides 1 to 223 (Figure 4) was determined by rapid amplification of the cDNA 5′-end using the RACE method. An RGR-specific antisense primer, A290 (Figure 4), was used in the reverse transcription of pooled human retina poly(A)$^+$RNA. Following the ligation of a single-stranded oligonucleotide anchor directly to the 3′-end of the first-strand cDNAs, the 5′-end of the RGR cDNA was amplified using a primer complementary to the anchor and the RGR-specific primer, A290. The results of this primer extension and PCR were the amplification of a single major DNA fragment about 380 bp in length (Figure 5). The 5′-end cDNA fragment was then cloned, and several copies were sequenced. Multiple clones of the amplified 5′-end fragment ended with the adenosine at nucleotide position 1; thus, this site may mark the major 5′-terminus of human RGR mRNA. About half (13/23) of the RACE clones contained a cytidine residue at nucleotide position 65 of the cDNA sequence and the other half (10/23) contained thymidine. These results suggest that there is at least one common polymorphic difference in the human *rgr* gene at the DNA level. The alternative codons both encode threonine.

The complete nucleotide sequence of the human RGR cDNA, derived from cDNA clone HRGR1-2 and the 5′-end PCR fragment, is 1414 nucleotides long, excluding the poly-(A) tract (Figure 4). The overlapping sequences between the cDNA clone HRGR1-2 and the amplified 5′-end cDNA fragment were identical. Translation of the human RGR cDNA from its 5′-most ATG codon to the in-phase stop codon yields an open reading frame of 291 amino acids, with a cumulative molecular weight of 31 872. The 5′-untranslated region is 38 nucleotides long, and the putative translational initiation codon conforms to a consensus translational start site (Kozak, 1991). This initiation codon is preceded by an in-frame termination codon located 15 nucleotides upstream from the ATG sequence. A poly(A) tract of 12 adenosine residues was located at the 3′-end of the cDNA clone.

Prior to amplification of the RGR cDNA 5′-end by the RACE method, several other cDNA clones were characterized in an attempt to obtain a complete cDNA insert. One of these clones, HRGR33, contained a cDNA sequence that was in essence identical to that of the cDNA clone HRGR1-2, except that an insertion of 12 nucleotides was found after nucleotide position 274 (see legend to Figure 4). The insertion did not terminate or shift the original open reading frame.

Structure of the Human rgr Gene. The structure of the human *rgr* gene was determined by restriction enzyme mapping, exon–intron mapping, and nucleotide sequencing. Thirteen genomic clones with inserts of about 20 kb were isolated from a λFIXII library by probing with a human RGR cDNA. The complete gene was characterized by analysis of two overlapping genomic clones, λrgr12-1 and λrgr13-2. The λrgr12-1 clone contained the first four exons and 6.8 kb of the 5′-flanking region, but lacked the 3′-end of the gene. The λrgr13-2 clone contained the entire gene.

A restriction map of the human *rgr* gene was determined for *Bam*HI, *Eco*RI, and *Sac*I cleavage sites (Figure 6). The gene spans about 14.8 kb and is split into seven exons, identified by nucleotide sequence (Figure 7). The transcriptional initiation site and the 5′-end of exon 1 were inferred from the 5′-terminal cDNA sequence, as determined by the RACE method. Exon 7 encodes the carboxyl-terminal 43 amino acid residues of the protein and includes the contiguous 3′-untranslated cDNA sequence. The polyadenylation cleavage site was inferred from the cDNA clone HRGR1-2 and the site of its 3′-poly(A) tract. The polyadenylation site was not preceded by a canonical recognition sequence, AATAAA, characteristic of mammalian poly(A) signals (Gilmartin & Nevins, 1989).

The nucleotide sequence of the exons is identical to the cDNA sequence, except at two positions. The sequence at nucleotide position 979 of the cDNA contains guanosine,

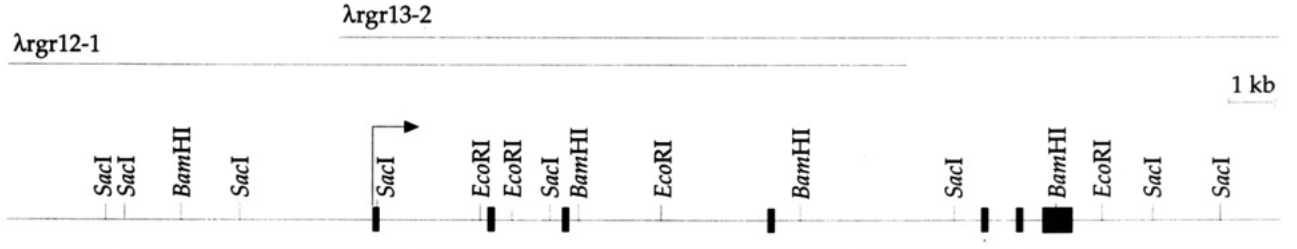

FIGURE 6: Structure of the human *rgr* gene. The restriction map of the human *rgr* gene for *Bam*HI, *Eco*RI, and *Sac*I sites was determined from overlapping genomic DNA clones λrgr12-1 and λrgr13-2. The transcriptional initiation site is marked by an arrow, and the exons are represented as solid boxes.

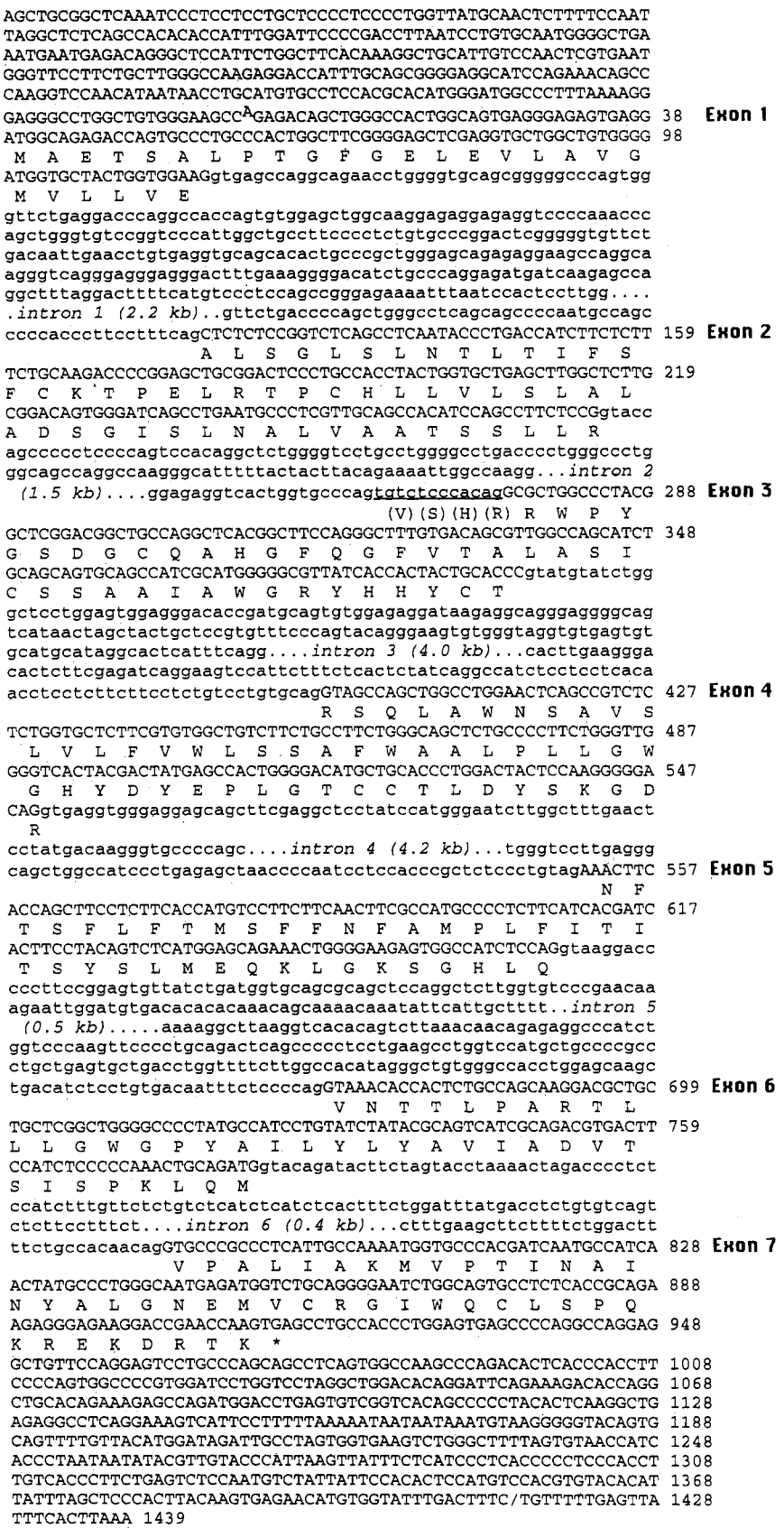

FIGURE 7: Nucleotide sequence of the human *rgr* gene. The intervening sequences are shown in lowercase letters. The major transcriptional initiation site is indicated as a superscript and is numbered +1. Only the exonic sequence is numbered on the right, and the 5′-flanking sequence is numbered negatively on the left. The underlined nucleotide sequence at the 3′-end of intron 2 was found as an insertion in human RGR cDNA clone HRGR33. A polyadenylation cleavage site is indicated by the backslash (/). The deduced amino acid sequence is written below the coding DNA sequence (GenBank accession number U14911).

however, adenosine is found at this position in the genomic sequence (Figure 7). The codon specifying tyrosine[153] is TAT in the cDNA clone, but in the genomic clone the codon sequence is TAC. These sequence differences between the

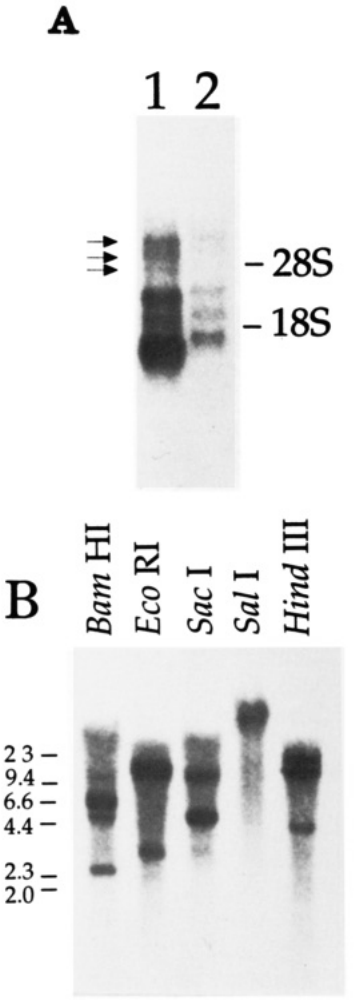

FIGURE 8: RNA and DNA blot hybridizations. (A) Northern blot hybridization of human retinal RNA to RGR cDNA probe HRGR1-2. Lanes: 1, pooled retinal poly(A)$^+$RNA (1 μg) (ages 16–70); 2, total RNA from the postmortem retina of a single donor (10 μg). The three arrows point to relatively faint mRNA transcripts. Ribosomal 28S and 18S RNAs were used as markers. (B) Blot hybridization of human lung DNA (20 μg per lane) after digestion with the indicated restriction enzymes. The DNA was hybridized to human RGR cDNA probe HRGR1-2. *Hin*dIII fragments of phage λ DNA were used as markers.

cDNA and gene clone may represent additional DNA polymorphisms in the human *rgr* gene. All of the intron–exon boundary sequences conform to consensus splice junctions by adherence to the GT/AG rule for intron termini.

Human rgr Gene Expression in Retina. In human retina, three major mRNAs, 1.5, 2.4, and 3.5 kb in length, and three larger faint transcripts were detected by hybridization to a cDNA fragment from clone HRGR1-2 (Figure 8A). The 1.5- and 3.5-kb major mRNAs are similar in size and relative intensity to the *rgr* mRNA transcripts in bovine RPE and retina. The results of the hybridization were similar whether the retinal RNA was from a group of donors and or from a single individual.

To investigate the possibility of multiple *rgr* genes, a human RGR cDNA probe was hybridized to human lung DNA cut with various restriction enzymes (Figure 8B). The pattern of genomic fragments that hybridized with the cDNA probe was consistent with the restriction map. Only the approximately 2.8-kb *Bam*HI fragment was not predicted from the map of the cloned gene. This difference may be due to a restriction fragment length polymorphism exhibited between the cloned *rgr* gene and the alleles from the isolated tissue.

DISCUSSION

In these studies we have identified a novel retinaldehyde receptor in bovine retina, deduced the complete amino acid sequence of the protein in humans, and determined the structure of the human gene that encodes this additional retinal opsin. We have also provided evidence for an alternatively spliced mRNA that encodes a variant of human RGR.

Ligand-Binding Function of RGR. The homology between RGR and visual pigments begat the prediction that covalent binding of *all-trans-* or 11-*cis*-retinal to RGR may be demonstrated in an assay that involves the reduction of potential Schiff base linkages by means of sodium borohydride (Bownds, 1967). This hypothesis was tested, and the results were consistent with the formation of a Schiff base linkage between the retinaldehyde isomers and a 32-kDa protein in the RPE microsomes and subsequent reduction of the bond to a stable secondary amine. The detection of noncovalently bound ligands would not be expected in this assay involving conventional SDS–PAGE and fluorography. Although the aldehyde group of retinal may react nonspecifically with primary amines, it reacts only very slowly in neutral solution and at low substrate concentrations (Ball et al., 1949). Therefore, the observation that a single RPE microsomal protein was strongly labeled suggests that the protein has a high-affinity reactive site for the retinaldehyde isomers. The labeled protein is similar in size to bovine RGR and comigrates with RGR detected by immunoblot analysis.

The labeled protein is unlikely to be rhodopsin, which is present as a contaminant of the RPE microsomes. It differed in size from rhodopsin, as detected by Western immunoblot, and did not aggregate upon boiling the protein samples prior to gel electrophoresis. Indeed, labeled rhodopsin may not have been detected because of its low amount and its susceptibility to degradation in the presence of sodium borohydride (Wang et al., 1980). The reduction of the Schiff base linkage between the retinylidene chromophore and rhodopsin by means of sodium borohydride is enhanced by light-induced isomerization of the protein-bound 11-*cis*-retinal to *all-trans*-retinal (Bownds, 1967). In contrast, the 32-kDa protein was labeled as well in the dark as it was upon illumination. Verification that RGR is the labeled protein was achieved by its specific immunoprecipitation with an antipeptide antibody directed against the carboxyl terminus of bovine RGR.

The RGR protein bound both *all-trans*-retinal and 11-*cis*-retinal. Since the exclusive function of 11-*cis*-retinal in vertebrates is its role in vision, RGR is probably directly involved in an aspect of the visual process. The 32-kDa receptor bound *all-trans*-retinal more extensively than it bound the 11-*cis* isomer. The preferential labeling of RGR with *all-trans*-retinal, rather than 11-*cis*-retinal, is surprising because although opsin binds many isomeric analogues of retinal with little stereoselectivity, it is known to bind the *all-trans* isomer quite poorly or at a significantly reduced rate (Liu et al., 1984). Since RGR appears to bind *all-trans*-retinal preferentially, one of its functions may be to catalyze isomerization of the chromophore by a mechanism that is similar to the invertebrate mechanism of isomerization, which involves retinochrome (Hara & Hara, 1987). Like RGR, retinochrome binds both *all-trans-* and 11-*cis*-retinals. It would be improbable that the conformation of the bound retinylidene on RGR is not subject to transformation by light, since directional photoisomerization is a conserved feature of all opsin-related receptors, including bacteriorhodopsin. Further evidence is required to determine whether RGR par-

ticipates in a pathway for the isomerization of *all-trans*-retinal to 11-*cis*-retinal.

Comparison of Human and Bovine RGR. The deduced amino acid sequence of human RGR is 86% identical to that of bovine RGR and precisely aligns with the bovine amino acid sequence. The two proteins are 291 amino acids long and have homologous coterminal amino and carboxyl ends. The hydrophilic lysine^{255} residue within the putative seventh transmembrane domain of RGR is conserved in both species and may be the high-affinity binding site for retinal by analogy to homologous opsins. Other amino acid residues in RGR conform to conserved sequence motifs of G protein-coupled receptors (see the legend to Figure 4).

RGR differs from the vertebrate visual pigments in that a negatively charged residue, homologous to the glutamate^{113} counterion in rhodopsin (Nathans, 1990; Sakmar et al., 1989; Zhukovsky & Oprian, 1989), is not conserved as a counterion for a potential protonated Schiff base in RGR. Instead, histidine^{91} is found in the corresponding position of RGR, and the nearest acidic residue is aspartate^{86} in human RGR and glutamate^{86} in bovine RGR. The rhodopsin and retinochrome in squid also lack the conserved glutamate residue as a counterion near the amino-terminal portion of transmembrane helix III (Hara-Nishimura et al., 1990; Hall et al., 1991). RGR also has short amino- and carboxyl-terminal domains and a short connecting loop between transmembrane domains V and VI.

The divergent substitutions between human and bovine RGR are more numerous in the amino- and carboxyl-terminal domains and in the connecting loops between the putative transmembrane segments, particularly in the third connecting loop on the side of the carboxyl terminus. These regions of RGR are analogous with the third cytoplasmic loop and the carboxyl-terminal domain of other receptors and have been shown in the homologous receptors to interact with G proteins (Kobilka et al., 1988; König et al., 1989). Despite a level of divergence between human and bovine RGR in these regions of the receptor, it would be of great interest to determine whether G protein coupling has been conserved in RGR and whether or not the opsin homologue is involved directly in a signal transduction process.

Structure and Expression of the Human rgr Gene. The gene that encodes human RGR is made up of seven exons, each of which contains part of the protein-coding region. The exons may correspond to specific structural and functional domains of the receptor. The first exon encodes the amino-terminal domain and part of the first hydrophobic segment. The second exon encodes the remainder of the first and the entire second hydrophobic segment. Exons 3, 4, 5, and 6 encode hydrophobic segments III, IV, V, and VI, respectively. The seventh exon encodes most of the seventh hydrophobic segment, the carboxyl-terminal domain, and the 3′-untranslated region. Each intron, except the first, splits the protein sequence within or close to a connecting loop between the putative transmembrane domains, and the number of introns coincides with the number of connecting loops. Thus, in the human *rgr* gene, most of the exons correlate with a specific transmembrane domain.

The structure of the human *rgr* gene differs from that of the known visual pigment genes, which are evolutionarily related by a common exon–intron arrangement and often exact correspondence between sequences in specific exons (Nathans et al., 1986). The introns of the *rgr* gene differ in both number and relative positions from the introns found commonly in rhodopsin and other visual pigment genes. This distinct

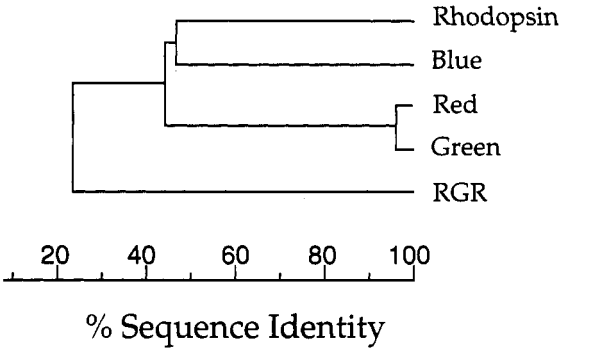

FIGURE 9: Percent amino acid sequence identity between RGR and visual pigments. The percent amino acid sequence identities between different members of the human opsin gene family (Nathans et al., 1986) are indicated by the nodal points.

arrangement of exons and introns supports the notion that the *rgr* gene evolved from an early independent branch of the opsin gene family.

The organization of exons and introns in the coding region of the *rgr* gene is also elaborate in comparison with that of many G protein-coupled receptor genes, some of which completely lack introns or contain only a single intron in the coding region (O'Dowd et al., 1989). Receptors that are encoded by genes with introns in the coding region possess a mechanism for diversifying receptor subtypes by alternative splicing of precursor mRNA. Alternatively spliced mRNAs have been reported for the D_2 dopamine receptor (Giros et al., 1989; Monsma et al., 1989) and the thyrotropin-releasing hormone receptor (de la Pena et al., 1992), for which there are variant receptors that differ in the region of the third cytoplasmic loop and the carboxyl terminus, respectively. Characterization of the human *rgr* gene and cDNA clones predicts an alternatively spliced variant of human RGR that contains an insertion of four amino acids (valine-serine-histidine-arginine) in the connecting loop between the second and third hydrophobic domains. This variant of RGR may result from the alternative use of an internal acceptor splice site within intron 2, as indicated by the sequence of the *rgr* gene (Figure 7). The placement of introns between the transmembrane domains allows a mechanism for the connecting loops to evolve by intron shuffling. The abundance, subcellular localization, and functional difference, if any, of the RGR variant are unknown.

The expression of the *rgr* gene in human retina appears more complex than that in bovine retina or RPE and gives rise to at least six transcripts detectable by Northern blot hybridization. The exact relationship between the mRNA transcripts and the human *rgr* gene sequence has not been determined; however, Southern blots of human genomic DNA do not indicate the existence of a large *rgr* gene family and suggest that the transcripts are derived from a single-copy gene. Primer extension and amplification of the 5′-end of retinal mRNA by the RACE method suggest that transcription of the *rgr* gene is initiated from a single major proximal site, although very distal transcriptional initiation sites cannot be excluded. Some of the mRNA transcripts may result from cleavage and termination at different downstream polyadenylation sites, as noted for mouse rhodopsin mRNAs (Al-Ubaidi et al., 1990).

The *rgr* gene is the most distant evolutionary branch of the visual pigment tree and has a distinct organization of exons and introns. Whereas vertebrate rhodopsin and cone visual pigments are about 45% identical in amino acid sequence, RGR and rhodopsin are only 25% identical (Figure 9). The

organization of the *rgr* gene and the amino acid sequence difference between RGR and the visual pigments suggest that the *rgr* gene evolved before the radiation of the visual pigment lineage. RGR binds the *all-trans*-retinal isomer preferentially. Native *all-trans*-retinal-binding chromoproteins, which include retinochrome and bacteriorhodopsin (Stoeckenius & Bogomolni, 1982), may have appeared early in evolution before the true visual pigments, in keeping with the higher stability of *all-trans*- over 11-*cis*-retinoid molecules and the more specialized role that the 11-*cis* isomer has in vision biology. Some of the biochemical properties of RGR may reflect novel features of the ancient common precursor of the present-day visual pigments.

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