A subtractive cDNA cloning strategy was used to isolate a 1381-base pair human retina-specific cDNA, human retinal gene 4 (HRG4), which hybridized to a 1.4-kilobase message in the retina and encoded a 240-amino acid acidic protein with a calculated molecular mass of 26,964 Da. The proximal ¾ of the conceptual protein sequence was rich in glycine (18%) and proline (20%), had a predicted secondary structure of turns, and showed a loose similarity (19–24%) to various α-collagen sequences, while the distal ¼ consisted of a mixture of α-helices, β-sheets, and turns. Genomic Southern analysis with HRG4 showed cross-hybridizing sequences in six different species, and HRG4 was 92% homologous with a 1264-base pair rat cDNA (rat retinal gene 4; RRG4) at the protein level. The region of 100% identity between the two sequences corresponded to the distal ¾ of the protein sequence consisting of mixed secondary structures, suggesting a functionally important domain.

In vitro transcription and translation corroborated the open reading frames corresponding to HRG4 and RRG4 in the cDNAs. Expression of HRG4 in the retina was localized to the photoreceptors by in situ hybridization. Developmentally, RRG4 began to be highly expressed around postnatal day 5 in the rat outer retina when the photoreceptors begin to differentiate and rapidly increased in expression to reach the mature adult level by postnatal day 23. No diurnal fluctuation in expression of RRG4 was seen.

Isolation and characterization of mammalian genes specifically or abundantly expressed in the retina have contributed greatly toward understanding of the retina and disease. Such genes include rhodopsin, cGMP-phosphodiesterase, transducin, and arrestin that are involved in phototransduction (1–4); peripherin and cGMP-phosphodiesterase β, and rom-1 (possibly by itself and in combination with peripherin) to be implicated in retinopathies including retinitis pigmentosa and macular pattern dystrophies (8–16).

In order to expand our knowledge of retinal biology and to identify additional retinal genes that may be involved in retinal diseases, we have been isolating new retinal genes by a subtractive cDNA cloning strategy. This strategy has already resulted in the isolation and characterization of the cDNA and gene for human recoverin, a protein previously thought to be involved in the recovery of the depolarized state in phototransduction (17), and X-arrestin, a new retinal arrestin that appears to play a desensitization role in a yet to be defined retina-specific signal transduction (18). We now report another cDNA obtained by this strategy that represents a new retina-specific gene that is highly conserved in human and rat and that begins to express an acidic, hydrophilic protein with some similarity to collagen specifically in photoreceptors at the time of outer retinal maturation and continues to express this protein throughout adult life.

MATERIALS AND METHODS

Subtractive cDNA Cloning

A retina-enriched cDNA library was prepared as described previously (17).

Northern Blot Analysis

Human Tissue Blot—Total RNA was isolated from human retina, cornea, iris, cultured retinal pigment epithelial cells, and fibroblasts using guanidine thiocyanate (19). Human brain, lung, and kidney total RNAs were obtained from Clontech (Palo Alto, CA). Ten micrograms of each RNA sample was electrophoresed in denaturing agarose gels (20) and transferred onto a nylon membrane by the Southern blotting method (21). The blot was hybridized overnight at 42 °C in a buffer containing 50% formamide, 10 mM Tris-HCl (pH 8.0), 5× SSC, 4× Denhardt’s solution, 2% SDS, 100 μg/ml denatured fish sperm DNA, and 32P-labeled HRG4 probe. The hybridized blot was washed finally with 0.1 × SSC at 52 °C and autoradiographed. Hybridization with a human full-length β-actin cDNA was performed to check the quality and quantity of RNA present in each lane of the blots.

Rat RNA Blots—To examine expression in various tissues, total RNA was isolated from adult rat lung, liver, kidney, spleen, brain, testis, adrenal, skeletal muscle, heart, and retina as described above. A Northern blot was prepared and hybridized with the full-length rat homologue cDNA of HRG4 (RRG4) or actin probe as described above. Final washing was done with 0.1 × SSC at 52 °C followed by autoradiography.

To study gene expression during retinal development, total retinal RNA was obtained from rats of various ages (postnatal day 0, 5, 7, 10, 15, 23, 30, 270, and 400). To avoid possible circadian variability in expression, all rats were sacrificed at about the same time of the day (4 p.m.). Northern blots with 8 μg of each RNA sample were hybridized with the full-length rat homologue cDNA (RRG4) or actin probe, washed at 52 °C, and autoradiographed as described above.

To examine possible diurnal variations in expression, total retinal RNA was prepared from adult rats at four different time points (7:00 a.m., 11:00 a.m., 7:00 p.m., and 11:00 p.m.). Rats were entrained to a cycle of 12 h of light starting at 8:00 a.m. and 12 h of dark starting at

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) U40998 and U40999.

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The abbreviations used are: RPE, retinal pigment epithelium; HRG4, human retinal gene 4; RRG4, rat retinal gene 4; bp, base pair(s); PND, postnatal day.

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**Cloning of the cDNA for a Novel Photoreceptor Protein**

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From the Bascom Palmer Eye Institute, University of Miami School of Medicine, Miami, Florida 33136 and the Department of Ophthalmology, National Defense Medical College, Saitama 359, Japan
8:00 p.m. Two-month-old rats from the same litter were divided into four groups, and each group was sacrificed at a different time point. In the dark period rats were killed and enucleated under dim red light, and retinas were kept from bright light until completely dissolved in the guanidine solution. Northern blots with 8 μg of each RNA sample were hybridized with RRG4, S antigen or actin probe as described above. A stretch of about 300 bp (1194–1482 bp) from the 3′ end of a rat S antigen cDNA (GenBank/EMBL, accession number X51781) was polymerase chain reaction-amplified from a cDNA clone obtained from our rat retinal cDNA library and used as a unique probe for S antigen. Final washing was done with 0.1 × SSC at 52°C followed by autoradiography.

Quantitation of mRNA Expression—Developmental and diurnal changes of expression were quantitated using the autoradiograms of the Northern blots. The intensity of the hybridized bands was measured using a F6910 Densitometer (Fisher Scientific, Pittsburgh, PA) with an integrator (HP3394A, Hewlett Packard Company, Avondale, PA). Films with different samples or exposure times were measured to ensure reproducibility.

In Vitro Transcription and Translation

The HRG4 cDNA clone was linearized in the 3′ noncoding region with Stul and transcribed into RNA using the T3 promoter located upstream of the cloning site of the pBluescript vector (Stratagene, La Jolla, CA). A transcription reaction using 1 μg of DNA template was carried out with T3 RNA polymerase at 37°C for 45 min. The RNA transcript was extracted with phenol and precipitated with ethanol, and 1–2 μg of the RNA template was translated in vitro with rabbit reticulocyte lysate (Stratagene, La Jolla, CA) with incorporation of [35S]methionine (DuPont NEN) at 30°C for 1 h. The translation products were analyzed in 12% SDS-polyacrylamide gels and visualized by autoradiography. The rat cDNA clone was linearized with Stul or MaeII, transcribed with T7 RNA polymerase, and translated as above. The translation reaction with no RNA was also carried out as a negative control.

"Zoo" Southern Blot Analysis

Genomic DNA from mouse, rabbit, pig, calf, and monkey was obtained from Clontech (Palo Alto, CA). Human genomic DNA was extracted with Phenol/chloroform and precipitated with ethanol. Digested genomic DNA was separated by agarose gel electrophoresis. After electrophoresis, the DNA was transferred to nylon membranes and hybridized with [32P]-labeled full-length cDNA probe of HRG4. The hybridization and washing conditions were as described above. Membrane autoradiography was performed with an intensifying screen.
tracted from normal skin fibroblasts (22). Ten micrograms of each DNA sample was digested with EcoRI, phenol/chloroform-extracted, ethanol-precipitated, electrophoresed in a 0.8% agarose gel, and transferred onto a nylon membrane by the Southern blotting method (21). The blot was hybridized with a full-length HRG4 cDNA probe as described for the Northern analysis. Final washing after hybridization was performed at 42 °C followed by autoradiography as described above.

**In Situ Hybridization**

In situ hybridization of HRG4/RRG4 riboprobes was performed in human or rat retina as described before (18) with some modifications. After fixation, frozen sectioning, and proteinase K treatment, the sections were acetylated by immersion in 0.25% acetic anhydride in 0.1M triethanolamine (pH 8.0) for 10 min at room temperature, followed by dehydration and hybridization. The sense (negative control) and antisense riboprobes were transcribed in vitro from the T3 or T7 promoter located on either side of the cloning site in the Bluescript vector with incorporation of [α-35S]rCTP (DuPont NEN). Riboprobes (1 x 10⁴ cpm/ml) for the human or rat tissue corresponded to a 569-base fragment in the 3'-noncoding region of the HRG4 cDNA, or a 360-base fragment in the 3'-noncoding region of the RRG4 cDNA, respectively. After hybridization, the slides were washed three times in 0.1 x SSC for 20 min at 65 °C and subjected to liquid emulsion autoradiography. After exposure for 4 days (8 days for rat retina of P0 and P10) at 4 °C, slides were developed and counterstained lightly with hematoxylin and eosin.

**Screening of cDNA Library**

A human retinal λgt11 cDNA library (23) was screened with a cDNA probe originally obtained from the retina-enriched library in order to isolate a full-length clone by standard procedures (24). Similarly, the full-length cDNA of HRG4 was used to screen our retinal λgt11 cDNA library at low stringency. Several clones were subjected to phage DNA preparation and subcloned into the pBluescript KS(+) vector for further analyses.

**DNA Sequencing**

DNA was sequenced by the dideoxy chain termination method (25) using the fmol DNA Sequencing System (Promega, Madison, WI).

**Probe Labeling**

DNA probes for hybridization were labeled with [32P]dCTP (DuPont NEN) by random priming (26) using the Oligolabeling kit (Pharmacia Biotech Inc.).

**Computer Analysis**

The DNA and conceptual amino acid sequences were analyzed by the IntelliGenetics and Genetic Computer Group software packages. DNA sequence data were assembled in the GEL program. Similarity searches were performed using the FASTDB, FASTA, or BLAST programs in any available DNA or protein data bases. Possible protein motifs in the conceptual amino acid sequence were determined using the MOTIFS program with the PROSITE dictionary. Predicted secondary structure, molecular weight, pl, and hydrophobicity of the protein sequences were determined using the PEP program. The human (HRG4) and rat (RRG4) protein sequences were aligned by the GENALIGN program.

**RESULTS**

Subtractive cDNA Cloning—Human retina-specific and -enriched cDNA clones were isolated through multiple liquid hybridization subtractions of a human retina cDNA library with human fibroblast cDNAs as described (17). Approximately 300 clones were initially obtained, and clones showing unique pat-
terns of hybridization in genomic Southern analysis were selected. These clones were in turn used as probes on tissue Northern blots to identify retina-specific and -enriched clones.

HRG4—One of the cDNA clones from the subtracted library showed a retina-specific pattern of expression when used as a probe on a Northern blot of RNA from various human tissues (Fig. 1). A message of approximately 1.4 kilobases was observed. Since the initial cDNA clone was a partial clone of approximately 570 bp, it was used as a probe to screen a human retina cDNA library to obtain a longer clone. Several positive clones were obtained (approximately 10% abundance in the retinal cDNA library of 0.05%), one of which contained a 1381 bp insert. The cDNA insert, designated human retinal gene 4 (HRG4) at the present time, was subcloned into pBluescript and sequenced.

The 1381-bp cDNA sequence contained an open reading frame of 720 bp, beginning at the first ATG codon, coding for a 240-amino acid protein with a pI of 5.96 and a calculated molecular mass of 26,964 Da (Fig. 2). The conceptual protein sequence showed a loose similarity to various collagen sequences (29 out of the first 30 best matches) when analyzed by the FastDB program (IntelliGenetics), although the matches were only in the range of 19–24%. The region showing the matches was mainly the proximal 1/4 of the protein high in proline and glycine content. A typical Gly-Xaa-Yaa repeat found in collagens, however, was not present in HRG4. The Chou-Fasman secondary structure analysis (IntelliGenetics) of the sequence demonstrated a stretch of turns in the glycine-
and proline-rich amino-terminal region of the protein, followed by alternating α-helix, β-sheet, and turn structures (Fig. 3). The Kyte-Doolittle hydropathy plot indicated the HRG4 protein to be relatively hydrophilic, and a search for protein motifs by the Quest program (IntelliGenetics) demonstrated a few frequently occurring phosphorylation sites for casein kinase II, protein kinase C, and tyrosine kinase. No signal sequence, transmembrane sequence, or glycosylation site was recognized.

Rat Homologue of HRG4—In light of the HRG4 clone appearing to represent a new human retina-specific cDNA, its conservation in different species was examined by a "zoo" genomic Southern blot analysis using the HRG4 cDNA probe. The result demonstrated the presence of hybridizing gene sequences in mouse, rabbit, pig, calf, and monkey, indicating sequence conservation (Fig. 4). In order to obtain a probe that would be useful in studying this gene in the rat and also to determine the exact degree of interspecies homology, a rat homologue of the HRG4 cDNA was cloned and sequenced (Fig. 5). The rat cDNA (RRG4) was 1264 bp in length, also containing an open reading frame of 720 bp, beginning at the first ATG codon, coding for a 240 amino acid protein like the HRG4 cDNA. The features of the rat protein were similar to HRG4 including a pI of 5.73, calculated molecular mass of 27,050 Da, hydrophilicity, and a predicted secondary structure consisting mostly of turns in the 50–60 amino-terminal residues rich in proline and glycine followed by a mixture of α-helix, β-sheet, and turns in the rest of the sequence. Unlike the human sequence, a potential glycosylation site was present at position 22 (asparagine) in the rat sequence.

A comparison of the human and rat conceptual protein sequences showed them to be highly homologous, with an overall homology of 92% (Fig. 6). A distinct pattern of homology was present with a region of uniqueness in the first 50–60 residues rich in proline and glycine (67% homology) while the rest of the sequence was 100% identical between the human and rat pro-
Cloning of a Novel Photoreceptor cDNA

In Vitro Expression of HRG4 and RRG4—In order to confirm that the open reading frame sequences present in the HRG4 and RRG4 cDNA clones are functional and code for translatable proteins, the clones were linearized downstream of the putative termination codons, and RNAs were transcribed from the human and rat cDNA clones are functional and code for translation products. This pattern of homology coincided with the distinct pattern of predicted secondary structure described above.

The retina specificity of expression of the rat cDNA was confirmed by a Northern blot analysis of various rat tissue RNAs using the rat probe. Expression of a 1.3-kilobase RRG4 message was observed only in the retina among 10 different tissues analyzed (Fig. 7).

In Vitro Expression of HRG4 and RRG4—To begin to probe the function of HRG4/RRG4, the time of its first appearance in the retina was determined. Since differentiation of the retina can be studied postnatally in the rat, developmental expression of RRG4 was investigated by Northern blot analysis and in situ hybridization in the rat retina. Analysis of the retinal RNAs from postnatal day (PND) 0, 5, 7, 10, 15, 23, 30, 270, and 400 rats by Northern blot hybridization with the RRG4 probe showed a barely detectable signal at PND 0, significant expression by PND 5, an exponential increase during the maturation of the photoreceptors in the following 20 days or so, and maximal stable expression by PND 23 (Fig. 10).

In agreement with the Northern analysis, in situ hybridization in the PND 0, 10, 30, and 270 rat retina demonstrated minimal signal at PND 0 but signal in the developing outer nuclear layer and the basilar layer, corresponding to the developing inner and outer segments, at PND 10 (Fig. 11). The hybridization signal was present in the outer nuclear layer and inner segment at PND 30, and this pattern was unchanged at PND 270.

Expression of RRG4 during the Diurnal Cycle—Since HRG4/RRG4 is photoreceptor-specific and a number of photoreceptor genes involved in phototransduction show diurnal changes in the level of expression (27, 28), the expression of RRG4 during the diurnal cycle was examined by Northern analysis of retinal RNA extracted as described at four time points in the light/dark cycle. The expression of S antigen was also examined for comparison with a phototransduction gene reported to show diurnal changes in expression (28). The results confirmed the previously reported fluctuation in S antigen expression (maximal expression in light and minimal expression in dark); the expression of RRG4, on the other hand, stayed relatively constant throughout the light/dark cycle (Fig. 12).

DISCUSSION

We have isolated a new retina-specific cDNA (HRG4/RRG4) using a previously described subtractive cDNA cloning approach (17). The encoded protein sequence showed an interesting two-domain structure consisting of a proximal 50–60-amino acid region rich in glycine and proline and forming turns and a distal 180–190-residue region made up of α-helices, β-sheets, and turns. Although classical collagen-associated Gly-Xaa-Yaa repeats were not present, the glycine- and proline-rich region contributed to a loose similarity to various α-collagen sequences upon sequence matching. The high degree of conservation between the human and the rat sequence suggests the importance of structural conservation for function of this retinal protein in different species. Moreover, the pattern of homology between the two protein sequences seemed to point to a functional basis of the putative two-domain structure described above. Whereas the glycine and proline-rich amino-terminal region consisting of 50–60 residues showed only 67% homology between the two sequences, the rest of the molecule, made up of a mixture of α-helices, β-sheets, and turns, was 100% identical. Its 100% conservation in human and rat seemed to indicate that this is the part of the protein strictly conserved structurally.

The open reading frame sequences present in the human and rat cDNA clones were the longest stretches of such sequence present and contained putative translation initiation codons satisfying the consensus sequence described for initiation codons (29). The in vitro transcription and translation, however, clearly indicated the functionality of the putative coding sequences in these clones. The 35-kDa HRG4 and 33-kDa
RRG4 proteins could only be made from the described open reading frame sequences in the clones. The sizes of the translation products in the SDS-polyacrylamide gel electrophoresis analysis were larger than the calculated sizes (27 kDa versus 35 and 33 kDa), but the larger than expected size was seen even for the rat clone that was linearized at only 44 bases downstream of the termination codon. Since the larger size cannot be explained even if there was a read-through to the linearized end in the rat clone (28 kDa expected) and post-translational modification does not occur in rabbit reticulocyte system (confirmed by Stratagene), the size discrepancy appears to be due to the primary characteristic of the proteins. It may be due to the proline-rich feature of these sequences, a phenomenon observed for other proteins (30, 31).

**Fig. 11. Developmental in situ hybridization of RRG4 in rat retina.** Retinal sections from rats of the indicated ages were hybridized with antisense (A) or sense (B) riboprobe of RRG4 and subjected to liquid emulsion autoradiography and counterstaining with hematoxylin and eosin. GCL, ganglion cell layer; NBL, neuroblastic layer; RPE, retinal pigment epithelium; INL, inner nuclear layer; ONL, outer nuclear layer; BL, bacillary layer; IS, inner segment; OS, outer segment.

**Fig. 12. Diurnal profile of RRG4 and S antigen expression.** A Northern blot with 8 µg each of total retinal RNA samples prepared at the four indicated time points was hybridized with RRG4 (A), S antigen (B), and actin (C) probes. D, densitometric analysis of the expression level of RRG4 (●) and S antigen (■). The intensity of the RRG4 and S antigen messages at each time point was plotted as the percentage of the 11:00 value after normalization with actin.
native proteins may have still a different size due to posttranslational modification.

In situ hybridization analysis of HRG4 transcripts narrowed down the site of expression of this gene to the photoreceptors in the retina. Both rod and cone photoreceptors appeared to be expressing this gene. The developmental profile of expression of this gene demonstrated that it begins to be significantly expressed at around PND 5 in the developing rat retina when the photoreceptor differentiation begins to take place with the formation of the outer plexiform layer, outer nuclear layer, and the inner and outer segments. The expression level increased rapidly throughout the remainder of the period of photoreceptor maturation and reached the maximal stable level by PND 23, when the retina was fully developed. The level remained the same thereafter. Throughout the developmental stage and adulthood, the site of expression was in the photoreceptors. This developmental pattern of expression is identical to that of other photoreceptor-specific genes such as rhodopsin (32), suggesting that HRG4/RRG4 may play a role in mature photoreceptors.

The function of the new photoreceptor-specific gene HRG4 cannot be clearly determined at this time. The hydrophilic nature of the encoded gene product suggests that it could be a soluble phototransduction protein, but it does not contain any recognizable sequence motif for such a protein, nor does its expression show fluctuation with the light/dark cycle, as has been shown for a number of phototransduction proteins including opsin, transducin, and S antigen (27, 28). This may argue against this gene product being involved in phototransduction. Given the stable expression pattern during the 12:12 light/dark cycle and its loose similarity to collagen at the primary sequence and secondary structural levels, an attractive possibility is that HRG4 may be a structural protein or a matrix component. The interphotoreceptor matrix between the retinal pigment epithelium and photoreceptors has been described as not containing collagen (33); however, HRG4 may be a collagen-like protein in the interphotoreceptor matrix. Alternatively, HRG4 might be a structural or matrix protein of the photoreceptor synapse. Production of antibody and precise immunolocalization should clarify this question. Although its function has yet to be determined, the high degree of conservation of this gene in human and rat, its strict photoreceptor-specificity, and its distinct pattern of expression, beginning with the differentiation of the photoreceptors and continuing throughout life, clearly suggest that HRG4 may be an important retinal gene.

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