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ISOLATION OF HUMAN RETINAL GENES: RECOVERIN cDNA AND GENE

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SUMMARY: A human retina cDNA library enriched for retina-specific clones was prepared by subtraction with a non-retina population of cDNA in combination with polymerase chain reaction (PCR) amplifications. A highly retina-specific cDNA clone (1190 bp) was obtained through this library encoding a 200 amino acid protein with three calcium binding sites and 87% homology to the bovine photoreceptor protein, recoverin, which has been shown to mediate the recovery of the dark current after photoactivation, and 58% homology to the calcium-binding chick cone protein, visinin. Analysis of the gene indicated a 9-10 kb single-copy gene with at least three exons and two introns. The three exons contained the entire coding sequence, and all of the calcium-binding EF-hand regions were in putative exon 1. The recoverin gene was mapped to human chromosome 17 by hybridization to a panel of human-rodent hybrid DNAs.

Isolation and characterization of retina-expressed and retina-specific genes have greatly enhanced our understanding of the biology of the retina and vision. Many of these genes are involved in the phototransduction cascade, and their isolation has allowed a closer examination of each of the components that make up this complicated process (1, 2, 3, 4). At least three of these retina-expressed genes, ornithine aminotransferase, rhodopsin, and peripherin, have been shown to be involved in human retinal degenerations, namely gyrate atrophy and autosomal dominant retinitis pigmentosa (5, 6, 7, 8, 9, 10).

Since isolation of new retinal genes may lead to identification of additional genes involved in important retinal functions or retinal degenerations, we have been attempting to isolate retina-specific genes by a differential cDNA cloning approach. A human retina cDNA library enriched for retina-specific clones was prepared by

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subtraction with a non-retina population of cDNA in combination with polymerase chain reaction (PCR) amplifications. This paper describes the isolation of a cDNA clone from the subtracted library which is specifically expressed in the retina and encodes a protein with three calcium binding sites and homology to the bovine photoreceptor protein, recoverin (11). A gene clone encoding the cDNA was obtained and shown to consist of at least three exons. The gene was tentatively mapped to chromosome 17.

MATERIALS AND METHODS

Preparation of retina-enriched cDNA library

Human retinal mRNA was prepared from neuroretinal tissue of an adult male using the FastTrack poly(A) RNA preparation kit (InVitrogen, San Diego, CA), and converted to double-stranded cDNA using the Moloney Murine Leukemia Virus reverse transcriptase (Bethesda Research Laboratories, Rockville, MD), RNase H, E. coli DNA ligase, and DNA polymerase (12) (New England BioLabs, Beverly, MA). Products greater than 300 bp were obtained by size-fractionation, and Eco RI "lone linkers" were ligated to the ends (13). All of the cDNA sequences in the mixture were amplified by 30 cycles of polymerase chain reaction (PCR) using a linker primer and annealing temperature of 52 °C (14). A similar cDNA mixture was prepared from human fibroblast mRNA, digested with Eco RI, and photobiotinylated (15). Six parts of biotinylated fibroblast cDNA and one part of retina cDNA were hybridized in 0.5 M sodium phosphate, pH 6.8, 0.15 % sodium dodecyl sulfate (SDS), and 5 mM of EDTA at 68 °C for 16 hrs. Double-stranded DNA containing the biotinylated cDNA including the fibroblast-retina hybrids were removed by the addition of Streptavidin (Bethesda Research Laboratories, Rockville, MD) in HB buffer (50 mM HEPES, pH 7.6, 2 mM EDTA, 500 mM NaCl), incubation at room temperature for 5 min., and phenol-chloroform extraction. The aqueous portions were pooled after 2-3 cycles of the Streptavidin extraction, and the DNA was precipitated. The hybridization-subtraction with the biotinylated fibroblast cDNA was repeated, the subtracted cDNA was amplified by PCR for 20 cycles, and the product was subjected to the same subtraction procedure as described. The final product was digested with Eco RI and ligated into pBluescript SK (Stratagene, La Jolla, CA). XL-1Blue bacteria was transformed with the recombinant plasmids, and colorless transformants were isolated in LB plates containing X-gal and IPTG (Bethesda Research Laboratories, Rockville, MD). Approximately three hundred recombinant clones were isolated.

Southern and northern hybridization

High-molecular-weight human genomic DNA was digested with restriction enzymes, electrophoresed in 0.8% agarose gel, transferred onto nitrocellulose or nylon filters by the Southern method (16), and hybridized to [^{32}P]-labeled cDNA probe. The hybridized filters were washed and autoradiographed as described (17). Total human retinal and fibroblast RNA was isolated from tissue or cells using guanidine thiocyanate (18). Human brain, liver, and lung RNA were obtained from Clonetech (Palo Alto, CA). The RNA was electrophoresed in denaturing agarose gel (19), transferred onto nylon membranes by the Southern blotting method (16) and hybridized with [^{32}P]-labeled DNA probe. The hybridized blots were washed and autoradiographed as described before (17). The quantity and quality of RNA on the blot were checked by hybridization with actin cDNA probe.

Screening of cDNA library

A human retinal λgt11 cDNA library (20) was screened with [^{32}P]-labeled cDNA originally obtained from the retina-enriched library in order to isolate a full-length clone by standard procedure (17).

Isolation and characterization of genomic clones

Approximately 10^6 clones from a cosmid (pWE 15) and a phage (Lambda Fix II) human placenta genomic library (Stratagene, La Jolla, CA) were screened with the human cDNA probe labeled with [^{32}P] (21), and positive clones were isolated by standard procedure (17). One cosmid and three phage recoverin gene clones were obtained. The gene clones were analyzed by restriction mapping with Eco RI, Bam HI, Xho I, Not I, and Hind III. Specific gene fragments were subcloned into pBluescript KS for sequencing and further analysis.

Chromosomal mapping

DNAs from hamster-human somatic cell hybrids containing specific human chromosomes were used in genomic Southern analysis with the [^{32}P]-labeled cDNA probe (Bios, New Haven, CT). The hybridization pattern observed in a panel of somatic cell DNAs indicated the chromosomal location of the gene.

DNA sequencing

DNA was sequenced by the dideoxy chain termination method using the Sequenase DNA sequencing kit (22) (United States Biochemical, Cleveland, OH). Sequences were analyzed by the IntelliGenetics (Mountain View, CA) and Genetic Computer Group (Madison, WI) software packages.

RESULTS

A systematic analysis of the cDNA clones in the retina-enriched cDNA library by northern analysis identified a clone which demonstrated a highly retina-specific pattern of expression. When this cDNA clone was used as a probe in northern analysis of RNA from the human retina, fibroblast, liver, brain, and lung, a transcript of approximately 1.4 kb was visible only in the retina (Fig. 1). The intensity of the hybridizing band in comparison to other retina-expressed transcripts such as rhodopsin and ornithine aminotransferase (data not shown) and the frequency of this clone in the cDNA library indicated that the abundance of this message in the RNA population is approximately 0.1-0.2%.

The original cDNA clone which was approximately 400 bp was used as a probe to isolate a 1190 bp clone from a λgt11 human retinal cDNA library. Sequencing of this cDNA clone demonstrated a sequence characteristic of mRNA with a poly(A) stretch of 16 adenine residues at the 3'end, a polyadenylation signal at position 1154, and a translation initiation AUG codon at position 188 followed by an open reading frame of 599 bp (Fig. 2). The encoded protein consisted of 200 amino acid residues and contained three regions identified as calcium-binding sequences (EF hands) (23).

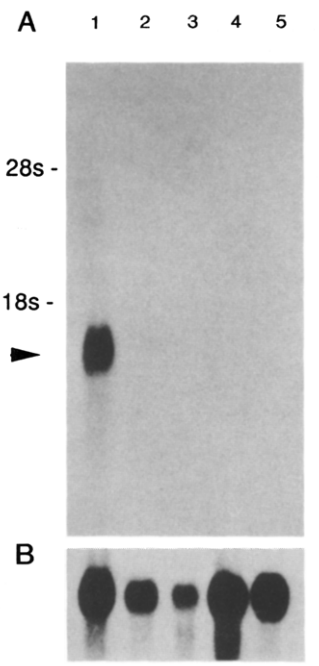

Figure 1. Northern blot analysis with the cDNA probe. Ten μg of total RNA from various human tissues was applied in each lane: 1. retina; 2. brain; 3. lung; 4. liver; 5. skin fibroblasts. A, Hybridized with [^{32}P]-labeled cDNA clone; arrow points to the transcript of approximately 1.4 kb seen only in the retina. B, Hybridized with the human β-actin probe to check on the quantity and quality of RNA present in each lane. Positions of the 28s and 18s ribosomal RNA are shown.

Comparison of the cDNA sequence with sequences in the GenBank did not show any matches, but the sequence demonstrated a 77% homology with a recently-published bovine calcium-binding photoreceptor gene, recoverin, which has been shown to modulate guanylate cyclase activity in phototransduction (24). Comparison of the cDNA-encoded amino acid sequence with sequences in the Protein Identification Resource data bank demonstrated a 87% homology with bovine recoverin and 58% homology with the chicken calcium-binding retinal cone protein, visinin (11, 25) with conservation of the three calcium-binding regions (Fig. 3). In view of this result, our cDNA clone was determined to be that of human recoverin and named pHUMRECO.

Southern blot analysis of human genomic DNA using the coding region of human recoverin as a probe demonstrated a single hybridizing band of 9-10 kb with three different enzymes, indicating a single-copy gene (Fig. 4). Phage and cosmid gene clones of recoverin were obtained and analyzed. The human recoverin gene was approximately 9 kb in size and consisted of at least two introns and three exons (Figs. 2, 5, 6). Putative exon 1 was at least 568 bp long, and the size of the mRNA from the northern analysis indicated that the start of this exon should be at least 100-200 bp

```
          10                  30                  50
GCCCTCCACAAAGCTCCTGGGCCCCTCCTCCCTTCAAGGATTGCGAAGAACTGGTCGCAA
          70                  90                 110
ATCCTCCTAAGCCACCAGCATCTCGGTCTTCAGCTCACACCAGCCTTGAGCCCAGCCTGC
         130                 150                 170
GGCCAGGGGACCACGCACGTCCCACCCACCCAGCGACTCCCCAGCCGCTGCCCACTCTTC

         190                 210                 230
CTCACTCATGGGGAACAGCAAAAGTGGGGCCCTGTCCAAGGAGATCCTGGAGGAGCTGCA
  1       M  G  N  S  K  S  G  A  L  S  K  E  I  L  E  E  L  Q   18

         250                 270                 290
GCTGAACACCAAGTTCTCGGAGGAGGAGCTGTGCTCCTGGTACCAGTCCTTCCTGAAGGA
 19  L  N  T  K  F  S  E  E  E  L  C  S  W  Y  Q  S  F  L  K  D   38
                                                      ------

         310                 330                 350
CTGTCCCACCGGCCGCATCACCCAGCAGCAGTTCCAGAGCATCTACGCCAAGTTCTTCCC
 39  C  P  T  G  R  I  T  Q  Q  Q  F  Q  S  I  Y  A  K  F  F  P   58
     ---------------------------

         370                 390                 410
CGACACCGACCCCAAGGCCTACGCCCAGCATGTGTTCCGCAGCTTCGATTCCAACCTCGA
 59  D  T  D  P  K  A  Y  A  Q  H  V  F  R  S  F  D  S  N  L  D   78
                                                ------------------

         430                 450                 470
CGGCACCCTGGACTTCAAGGAGTACGTCATCGCCCTGCACATGACCACCGCGGGCAAGAC
 79  G  T  L  D  F  K  E  Y  V  I  A  L  H  M  T  T  A  G  K  T   98
     -------------------

         490                 510                 530
CAACCAGAAGCTGGAGTGGGCCTTCTCCCTCTACGACGTGGACGGTAACGGGACCATCAG
 99  N  Q  K  L  E  W  A  F  S  L  Y  D  V  D  G  N  G  T  I  S  118
                                 --------------------------------

         550                 **                  590
CAAGAATGAAGTGCTGGAGATCGTCATGGCTATTTTCAAAATGATCACTCCCGAGGACGT
119  K  N  E  V  L  E  I  V  M  A  I  F  K  M  I  T  P  E  D  V  138
     ------------

         610                 630                 650
GAAGCTCCTTCCAGACGATGAAAACACGCCGGAAAAGCGAGCCGAGAAGATCTGGAAGTA
139  K  L  L  P  D  D  E  N  T  P  E  K  R  A  E  K  I  W  K  Y  158

         670         **      690                 710
CTTTGGAAAGAATGATGATGATAAACTTACAGAGAAAGAATTCATTGAGGGGACACTGGC
159  F  G  K  N  D  D  D  K  L  T  E  K  E  F  I  E  G  T  L  A  178

         730                 750                 770
CAATAAGGAAATTCTGCGACTGATCCAGTTTGAGCCTCAAAAAGTGAAGGAAAAGATGAA
179  N  K  E  I  L  R  L  I  Q  F  E  P  Q  K  V  K  E  K  M  K  198

         790                 810                 830
GAACGCCTGATGCCAACTGTTCAGCTGTCCTCCCTCCACCTACCACTCACATGACACCCG
199  N  A  -                                                     200

         850                 870                 890
TGAGCGCCTGTGCACACACACACACATGCACACACACGCGCGCGCACACACACACACACA
         910                 930                 950
CATCCACCCCAGGGCCAAGAGAAAGGCCTGCACACAAGCCCACAGCACAGCTCCCTGCCA
         970                 990                1010
AACTGAAGCATCTGTAGTGACCCACTGGTTCCTTCTTCCTGGGTCTTCAGCATTCCCTCC
        1030                1050                1070
CATCATGCCCGGTCCCACCCCTCCCTCTGTCCACCAGCCCATGTCCCTGTGCTAATCCCA
        1090                1110                1130
GGATTAGGCCATAGGAGTCCTAAGTGTCACCCCGCTGTAAGCTCCTTTGTGGAGTGCTGG
        1150                1170                1190
GTAAGCAGTTTCCAATAAACGCAAGCTGAGCTGGAAAAAAAAAAAAAAAA
```

Figure 2. Nucleotide and deduced-amino acid sequence of human recoverin cDNA. The amino acid residues (1-200) are in single letter codes below the nucleotide sequence starting with the initiation AUG codon at position 188. A line is drawn over the polyadenylation signal (AATAAA) at position 1154. The three calcium-binding EF-hand regions are indicated by broken underlines. The positions of the two introns are indicated by two asterisks at position 568 and 680. The numbers above the sequence refer to nucleotide positions and numbers at the side refer to amino acid residues.

```
RECO$CHICK     1 MGNSrSsALSrEvLqELrasTrytEEELsrWYegFqrqCsdGRIrcdeFerIYgnFFPnsePqgYArHVF
RECO$HUM       1 MGNSKSGALSKEILEELQLNTKFsEEELcSWYQSFLKdCPtGRITqQqFQsIYaKFFPdtDPKAYAQHVF
RECO$BOVIN     1 MGNSKSGALSKEILEELQLNTKFtEEELsSWYQSFLKeCPsGRITrQeFQtIYsKFFPeaDPKAYAQHVF
 consensus       MGNSkSgALSkEiLeELqlnTkftEEELssWYqsFlk-Cp-GRIt-qeFq-IY-kFFP--dPkaYAqHVF

RECO$CHICK    72 SFDtNdDGTLDFrEYiIALHlTssGKThlKLEWAFSLfDVDrNGevSKsEVLEIitAIFKMIpeEerlqL
RECO$HUM      72 SFDsNlDGTLDFKEYVIALHMTtAGKTNQKLEWAFSLYDVDGNGTISKNEVLEIVmAIFKMItPEDvKlL
RECO$BOVIN    72 SFDaNsDGTLDFKEYVIALHMTsAGKTNQKLEWAFSLYDVDGNGTISKNEVLEIVtAIFKMIsPEDtKhL
 consensus       SFD-N-DGTLDFkEYvIALHmTsaGKTnqKLEWAFSLyDVDgNGtiSKnEVLEIvtAIFKMI-pEd-k-L

RECO$CHICK   143 eDENsPqKRAdKlWaYFnKgenDKiaEgEFIdGvmkNdaImRLIQyEP        K K
RECO$HUM     143 dDENTPEKRAEKIWkYFGKnDDDKLTEKEFIEGTLANKEILRLIQFEPQKVKE  KmKna
RECO$BOVIN   143 eDENTPEKRAEKIWgfFGKkDDDKLTEKEFIEGTLANKEILRLIQFEPQKVKEklKeKkl
 consensus       eDENtPeKRAeKiW-yFgK-ddDKltEkEFIeGtlaNkeIlRLIQfEPqkvkeklK-K--
```

Figure 3. Alignment of human, bovine, and chick recoverin sequences. The human (RECO$HUM), bovine (RECO$BOVIN), and chick (RECO$CHICK) recoverin (the chick protein originally called visinin) amino acid sequences were aligned by the GENALIGN program (GENALIGN is a copyrighted software product of IntelliGenetics, Inc.; the program was developed by Dr. Hugo Martinez of the Univestiy of California at San Francisco). Bars represent identical residues. A consensus sequence is shown at the bottom with uppercase letters indicating completely conserved residues. The three calcium-binding regions are overlined.

further upstream, or it may be interrupted by another intron. A typical promoter sequence such as TATA could not be found in the 500 bp region upstream of the cDNA start. Putative exons 2 and 3 were 113 and 493 bp long, respectively.

The chromosomal location of the recoverin gene was determined by hybridization of the recoverin coding sequence probe to a panel of DNA from somatic hybrids containing specific human chromosomes. The hybridization pattern indicated the recoverin locus to be on chromosome 17 (Table 1).

DISCUSSION

Our attempt to isolate retina-specific genes by a differential cDNA cloning approach resulted in the isolation of the human recoverin gene. Recoverin is a calcium-binding protein expressed in both the rod and cone photoreceptor cells. It has been shown to modulate the activity of guanylate cyclase depending on the concentration of calcium (11, 26). Recoverin stimulates guanylate cyclase at low calcium concentration which results from the photoactivation-induced hydrolysis of cyclic GMP and closing of the cation channel. The stimulated guanylate cyclase leads to regeneration of cyclic GMP, opening of the channel, and reestablishment of the dark current. A related calcium-

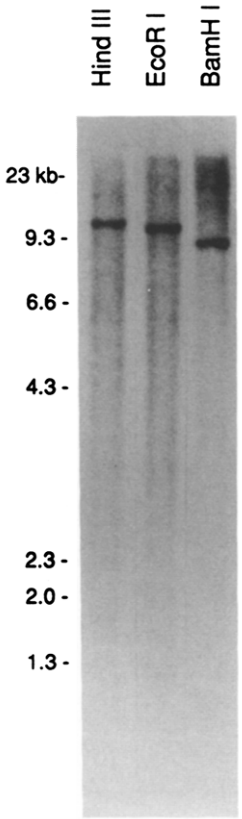

Figure 4. Genomic Southern blot analysis. Ten μg of human genomic DNA was digested with Hind III, Eco RI, or Bam HI, electrophoresed, and blotted. The blot was hybridized with [^{32}P]-labeled human recoverin cDNA (only the coding region was PCR-amplified and used to minimize background hybridization due to repeat sequences). Size markers are Hind III-digested λ DNA.

binding protein, visinin (chicken recoverin), which most likely has a similar function in the chicken retina, has been isolated from the chicken cone photoreceptors (25). Recoverin belongs to a group of more than 150 calcium-modulated proteins containing the EF-hand sequence which was originally described in calmodulin (23).

The human recoverin gene was shown to consist of at least three exons. The transcriptional start site has not been determined yet, so that our putative exon 1

	568		569	
Exon 1	...ATCGTCATG/ gtcagtctc.....	Intron 1	.....ttggtacag /GCTATTTTC...	Exon 2
	680		681	
Exon 2	...ATGATGATG/ gtgaattcc.....	Intron 2	.....aatgcacag /ATAAACTTT...	Exon 3

Figure 5. Exon/Intron borders of the human recoverin gene. The exon/intron junctional sequences of introns 1 and 2 are shown. The number refers to the position of the base in the cDNA sequence.

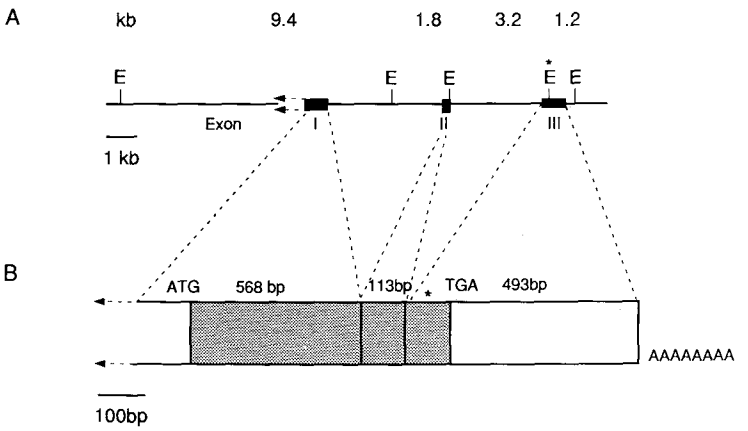

Figure 6. Human recoverin gene and cDNA. A, Recoverin gene. The putative exons I, II, and III are represented as filled boxes. The arrows at the left of exon I indicate that this exon may be longer upstream or interrupted by another intron. Eco RI sites are designated by E (asterisk indicates an RI site within an exon), and the numbers refer to the distances between the sites in kilobases. B, cDNA. ATG is the translational initiation codon, and TGA is the termination codon. Coding region is shaded. The exonic divisions of the sequence are indicated, with the arrows at the left end representing continuation of the exon upstream. The sizes of the exons are shown in basepairs.

Table 1. Chromosomal Mapping of the Human Recoverin Gene

Cell Line	Recoverin	Chromosome 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y
867	-	+	-	-	-	+	-	-	-	-	-	-	-	+	+	-	-	-	+	+	-	-	-	-	-
854	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
423	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
860	-	-	-	+	-	+	+	-	-	-	+	-	-	-	-	-	-	-	-	+	-	+	-	-	-
803	-	-	-	-	+	+	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	+	+	-
909	-	-	-	-	-	D	+	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-
1006	-	-	-	-	+	+	-	+	+	-	-	-	-	+	-	+	-	-	-	+	-	+	-	-	-
811	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-
967	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
734	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
968	-	-	-	-	-	+	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-
683	-	-	-	-	-	+	-	-	-	-	-	+	+	-	+	-	-	-	-	+	-	+	+	-	-
507	-	-	-	+	-	+	-	-	-	-	-	-	+	-	+	-	-	-	-	-	+	-	+	-	+
750	-	-	-	-	-	D	-	-	-	-	-	-	-	+	+	+	-	-	-	+	-	-	-	-	-
1099	-	+	-	-	-	D	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	+	+	-	-
324	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
940	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
983	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Discordancy (%)		22	11	22	17	89	17	11	22	17	17	17	17	33	33	17	11	0	17	39	17	28	28	22	11

For each hybrid cell line the presence (+) or absence (-) of each of the human chromosomes in the hybrid is indicated in the columns under Chromosome. D = Deletion at p15.1-15.2. In the column labeled Recoverin the presence (+) or absence (-) of human recoverin-hybridizing band(s) in each cell line is indicated. The hybridization pattern and the human chromosome contents of the cell lines are used to calculate the %discordancy for each chromosome.

could simply be longer by approximately 100-200 bp at the 5' end or interrupted by another intron upstream. The entire coding sequence, however, appeared to be contained in the three exons described. It is interesting that all three calcium-binding EF-hand regions are in one exon, exon 1. In view of the occurrence of these sequences in many calcium-binding proteins, the exon 1 sequence may represent a calcium-binding "unit" that could have been derived from a common precursor sequence after gene duplication (23). It is notable that in other EF-hand-containing proteins such as the Spec proteins of sea urchin, rat and Drosophila calmodulin, and human calpain, the gene sequences that contain the EF-hand domains are separated by introns (27, 28). The EF-hand domains of the recoverin gene appear to have evolved by duplication without "intron insertion" suggested for many other genes containing the EF-hand domains (28).

Bovine and chicken recoverin protein and cDNA have been described (11, 24, 25). The human recoverin cDNA and gene should be useful in studying the function and expression of this protein in human photoreceptors. The clones should also be useful in investigating the possible relationship of recoverin to human retinopathies since a defect in a critical component of phototransduction, such as recoverin, is likely to create problems in vision. In this regard, it is interesting that antibody against recoverin has been identified in patients with cancer-associated retinopathy (29). The retinal degeneration observed in these patients may be, in fact, the result of recoverin dysfunction, although that the antibody may be secondary to the retinal degeneration needs to be ruled out. The mapping of the recoverin gene to chromosome 17 should also aid in considering it as a candidate gene for ocular diseases that map to the same chromosome.

Finally, the isolation of the recoverin clones appears to validate our approach of isolating important retina-specific genes. Similar approaches using subtractive cloning have been reported. One approach has resulted in isolation of a retinal gene containing a basic motif/leucine zipper domain in the encoded protein (30, 31) and another in the photoreceptor protein, peripherin, which is mutated in the retinal degeneration slow (rds) mouse (32). Our approach should continue to yield retina-specific genes that may be useful in understanding visual processes and ocular diseases.

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