

Gene Expression Profile of Native Human Retinal Pigment Epithelium

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PURPOSE. To generate a profile of genes expressed in the native human retinal pigment epithelium and identify candidate genes for retinal and macular diseases.

METHODS. Two cDNA libraries (one amplified, the other unamplified) were constructed using RNA isolated from native human RPE sheets. The sequence from the 5' end was obtained for randomly selected clones from the two libraries. Of these, more than 2000 expressed sequence tags (ESTs) were analyzed for similarity to sequences and gene clusters in public databases.

RESULTS. EST analysis revealed several known RPE-expressed genes and more than 500 genes that have been characterized previously but were not known to be expressed in the RPE. Transthyretin and 90-kDa heat shock protein represent the most abundant transcripts identified in these RPE libraries. More than 200 novel ESTs and putative proteins were identified. An additional 344 sequences matched only the human genomic sequence.

CONCLUSIONS. High-complexity cDNA libraries were generated from native human RPE. Analysis of ESTs generated from these libraries has yielded a profile of genes expressed in the native RPE. Several of the identified genes are known to play a significant role in the RPE. Novel ESTs, putative proteins, and genomic hits may represent as yet unidentified RPE-expressed genes and many of these, mapping in the region of retinal disease loci, may serve as candidate genes. In addition, the nonredundant set of more than 1100 genes and ESTs described herein will be a valuable resource for generating gene microarrays, which can assist in delineating RPE expression profiles during human disease pathogenesis. (*Invest Ophthalmol Vis Sci.* 2002;43:603–607)

Phototransduction and the integrity of photoreceptors depend on several highly specialized functions of the adjacent retinal pigment epithelium (RPE). These include storage, transport, and conversion of retinol, phagocytosis of photore-

ceptor outer segments; synthesis of the interphotoreceptor extracellular matrix; selective transport of ions, nutrients, and metabolites to and from the neural retina; and maintenance of the blood-retinal barrier.^{1,2} Given the critical role of the RPE, mutations of genes expressed in this epithelium or alterations in their expression secondary to environmental changes are likely to contribute to disorders of the retina. Indeed, there is strong evidence that the RPE is involved in the etiology of a number of retinal dystrophies, including Stargardt disease, Best disease, Leber congenital amaurosis, and age-related macular degeneration.^{3–9} In a few instances, the defective gene has been identified and shown to be preferentially expressed in the RPE (e.g., retinaldehyde binding protein, bestrophin, and *RPE65*). In general, however, the molecular components and biochemical pathways underlying RPE function and pathophysiology have been only partially characterized.

Expressed sequence tag (EST) analysis, together with the sequencing of human genome, has been a powerful tool for discovering novel and uniquely expressed genes.^{10–12} The collection of ESTs from a specific tissue or cell type provides an expression profile including information about the level and complexity of gene expression in that tissue or cell type.^{13–16} Several laboratories have published ESTs originating from eye tissues and cell lines.^{17–23} ESTs from RPE have been reported earlier,^{24–27} but these were derived from RPE cells in culture or RPE-choroid (available at neibank.nei.nih.gov), and therefore, are not necessarily representative of the native human RPE. In addition, the reported ESTs are not appropriately annotated, making their usage in microarray studies more difficult.

With the goal of generating a comprehensive profile of genes expressed in native human RPE, creating a nonredundant set of genes for custom RPE gene microarrays, and enhancing the pool of candidate genes for retinal and macular diseases, we constructed high-complexity cDNA libraries from freshly isolated human RPE sheets. Our analysis of random ESTs from these libraries provides a nonredundant set of more than 1100 genes. In addition to cDNAs for known RPE-expressed genes, we report a large compilation of ESTs for genes encoding proteins of yet unknown physiological functions.

MATERIALS AND METHODS

Human RPE Tissue and RNA Preparation

One pair of human eyes from a 53-year-old male donor (library 1) and one pair from a 12-year-old female donor (library 2) were obtained from the Michigan Eye Bank within several hours of death. The donor eyes were acquired for research purposes with family consent and processed in compliance with University of Michigan regulations. After dissecting the anterior segment and removing the vitreous and neural retina, the resultant eye cups were filled with 1% Dispase II (Roche Molecular Biochemicals, Indianapolis, IN) in HEPES-buffered Ringer (5 mM KCl, 135 mM NaCl, 10 mM Glucose, 1.8 mM CaCl₂, 1 mM MgCl₂, and 10 mM HEPES [pH 7.4]) and incubated for 2 hours at 37°C. RPE sheets were then dislodged from Bruch's membrane by directing a gentle stream of Ringer with a Pasteur pipette, collected, and washed several times. Poly(A)⁺ RNA was obtained by extracting total RNA

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from dissociated RPE sheets with extraction reagent (TRIzol; Life Technologies, Rockville, MD) and then passing it through an oligo-dT column (Amersham Pharmacia Biotech, Piscataway, NJ).

cDNA Library Construction

Human RPE poly(A)⁺ RNA (40 ng) was used for generating the first cDNA library, with a kit (SMART cDNA Library Construction Kit; Clontech, Palo Alto, CA), according to the manufacturer's instructions. Two microliters of cDNA from the first-strand reactions was used for a template, together with primers and reagents from the kit, in 24 cycles of amplification, using primers and reagents from the kit. The double-stranded cDNA was ligated to λ TriplEx2 vector arms (Clontech) and an aliquot of the ligation mixture was packaged (Gigapack III Gold kit; Stratagene, La Jolla, CA) to generate the cDNA library. Plating and titration of λ TriplEx2 recombinants were performed on *Escherichia coli* strain XL1-Blue MRF', as suggested by the manufacturer. Phagemids were obtained through mass in vivo excision from the phage library using *E. coli* strain BM25.8.

A second unamplified cDNA library was generated in the pSPORT1 vector (SuperScript Plasmid System; Life Technologies) using total RNA. Briefly, first-strand cDNA was synthesized with a *NotI*-oligo(dT) primer-adaptor. After second-strand synthesis and ligation of *Sall* adaptor, cDNA was digested with *NotI*, generating cDNAs with *Sall* sites at the 5' end and *NotI* sites at the 3' end. cDNAs were size fractionated, and those of 0.5 to 2 kb were ligated to pSPORT1. The ligated sample was used to transform *E. coli* (ElectroMax DH-5 α ; Invitrogen, San Diego, CA) by electroporation.

Sequencing

Double-stranded phagemid DNA templates were prepared for sequencing from randomly selected clones (CONCERT Rapid Plasmid Miniprep System; Life Technologies). The recombinant clones from library 1 were sequenced (Cy5/Cy5.5 Dye Primer Cycle Sequencing Kit; Visible Genetics, Toronto, Canada) with 5'TriplEx-labeled primer, and the products were analyzed by an automated system (OpenGene; Visible Genetics). For the clones from library 2, DNA sequencing was performed with a high-throughput automated sequencer (Applied Biosystems, Inc. Foster City, CA). BLAST search was performed against GenBank and dbEST for each sequence (BLAST, GenBank, and dbEST are provided in the public domain by the National Center for Biotechnology Information [NCBI], Bethesda, MD, and are available at <http://www.ncbi.nlm.nih.gov/BLAST/>, [/genbank/](http://www.ncbi.nlm.nih.gov/GenBank/), and [/dbEST/](http://www.ncbi.nlm.nih.gov/dbEST/), respectively).

RESULTS

Characterization of the Human RPE cDNA Libraries

Library 1 contains 2.3×10^6 independent plaque-forming units. Of these, more than 90% are recombinant clones, as determined by a lacZ α -complementation system. Screening of cDNAs by PCR amplification (using λ TriplEx2 LD-Insert Screening Amplimers; Clontech) revealed that more than 90% of the clones contain inserts in the range of 400 to 1100 bp.

Library 2 clones are 60% recombinant by restriction enzyme analysis, with inserts in the size range of 500 to 2000 bp.

Sequence Analysis and EST Identification

Typically, 200 to 400 bp of unambiguous sequence data were obtained from 487 independent cDNA clones from the amplified RPE library 1. A summary of the comparison of these sequences to those in NCBI databases is provided in Table 1. Sequence comparison analysis resulted in identification of 322 known genes. Among these, transthyretin (26 clones) and 90-kDa heat shock protein (HSP90; 13 clones) appear to represent abundant transcripts in the RPE. Several known RPE-expressed genes were also identified, including retinol-binding

TABLE 1. Results of Sequence Comparison Analysis

Library 1 (Amplified)	
Total number of analyzed sequences	487
ESTs matching known genes in database	322 (220 nonredundant)
ESTs corresponding to putative proteins	82
Novel ESTs (no match to database)	29
Mitochondrial DNA	12
Ribosomal RNA	2
Repetitive DNA	17
Vector alone or short sequences	23
Library 2 (Unamplified)	
Total number of analyzed sequences	1614
ESTs matching known genes in database	477
ESTs corresponding to putative proteins	111
Number matching genomic sequence	344
Number without significant matches to database	110
Mitochondrial DNA	177
Ribosomal RNA	117
Repetitive DNA	215
Vector alone or short sequences	63

protein (five clones), *RPE65* (two clones), pigment epithelium-derived factor (PEDF; four clones), and cystatin C (three clones). In addition, several genes known to be functionally important in the RPE, such as cellular retinaldehyde binding protein (*CRALBP*), and *TIMP3*, were detected. Of the remaining 111 clones, 82 represented ESTs identified in other cDNA libraries, including brain and retina or matched human genomic sequences. Twenty-nine ESTs did not show any significant sequence homology with current database entries or to each other. These sequences may represent novel genes expressed in the RPE. Sequences from all clones have been deposited to GenBank (accession numbers BG108623-BG108956).

An average of 400 bp of cDNA sequence was obtained from the 5' end of 1614 clones isolated from the unamplified library (library 2). The results of sequence comparison analysis with the NCBI databases are summarized in Table 1. In this library, 477 known genes were identified and classified based on their (putative) function and cellular location (Fig. 1). Transthyretin (15 clones) and *HSP90* (4 clones) were again highly represented. In addition, prostaglandin D₂ synthase (9 clones), *PEDF* (7 clones), target of *myb1* (chicken) homolog *TOM1* (13 clones), and tyrosine-related protein *TYRPI* (8 clones) were frequently identified. Other known RPE-expressed genes include *RPE65*, *CRALBP*, cystatin C, arrestin 3, bestrophin, PH domain containing protein in retina 1 (*PHRETI*), retinoic acid receptor responder-1 and -2, RPE-retinal G-protein-coupled receptor (*RGR*), inwardly rectifying K⁺ channel Kir7.1, and monocarboxylate transporter 3 (*MCT3*). A total of 344 clones matched only human genomic sequences, and several of these corresponded to genomic intervals to which retinal disorders have been mapped (Table 2). In addition, 111 clones were identical with ESTs identified in other cDNA libraries and 110 clones did not have a significant match to sequences in the database. All sequences have been deposited to GenBank (accession numbers BI480618-BI481531).

A complete list of all clones from the two RPE libraries is available on our web site (www.umich.edu/~retina/RPE.html), which includes an alphabetical list of the known genes along with their accession numbers, chromosomal locations, and the number of times they were detected in the unamplified library (quantitative expression level).

DISCUSSION

We first constructed a high-complexity oligo-dT primed cDNA library of 2.3 million independent clones from the native hu-

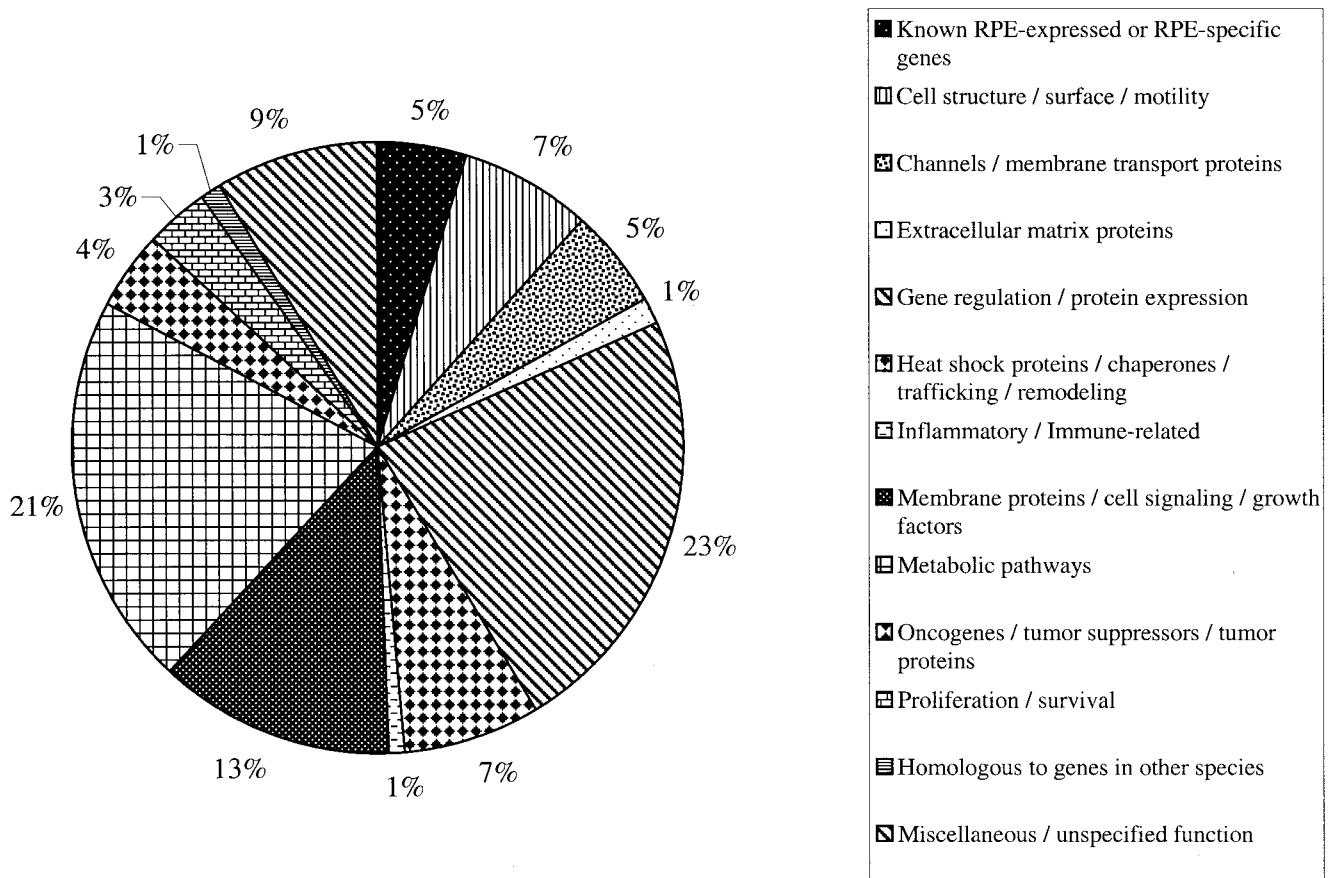


FIGURE 1. The known human genes identified among human RPE ESTs are grouped according to (putative) function or cellular localization.

man RPE. Despite 24 cycles of amplification for generating double-stranded cDNAs, we did not observe much redundancy in ESTs. Only eight clones were detected three or more times. Because PCR amplification may result in the loss of representation of moderately expressed sequences, a second unamplified library was generated. Altogether, a nonredundant set of more than 1100 genes was produced, pro-

viding an overview of the gene expression profile of native human RPE. Even though a large number of clones were analyzed (more than 2000), the level of redundancy (repetitious hits) was still relatively low. This suggests that sequencing of additional ESTs (especially the unamplified library 2) may be needed to develop a more comprehensive RPE gene profile.

TABLE 2. RPE cDNAs that Map in the Chromosomal Regions of Retinal Disease Loci

Clone ID	Accession Number	Chromosomal Location	Disease
H2RPE-1261	Z99943	1q24	Recessive cone-rod dystrophy
H2RPE-1237	AL022171	1q24	Recessive cone-rod dystrophy
H2RPE-1120	AC008122	2q31-33	Recessive RP
H2RPE-1217	AL035467	6q12-13	Recessive Leber congenital amaurosis
H2RPE-0089	AL121966	6q15-16.1	Dominant MD, recessive Leber congenital amaurosis
H2RPE-2232	AL050337	6q24.1-25.2	Dominant retinal cone dystrophy 1
H2RPE-0083	AL033392	6q24.1-25.2	Dominant retinal cone dystrophy 1
H2RPE-1923	AC007444	7p14-15	Dominant RP, dominant MD
H2RPE-0650	AC003074	7p15	Dominant RP, dominant MD
H2RPE-1707	AC004130	7p15-p21	Dominant MD
H2RPE-1658	AC004940	7p21	Dominant MD
H2RPE-2014	AL109624	11p13	Dominant familial exudative vitreoretinopathy
H2RPE-2115	AL049629	11p13	Dominant familial exudative vitreoretinopathy
H2RPE-1718	AC000353	11q13	Recessive Bardet-Biedl syndrome, dominant neovascular inflammatory vitreoretinopathy
H2RPE-0496	AC004106	Xp22	X-linked RP
H2RPE-0519	AC002477	Xq25-q26	X-linked RP
H2RPE-2022	AL080272	Xq26.3-28	X-linked RP, X-linked progressive cone dystrophy 2

The location of disease loci is based on RetNet (<http://www.sph.uth.tmc.edu/Retnet/>). RP, retinitis pigmentosa; MD, macular degeneration.

The identification of RPE- and retina-expressed genes will enhance the success and the pace of studies attempting to identify the genes responsible for many retinal and macular disorders. Herein, we report the isolation of a large number of ESTs from native RPE that match anonymous sequences or clusters identified from other cDNA libraries or the human genomic sequence. Several of these map to the chromosomal regions of previously localized disease loci (Table 2) and thus qualify as candidate disease genes. In addition, we identified numerous novel ESTs, some of which may represent RPE-specific transcripts. Their identification should provide valuable insight into the molecules necessary for normal RPE function.

We identified more than 500 genes that had previously been characterized. Some of these are known RPE-specific or RPE-expressed genes and include genes involved in visual pigment transport and metabolism (*RPE65*, *CRALBP*, retinol-binding protein); growth and development (*PEDF* and cystatin C); and transport (monocarboxylate transporter-1 [*MCT1*] and -3 [*MCT3*],²⁸ GABA transporter, α I and β I subunits of Na,K-ATPase, and Kir7.1²⁹). In the unamplified library, a substantial fraction of the known genes represents genes involved in metabolism (~21%) and in gene regulation and protein expression (~22%). Approximately 13% of the reported genes are involved in cell-signaling pathways or are growth factors and membrane proteins; 7% are involved in cell structure, cell surface, and motility; 7% in protein trafficking and remodeling; and 5% in membrane transport (Fig. 1). This is consistent with the role of RPE in synthesis of various enzymes, growth factors, and pigments, transport of nutrients and ions, and maintenance of the interphotoreceptor matrix.

Although cDNA libraries have previously been constructed from cultured fetal RPE cells²⁶ and from RPE cell lines,^{24,25} this is the first report of a library derived from native human RPE sheets. Based on previous studies,³⁰⁻³³ the gene expression profile of native RPE might be expected to be different from that of cultured RPE cells. Indeed, the analysis of ESTs indicates that many RPE-specific genes are not represented in two cDNA libraries derived from cultured RPE.^{24,26}

It is estimated that 35,000 to 120,000 genes are encoded by the human genome,^{34,35} and only a fraction of these (10%–20%) may be expressed in any single differentiated cell type. As the first step toward defining the expression profile of native human RPE, we describe at least 1100 genes expressed in the RPE. We compared the gene profile of native RPE to that of the ciliary epithelium.³⁶ These two epithelia share a common embryonic origin and therefore similarities in gene expression might be expected. Of the 220 nonredundant clones from library 1 that match genes in databases, 12% were similar to those in the subtracted library from ciliary body. These include genes for extracellular proteins, antioxidants, apoptotic as well as stress proteins, and transcription and translation factors. Specifically, PEDF, 90-kDa heat shock protein, prostaglandin D₂ synthase, calmodulin, inositol-1,4,5-triphosphate receptor, and cathepsin D are present in both libraries. In contrast, a comparison of the native RPE gene profile with that of the corneal epithelium¹⁸ shows little similarity. Although apolipoprotein J, a frequently detected gene in the corneal epithelium library is detected in the second RPE library, overall a different set of receptors, transporters, and secretory and cytoskeletal proteins are present in the two epithelia. Not surprisingly, the native RPE gene profile is also distinct from that of the neural retina.^{19,23,37} These findings suggest that the highly specialized functions of the RPE required for the maintenance of photoreceptor integrity are the result of a unique gene expression pattern. The similarity between the RPE library and libraries from the retina and the corneal epithelium is limited primarily to the presence of housekeeping genes

including ribosomal proteins and those involved in metabolism and energy production.

The comparison of RPE profile with genes expressed in nonocular tissues, such as cochlear¹⁵ and hematopoietic tissues,¹² reveals that only constitutively expressed genes, such as those involved in protein synthesis and metabolism, are shared between these tissues. However, many differences among the libraries are notable: For example, different types of collagen are among the most frequently identified genes in the cochlea, whereas no collagen form has been observed among the RPE clones thus far. Furthermore, approximately 13% of the known genes in the unamplified RPE library appear to be in the cell-signaling and growth factor category, whereas only approximately 5% of the genes from the cochlear library belong to this category.

The identification of RPE-expressed genes will be beneficial to future studies of gene expression profiles in RPE and generation of DNA microarrays that are fast becoming the technology of choice for identifying global changes in gene expression patterns during development and disease.³⁸⁻⁴⁰ Gene arrays of specific tissues or cell type of interest should greatly facilitate such investigations because of greater focus, relatively low expense, and better methods for data analysis. Mouse gene microarrays that contain more than 3000 ESTs from mouse eye-retina cDNA libraries have already been generated.⁴¹ The nonredundant set of more than 1100 human RPE cDNAs described herein provides a starting point for producing human gene microarrays that include RPE-expressed genes. Investigations using such microarrays will lead to better understanding of the genes and pathways involved in the maintenance of photoreceptor function and will provide candidate genes for retinal and macular diseases.

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