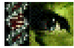
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EST analysis of mouse retina and RPE/choroid cDNA libraries

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Purpose: cDNA libraries from the mouse retina have recently been reported, but no well characterized library from the retinal pigment epithelium (RPE) or choroid of the mouse has yet appeared in the literature. To complement these libraries and to provide the first mouse RPE/choroid library, we used freshly dissected tissue from adult C57BL/6J mice to construct new retina and RPE/choroid libraries.

Methods: Eyes from 100 six to eight week old C57BL/6J mice were dissected in groups of 10. The whole retina and RPE/choroid were isolated individually and then homogenized before RNA isolation. Over 5000 clones each were sequenced from the unamplified and un-normalized retina and RPE/choroid libraries. All sequences were analyzed using GRIST (GRouping and Identification of Sequence Tags), a bioinformatics program for gene identification and clustering.

Results: The RPE/choroid library contained 3145 clusters with 76% of the clusters representing single clones. Nearly 87% of the clusters corresponded to named genes in GenBank, and 8% of the RPE clusters remain unidentified. The retina library contained 3190 clusters of which 78% represented only one clone. Approximately 85% of the clusters matched sequences in GenBank, and 9% of the clusters remain unidentified. The clones most abundant in each library were all well-known sequences and both libraries contained a number of tissue specific or tissue-enhanced genes.

Conclusions: These new libraries should provide a valuable resource for gene discovery and cDNAs for expression analysis and functional studies.

The mouse serves as an excellent model to further define genes important to the health of the posterior pole of the eye. Invaluable information about the molecular and pathological basis of several retinal diseases has come from elucidating the gene mutations in mouse retinal degeneration models [1-5]. Sixteen mutations causing retinal degeneration in the mouse have been identified, and several studies have appeared on retinal degeneration associated with age and environmental factors such as light and oxidative stress [1,6-11]. Other advantages to using the mouse include the ability to isolate fresh RNA preparations, the ability to control environmental factors, and the ability to experimentally manipulate the genome using techniques such as transgenics and the construction of knock-out or knock-in lines.

cDNA libraries from mouse retina and retinal pigment epithelium (RPE) should provide valuable tools for identifying new disease loci and studying gene expression during disease development. Most notably, cDNA libraries of high quality from the developing mouse eye and adult retina have recently been developed using freshly dissected tissue [12,13]. To complement these libraries, and to provide the first mouse RPE/choroid specific library, we undertook the construction of new retina and RPE/choroid libraries using freshly dissected tissue from adult C57BL/6J mice. Expressed sequence tag (EST) analysis of over 5000 clones from each library identified a large dataset of non-redundant gene clusters. The data provided good estimates for the most highly expressed sequences in each tissue and revealed potentially novel or previously unidentified ESTs.

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METHODS

RNA isolation from RPE/choroid and retina: Approximately 200 eyes from six to eight week old male C57BL/6J mice were dissected in groups of 10. Animal care guidelines comparable to those published by the US Public Health Service (Public Health Service Policy on Humane Care and Use of Laboratory Animals) were followed. Globes were removed and placed immediately into a petri dish kept on ice and containing 0.5 M EDTA/PBS (without calcium chloride and magnesium chloride, pH 7.4). Eyes were dissected in an RNase free environment using a stereo zoom microscope (Nikon SMZ800, Tokyo, Japan). The optic nerve head was cut and the anterior segments were removed with a circumferential incision of the eye. The whole retina was peeled away, and then the pigmented layer including RPE/choroid was peeled gently from the sclera as a sheet in PBS/EDTA (Figure 1). The RPE/choroid was processed in batches of 5 eyes and the retina in batches of 2 to 4 eyes. Each batch was placed in a tube containing 600 μl RLT lysis buffer (QIAgen, Valencia, CA). Before RNA isolation, tissues were first homogenized with a 22 gauge needle and a QIAshredder spin column. Total RNA was isolated using the QIAgen RNeasy kit (QIAgen) following the manufacturer's protocol. The purity and yield

of RNA were determined by measuring the optical density at 260 and 280 nm. The ratio of OD_{260} to OD_{280} measured consistently between 1.8 and 2.0. The typical yield of total RNA per mouse was approximately 1 µg from RPE/choroid and 5-10 µg from retina (both eyes). Total RNA preparations were pooled into groups of 10, and their integrity was determined by SYBR Gold stained agarose gels (Molecular Probes, Inc., Eugene, OR). RNA was visualized by scanning the gels in a phosphorimaging instrument (Storm 860, Molecular Dynamics, Sunnyvale, CA; image not shown).

Preparation of RPE/choroid/sclera for histology: In order to visually verify the specificity of dissection, a representative eye was prepared for examination by light microscopy. Just as the RPE/choroid sheet was being peeled away, the eyecup was placed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer. The whole tissue was postfixed with 1% osmium tetroxide/cacodylate and dehydrated through a series of graded alcohols. The tissue was embedded in poly/Bed 812 resin (Polysciences, Inc., Warrington, PA) and serial sections were cut 1 µm in thickness. Sections were stained with 2% toluidine-blue, and examined by light microscopy.

cDNA library construction: Complete details of library construction are described elsewhere [14]. Briefly, poly(A) RNA, isolated by oligo(dT) cellulose column chromatography, was used for the synthesis of cDNA. A *Not I* primer-adaptor [GAC TAG TTC TAG ATC GCG AGC GGC CGC $CC(T)_{15}$] and SuperScript II reverse transcriptase (Invitrogen Corp., Carlsbad, CA) were used for first strand synthesis. After second strand synthesis with *E. coli* DNA polymerase, cDNA fragments > 500 bp were fractionated on a Sephacryl S-500 column. Libraries were made from the first two 35 µl fractions containing cDNA. *Sal I* linkers were ligated onto the blunt ends of the cDNA. The *Not I*/*Sal I* fragments were directionally cloned into the *Not I*/*Sal I* sites of the pSPORT1 vector (Invitrogen). Plasmids were transferred by electroporation into *E. coli* DH10B cells.

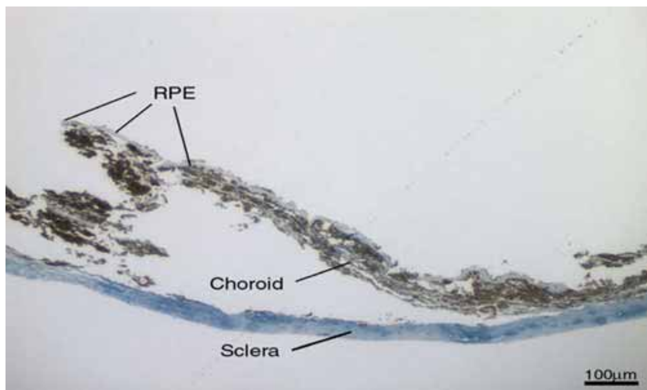

Figure 1. Isolation of the RPE/choroid from the eyecup. The neural retina was removed from the mouse eyecup and the RPE/choroid was scraped as a sheet from the scleral wall. The photograph shows the RPE/choroid layer as it is peeled away from the sclera as a sheet.

cDNA sequencing and bioinformatics: High throughput sequencing was performed on more than 5500 individual RPE and more than 5600 individual retina clones at the NIH Intramural Sequencing Center (NISC). Data were analyzed as described elsewhere [14]. EST sequences were identified and clustered using GRIST (GRouping and Identification of Sequence Tags), a bioinformatics program that uses sequence match parameters derived from BLAST program [15].

Statistical estimation of library depth: Our analysis of library depth makes use of the well-known problem of estimating numbers of species given a fixed area in which sampling is performed. Results from the estimator/model ACE (Abundance-based Coverage Estimator) [16] are reported. The total number of samples is expressed as the sum of a sequence of observed numbers. Let N_1 represent the number of species where only one individual was observed, N_2 equals the number of species where 2 individuals were observed, up to N_n equal to the number of species for which n individuals was observed. With this formulation, N_0, the number of species that exist for which no individual has yet been found, can be estimated by fitting expressions to the curve for species up to N_{10}. The ACE approach uses the frequency of observed rare species to estimate the number of missing species (N_0).
This problem statement is analogous to the problem of sampling a large library of ESTs to find the number of unique sequences that exist in the library. If we let a specie be a unique cluster in the library and the number of individuals in that specie be equal to the number of clones in the resulting cluster, then a series expression can also be written for the abundance of unique clusters in the library. As Chao et al. have shown [17], very good estimates of N_0 can be made by using the data for $N_1 ... N_{10}$, and the SPADE (Species Prediction And Diversity Estimation) algorithm [18] provides a simple means for calculating the estimated value of N_0 with 95% confidence limits.

Table 1. Distribution of clusters containing 10 or fewer clones

Number of clones	RPE/choroid	Retina
1	2402	2497
2	460	412
3	126	130
4	55	58
5	31	30
6	14	14
7	10	8
8	5	7
9	4	5
10	3	5

The number of clusters from the RPE/choroid and retina libraries listed by the number of clones in the cluster.

RESULTS

Library statistics: During synthesis of the RPE/choroid and retina libraries, cDNA was fractionated into two size ranges and subcloned into libraries designated *mi* and *mj* for the RPE/choroid and *mk* and *ml* for the retina. The *mi* library contained 16x10^6 primary recombinants with an average insert size of 1.8 kbp. Out of over 2870 quality sequence reads with an average size of 573 bp, less than 2% contained no inserts, 2.3% contained mitochondrial genome sequences, and 1% contained rRNA. For *mj*, there were 10.2x10^6 primary recombinants with an average insert size of 1.5 kbp. From over 2700 reads with an average size of 554 bp, no inserts were found in 2.2% of the sequences, 3.8% contained mitochondrial genome, and less than 1% had rRNA. The *mk* library from the mouse retina contained 16x10^6 primary recombinants with an average insert size of 1.9 kbp. Of more than 2700 quality sequence reads with an average size of 559 bp, only 1.9% contained no insert, 3.6% had mitochondrial sequences, and less than 1% had rRNA. For *ml*, there were 17.6x10^6 primary recombinants with an average insert size of 1.2 kbp. From over 2900 quality reads with an average size of 564 bp, no inserts were found in 1.6% of the sequences, 6.1% contained mitochondrial genome sequence, and less than 1% rRNA. In summary, normally >91% of recombinants contained inserts that appeared to be from the source material, and hence contained valuable clones.

High quality reads from both libraries were analyzed using GRIST [15]. GRIST identified and grouped ESTs into clusters that represent transcripts from the same gene. The analysis yielded 3145 gene clusters in the RPE/choroid library, of which 76% contain single clones, and thus potentially unique genes (Table 1). Three percent of the clusters match sequences from UniGene but not GenBank, 1% match ESTs in dbEST, 1% do not match other DNA sequences but do match possible ORFs, and 8% of the RPE clusters remain unidentified (Table 2). The retina library contains 3190 gene clusters, with 78% of the clusters containing single clones (Table 1). Approximately 9% of the retina clusters remain unidentified, 4% match sequences from UniGene but not GenBank, 1% match ESTs from dbEST, and 1% do not match other DNA sequences, but do match possible ORFs (Table 2).

Both libraries show that only 9% of the clusters appear more than twice (Table 1). The estimator/model ACE was used to estimate the number of missing clusters (species) for both libraries [16]. The ACE approach uses the frequency of observed rare species to estimate the number of missing species. For the combined RPE/choroid libraries, the number of missing clusters was calculated to be 7745. For the combined retina libraries, it was 9176. Thus, the total number of clusters in the combined RPE/choroid libraries is estimated to be 10,896 with a 95% confidence interval of (10400 to 11500). In the combined retina libraries, the total number is estimated to be 12,346 with a 95% confidence interval of (11700 to 13000).

Tissue specific clones: The mouse eyes were dissected such that the whole retina could first be peeled away from the posterior pole and then the RPE/choroid peeled away from

Table 2. Results of EST sequence analysis for RPE/choroid and retina libraries

Library	RPE/choroid	Retina
Total number of gene clusters	3145	3190
Match to GenBank	2726	2718
UniGene match but no GenBank match	96	131
Match with other EST, but no GenBank or UniGene match	32	36
No match to other DNA sequences, but possible ORF match	37	29
Unidentified	254	276

Library sequences were clustered and identified using GRIST (GRouping and Identification of Sequence Tags). ESTs were matched to sequences from GenBank, UniGene, other EST databases, possible ORFs, or remained unidentified. The numbers of various types of gene clusters in each category are shown for both mouse libraries.

Table 3. Characteristic and abundant clones in the RPE/choroid library

Rank	Description	GenBank	Chromosome	Number of clones	M/H
1	prostaglandin D2 synthase	AB006361	2	78	4
2	RGR opsin	AF076930	14	68	200
3	transthyretin	D89076	18	66	58
4	transferrin	NM_133977	9	35	12
5	apolipoprotein E	M73490	7	33	19
6	CRALBP	NM_020599	7	30	9
7	clusterin	AF248057	14	26	2
8	cystatin C	M59470	2	26	8
9	dopachrome tautomerase	NM_010024	14	25	11
10	tyrosinase-related protein 1	NM_031202	4	24	2
11	elongation factor 1 alpha 1	BC018223	9	23	0.4
12	prosaposin	XM_192668	10	21	2
13	IGFBP5	L12447	1	21	2
14	SPARC/osteonectin	NM_009242	11	21	4
15	retinol dehydrogenase 5	AF033196	10	20	*
16	ferritin light chain 1	NM_010240	7	20	1
17	basigin	D00611	10	19	11
18	Npps2	AF123542	15	18	4
19	Pmel17	NM_021882	10	17	1
20	AIP-6	AF172088	7	16	6
21	cathepsin D	NM_009983	4	15	5
22	RPE65	AF410461	3	15	13
23	alpha-tubulin	M13445	15	15	26
24	cathepsin B	NM_007798	14	13	5
25	ubiquitin C	XM_287520	5	12	2
26	TIMP3	AB041611	10	12	1
27	LRAT	AF255061	3	11	*
28	ubiquitin B	NM_011664	11	11	20
29	solute carrier family 25	XM_134169	8	11	*
30	heat shock protein 1, beta	M36829	17	11	2
31	actin, beta, cytoplasmic	J04181	5	11	1
32	putative phosphatase	NM_008916	11	11	*
33	pyruvate kinase, muscle	D38379	9	11	2
34	rhodopsin	NM_145383	6	11	10
49	oculospanin	NM_145363	11	7	4
52	OTX2	NM_144841	14	7	12
74	peropsin	AF012271	3	5	*
2402	MCT3	AF178956	15	1	*

All ESTs represented by more than 10 clones in the RPE/choroid library are listed. Characteristic RPE markers are also listed and indicated in red in the description column. Representative GenBank entries are given with chromosomal location and the number of clones identified. The M/H column gives the ratio of frequency of occurrence of each gene in the mouse (M) and human (H) libraries. An asterisk ("*") indicates no clones were found in the human library.

the sclera (Figure 1). Our data analysis indicated however that there was some contamination of the RPE library with retina cDNA. For the most part, the most abundant photoreceptor transcripts are present in the RPE library at less than 10% of the level that they are seen in the retina (data not shown). No contamination of the retina by the RPE was evident from the library data. Except for CRALBP, none of the abundant RPE transcripts was seen in the retina library (data not shown).

The most highly represented clones in the RPE/choroid and retina libraries are listed in Table 3 and Table 4, respectively. These lists are derived from the contents of NEIBank in August 2003. A complete list of the Mouse Retina and Mouse RPE/Choroid libraries are available at the NEIBank web site. Table 3 and Table 4 also list characteristic RPE and retina markers found in our mouse libraries. These tissue specific markers are represented by both abundant and non-abundant cDNAs.

Mouse/Human Comparison: While cDNA libraries cannot be completely faithful representations of the normal abundance of transcripts, they can give some indications to the level of expression of abundant transcripts. Previously published NEIBank libraries for human lens and retina show that the expected tissue specific and tissue preferred genes are indeed well represented by cDNA. Using the NCBI Homologene database, we identified probable homologous genes represented in the mouse retina and RPE/choroid libraries and in the equivalent NEIBank human libraries made in a similar fashion without amplification. We then compared the frequency of abundant genes in one species with the other.

In Table 3 and Table 4, the M/H column shows the ratio of frequency of occurrence of each gene in mouse and human

Table 4. Characteristic and abundant clones in the retina library

Rank	Description	GenBank	Chromosome	Number of clones	M/H
1	rhodopsin	NM_145383	6	157	1
2	alpha transducin	NM_008140	9	112	4
3	peripherin 2	NM_008938	17	41	10
4	phosducin	L08075	1	38	37
5	GAPDH	XM_124295	6	28	0.5
6	elongation factor 1 alpha 1	AK050635	9	27	0.5
7	S-antigen	M24086	1	24	0.7
8	enolase 1	NM_023119	4	22	2
9	tubulin, alpha	XM_128256	15	21	2
10	aldolase 1	NM_007438	7	21	1
11	phosphodiesterase 6B	X60133	5	20	20
12	ubiquitin B	NM_011664	11	19	9
13	interstitial retinol binding protein	NM_015745	14	16	2
14	ROM1	NM_009073	19	16	0.7
15	pyruvate kinase, muscle	NM_011099	9	16	0.7
16	guanylate cyclase activator 1B	NM_146079	17	15	5
17	GCAP2	NM_008142	4	14	2
18	phosphoglycerate mutase 1	XM_124890	19	13	1
19	ornithine decarboxylase antizyme	NM_008753	10	11	2
20	RIKEN cDNA 2900052E22 gene	BC028989	7	11	4
21	vitronectin	NM_011707	11	11	*
22	heat shock protein 8	U27129	9	11	2
23	phosphodiesterase 6G	AF190928	11	10	0.2
26	unc119 homolog	AF030169	11	10	0.6
42	neural retina leucine zipper gene	L14935	14	8	0.4
77	phosphodiesterase 6A	NM_146086	18	5	1
102	retinoschisis 1 (X-linked, juvenile)	AF073780	X	4	2
171	recoverin	NM_009038	11	3	10
185	GCAP	L36860	17	3	0.3
551	IPM 150 proteoglycan	AF266478	9	2	0.5
1930	vax2	AF028715	6	1	1

ESTs with more than 10 clones in the retina library are listed. Characteristic retina markers are also listed and indicated in red in the description column. Representative GenBank entries with chromosomal location and the number of clones are shown. The ratio of frequency of occurrence of each gene in the mouse (M) and human (H) libraries is given in the M/H column. An asterisk ("*") indicates no clones were found in the human library.

libraries. In retina, the majority of abundant and tissue specific transcripts in mouse have similar abundance in human (M/H between 0.5 and 2). RPE/choroid, in contrast shows a much greater level of discordance between the two species. For example, retinal G protein coupled receptor (RGR) opsin is extremely abundant in mouse and is found at lower levels in human. Some gene transcripts that are highly abundant or moderately abundant in the mouse RPE/choroid library, such as retinol dehydrogenase 5 and LRAT, are not represented in the (larger) human collection. On the other hand, genes such as glutathione peroxidase 3, opticin, and bestrophin that are abundant in the human library are absent from the mouse collection. Indeed, none of the ESTs for the mouse homolog of bestrophin in dbEST and Unigene are from eye or head libraries, most come from testis [19].

DISCUSSION

This set of un-normalized libraries for the retina and RPE/choroid of the C57BL/6J mouse represents part of the ongoing efforts of the NEIBank program. Previous to this work, we could only find a single well characterized cDNA library for the adult C57BL/6 retina. In the works published by Farjo et al. and Yu et al., roughly the same percentage of unique sequences were found, and the library also appears to have the same extent of contamination by mitochondrial and ribosomal sequences [12,13]. It should be noted that the publicly available sequence data for the mouse was generated from the C57BL/6 mouse. The overall profile of the distribution of numbers of sequences found in each cluster is similar for our retina and RPE/choroid libraries and the vast majority (>75%) of sequences were found only once.

We used an approach that is well known in ecological studies for estimating the total number of species in a discrete area that is sampled in a random fashion. The correlate to our approach is to consider a cluster a species, and the number of clones found to be like the number of individuals counted in the sample. Using this approach, we were able to estimate that our set of observed sequences is likely to represent approximately 28% of the total numbers of clusters which could be found in both libraries given very much larger numbers of sequence reads. The numerical approach to this method of estimation utilizes data only from the number of clusters (species) having from 1 to 10 individuals counted. The development of this method is reviewed in [16]. It is interesting to note that a modeling approach has been used to estimate coverage for genomics clones in a library [20].

The clones most abundant in each library were all known sequences and both libraries contained a number of tissue specific or tissue-enhanced genes. There is good agreement between our list and the list of Yu et al. of the most highly expressed genes in the adult mouse retina [13]. Six of 12 genes in the list of Yu et al. are in our list of the top 10 clones found in our retina library. The mostly highly expressed sequence in the RPE/choroid library was prostaglandin D2 synthase. This gene is also expressed in the choroid plexus and has previously been shown to be expressed only in the RPE when ex-

amining the posterior pole of the eye [21]. These data appear to conflict with the data published by Sharon et al. [22]. In their work on SAGE libraries of human retina and RPE for example, the authors stated that of the fifty most common tags observed in the human RPE, 29 were orphan tags (did not correspond to any known gene), and of these 29, 22 appeared to be uniquely expressed in the RPE. This assertion was based on a sequence search of all available SAGE libraries at the time of the work. An examination of the published literature on the SAGE technique reveals several important issues. First, a large number of tags cannot be identified by comparison with cDNA libraries [23]. Second, attempts to validate SAGE tags against model transcriptomes derived from completely sequenced genomes from human, mouse, and Arabidopsis also lead to large numbers of orphan or unidentified tags [23,24]. At the present time, it is not clear whether these tags represent systematic methodological errors in the generation of the tags or incomplete annotation of the genomes with respect to alternative mRNA splicing, antisense gene expression, or problems in the assumptions of the size of 5' and 3' UTRs in generating model transcriptomes.

It is interesting to note that some tissue specific transcripts were observed only once among the total number of sequences in the library. MCT-3, for example, is the monocarboxylate transporter expressed only in the RPE. Some reported tissue specific transcripts such as bestrophin were not observed in our sequence reads. Bestrophin protein was first detected in mouse RPE at postnatal day P10, and its message was detected in whole eye with highest expression in early postnatal development [25]. It is likely that mouse the mouse bestrophin sequence might reside among the roughly 65% of the total number of transcripts in the library which have not yet been sampled. Interestingly, Yu et al. also did not find bestrophin in their library [13]. In addition, none of the ESTs for mouse bestrophin in dbEST or Unigene is from eye or head libraries (most are from testis) while in the NEIBank human RPE/choroid library transcripts for bestrophin are moderately abundant. This certainly suggests that this gene is expressed at lower levels in mouse than in human RPE. Finally, the different observations on the abundance of bestrophin in the mouse RPE may reflect a strain variation. The most recent observation of bestrophin expression was performed with BALB/c mice, while the libraries of Yu et al. and our own were derived from the C57BL/6J strain. A comparison of single nucleotide polymorphisms among several mouse strains indicates that BALB/c is in a cluster of strains including A/J and C3H/He, while the B6 strain is somewhat distant from this cluster in an evolutionary tree [26]. The C57BL/6J strain varies in a number of functional properties from BALB/c, including sensitivity to light-induced photoreceptor degeneration and age-related retinal degeneration.

Indeed, a number of other RPE expressed genes have quite different levels of abundance in mouse and human RPE/choroid libraries, while retina libraries seem to be more similar. This may reflect real functional differences between the rodent and primate RPE that could be relevant to diseases such as AMD that occur in human but not in mouse. Alternatively, this could be related to differences in tissue processing and differences between an inbred (mouse) and outbred (human) population.

Finally, these libraries should provide a valuable resource for continuing work in the mouse for studies ranging from in situ or northern expression analysis to the identification of novel splice forms which may have a tissue specific pattern of expression.

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