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Identification of novel bovine RPE and retinal genes by subtractive hybridization

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Purpose: Understanding of the specialized function of the retinal pigment epithelium (RPE) can be aided by the identification and characterization of genes that are preferentially expressed in the RPE. With this aim, we undertook a systematic effort to identify and begin characterization of such genes.
Methods: A subtracted bovine RPE cDNA library was generated through subtractive hybridization using a single-stranded circular bovine RPE cDNA library as target and biotinylated mRNA from bovine heart and liver as alternate drivers. Approximately one thousand of the resulting subtracted cDNA clones were partially sequenced and analyzed, and a non-redundant set of one hundred of these cDNAs were examined for tissue expression pattern using a mini-Northern blot procedure and for identity by sequence analysis.
Results: The subtraction method successfully allowed the enrichment of cDNAs that are preferentially expressed in the RPE. Out of the analyzed clones, expression of forty-five clones was verifiable by Northern blotting. Of these, a significant proportion of cDNAs were preferentially expressed in the RPE. We observed that the expression of some subtracted cDNAs was restricted to the retina and no expression was detected in the RPE. These retinal clones were obtained in addition to RPE clones presumably because the initial RPE RNA population was contaminated with a small proportion of retinal RNA. Two thirds of the identified RPE and retinal cDNAs are likely to represent novel genes because they do not have homology to known genes in the databases.
Conclusions: Genes that are specifically or predominantly expressed in the RPE/retina are likely to be important for retinal function. We have identified novel cDNAs from bovine RPE and retina by subtractive hybridization. These cDNAs can be used as starting material for the identification of corresponding human genes expressed in the RPE and retina. The human genes thus identified are likely to contain good candidate genes for retinal disease.

The retina and RPE, well-defined structures in the eye, perform highly specialized functions. These functions require the involvement of a number of highly specialized genes that are likely to be preferentially expressed in the retina and RPE. The identification of such genes is pivotal in understanding the molecular basis of structure and function in the retina and RPE, and is likely to provide good candidate genes for the study of retinal disease genetics. Screening methods such as subtractive-, differential-, or suppression subtractive-hybridization have been employed for the identification of retina-specific genes [1-5]. A number of retinal genes such as *NRL*, *AOC2*, HRG4, *mrdgB* and CRB1 have been identified using these methods [5-9].

Earlier studies reporting the identification of RPE-specific genes involved the generation of subtracted cDNA libraries from human RPE-cell line [10] or human RPE and choroid [3]. As only a limited amount of RPE is recovered from a human eye, this restricts the use of subtractive hybridization approach for the identification of RPE-specific human genes. To overcome this problem, we constructed a subtracted cDNA library from bovine RPE. Bovine eyes were used because of their ready availability for mRNA extraction. In this paper, we report the use of a modified subtractive hybridization method for efficient generation of a subtracted bovine RPE cDNA library. We also present the expression and sequence analyses data for a set of hundred subtracted bovine cDNA clones obtained from this library. This subtractive hybridization strategy allowed us to identify a number of novel bovine genes that are predominantly expressed in the RPE and are potentially important in the function and dysfunction of the retina.

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METHODS

Generation of single-stranded (ss) circular bovine RPE cDNA library: A unidirectional bovine RPE cDNA library with $5x10^6$ independent phages was constructed in Uni-ZAP XR vector (Stratagene, La Jolla, CA). The library was amplified once to attain the titre of $5x10^{10}$ pfu/ml. To generate ss circular cDNA, the library was excised in vivo with ExAssist helper phage (Stratagene) following the manufacturer's instructions with some modifications. The phage particles were precipitated with 30% PEG/1.6 M NaCl at 4 °C overnight. The residual bacterial cells were removed by repeated centrifugation. The resulting phage preparation was treated with 200 μg DNase I at

37 °C for 30 min to digest any double-stranded (ds) DNA. The ss phagemid DNA was isolated by phenol/ chloroform extraction of phage particles followed by chloroform extraction and subsequent ethanol precipitation. The ss DNA pellet was resuspended in TE buffer, pH 7.6.

Isolation and biotinylation of driver mRNA: Total RNA was extracted from bovine heart and liver by the guanidinium/ acid phenol extraction method [11]. The poly-A^+ RNA was isolated by passing total RNA through a poly-dT cellulose column (Gibco BRL, Life Technologies) and purifying according to the manufacturer's protocol. The recovery of poly-A^+ RNA after two rounds of purification was about 1.9% of the starting total RNA.

For biotinylation, 50 µg of driver mRNA was mixed with 50 µl of photobiotin (long arm, SP-1020, Vector Laboratories). The solution was irradiated for 20 min in an ice bath with the tube cap open and at a distance of 10 cm from a sunlamp (150 W, Flood). The reaction was stopped with a final concentration of 100 mM Tris-HCl pH 9.0. The unbound biotin was removed by extraction with water-saturated isobutanol four to seven times or until the organic phase was colourless. The biotinylated mRNA was then extracted with chloroform to remove isobutanol, and precipitated with ethanol. The biotinylation and ethanol precipitation were repeated and biotinylated driver mRNA was finally resuspended in 50 µl H_2O.

Subtractive hybridization and transformation: The ss RPE cDNA library was subjected to four rounds of hybridization with biotinylated heart or liver mRNA as alternate drivers. Typically, 2 ng of ss circular library cDNA containing >$1x10^7$ phagemids was mixed with 5 µg of biotinylated driver mRNA and ethanol precipitated. The cDNA/RNA pellet was dissolved in 5 µl H_2O and following addition of an equal volume of 2X hybridization buffer (100 mM HEPES pH 7.6, 500 mM NaCl, 4 mM EDTA, 80% formamide), the solution was covered with mineral oil and heated at 95 °C for 5 min. Hybridization was carried out at 52 °C for 24 h. After hybridization, mineral oil was removed and 40 µl of 1X hybridization buffer was added to the reaction mix. To separate the hybridized and unhybridized biotinylated mRNAs, 10 µl of streptavidin (SA5000, Vector Laboratories) was added to the hybridization reaction and incubated at room temperature for 10 min with frequent mixing. After phenol/ chloroform extraction, the organic phase was again extracted with 50 µl of 1X hybridization buffer and the two aqueous phases were pooled. The streptavidin extraction was repeated twice again by adding 10 µl of streptavidin each time. After two rounds of chloroform extraction, 5 µg of another lot of biotinylated driver mRNA was added to the solution and ethanol precipitated. The hybridization procedure was repeated four times using heart and liver biotinylated mRNAs as alternate drivers.

After the last round of phenol/chloroform extraction, subtracted ss circular cDNA was ethanol precipitated and resuspended in 20µl H_2O. The ss circular cDNA was electroporated into electrocompetent MC1061 strain of *E. coli* and plated onto LB agar plates suplemented with ampicillin, X-gal and IPTG. About 1000 transformants/µl of ss circular cDNA were recovered. The blue/white selection of β-galactosidase expression was used to differentiate between recombinant and non-recombinant colonies.

Sequencing and sequence analysis: The sequencing reactions were performed with the fluorescence-labelled dideoxynucleotide (Prism, Applied Biosystems) using M13 (-20) and M13 reverse primers, and analysed on an ABI model 3700 Version 3.6 automated sequencer (Applied Biosystems). Sequences were analysed at the NCBI (National Center for Biotechnology Information) against the non-redundant nucleotide (nr), protein (Swissprot), human Expressed Sequence Tag (hEST) and human genome sequence (htgs) databases using the Basic Local Alignment Search Tool (BLAST).

Northern blot analysis: Northern blot analyses were performed on mini-Northern blots with RNA from bovine retina, RPE, kidney/muscle, heart/liver and brain. The total RNA from each tissue was extracted using the RNAzol B reagent (Tel-Test, Inc. TX, USA). The RNA from kidney and muscle and from heart and liver were pooled prior to Northern blotting. Each RNA sample (7 µg) was size fractionated on a 1% formaldehyde-agarose gel, and transferred and immobilised onto Hybond XL membrane (Amersham Pharmacia Biotech). Multiple mini-Northern blots were simultaneously prepared in this manner. Each blot was hybridized in 3 ml hybridization solution (6X SSC, 5X Denhardt's solution, 1% SDS, 50% deionised formamide) at 42 °C for 18-20 h. Up to ten blots were hybridized with ten different probes at a time. Radiolabelled probes were prepared in a 20 µl reaction volume using the Megaprime DNA labelling kit (Amersham Pharmacia Biotech). Blots were washed in 2X SSC, 0.1%SDS and 0.2X SSC, 0.1%SDS at 42 °C. An additional wash in 0.2X SSC, 0.1%SDS at 65 °C was performed if required. The hybridized blots were exposed overnight on a PhosphorImager screen and scanned using the ImageQuant software (Molecular Dynamics).

RESULTS & DISCUSSION

Generation of subtracted bovine RPE cDNA library: We generated a subtracted bovine RPE cDNA library to identify genes specifically or predominantly expressed in mammalian RPE. Subtraction was performed between a ss circular bovine RPE cDNA library and biotinylated mRNA from bovine heart and liver. The heart and liver were chosen as driver tissues for subtraction as they are developmentally different from the posterior of the eye where RPE resides. This subtraction was expected to allow the enrichment of genes expressed in RPE, a tissue of neural origin. The subtraction method required only 2 ng of ss circular RPE cDNA library as a starting material, allowing the use of more than 1000-fold molar excess of driver mRNA to target cDNA, which helped in obtaining efficient subtraction. The subtracted ss circular library cDNA was electroporated into MC1061 *E. coli* cells without converting into ds DNA. Rubenstein et al. [12] reported about 100 to 1000 fold higher transformation efficiency of ds DNA as compared to ss DNA, depending upon the amount of DNA used for transformation, however, we found only 2-3 fold difference in the transformation efficiency between ds and ss DNA (data not shown).

Following the initial partial sequencing of approximately 1,000 subtracted bovine cDNA clones, a set of one hundred non-redundant clones was chosen for further analysis. Upon sequence analysis (which was initially done in 1999), two clones were found in duplicate and seven clones matched to known mammalian gene sequences. Out of these seven, one bovine clone was homologous to mammalian Stra6, expressed at the blood-organ barriers including the RPE [13]. Another cDNA clone was a variant of bovine rhodopsin [14]. Three cDNAs were homologous to genes of neuronal origin, including the human photoreceptor-specific nuclear receptor PNR [15], DRES9 expressed in the neural retinal and central nervous system [16], and DnaJ expressed in human brain [17]. Two bovine clones corresponded to housekeeping genes. Homology of the subtracted bovine cDNAs to Stra6 and neuronal genes indicates that the subtractive hybridization method allowed the enrichment of genes expressed in tissues of neural origin. After excluding the duplicate clones and those homologous to known genes, subsequent investigation was carried out on ninety-one subtracted clones.

Expression analysis: The expression of ninety-one subtracted bovine cDNAs was analyzed by mini-Northern blotting on total RNA from bovine retina, RPE, kidney/muscle, heart/liver and brain. The heart/liver RNA was included on the Northern blots to determine the efficiency of the subtraction protocol. Expression in kidney/muscle RNA would represent any non-ocular expression of the subtracted cDNAs. Northern blot analysis revealed the tissue distribution of fifty percent of the cDNA clones. Expression of half of the subtracted cDNAs was undetectable under the Northern hybridization conditions used in this study suggesting possibly a very low level of expression of their respective transcripts. North-

Table 1. Summary of the expression patterns of subtracted bovine cDNAs

Expression pattern	Number of clones
RPE	11
RPE and retina	11
RPE, retina and brain	2
RPE and kidney/muscle	1
RPE, retina and kidney/muscle	1
All tissues tested but high in RPE	1
All tissues tested but high in RPE and retina	2
Retina	13
Retina and brain	3
Total	45

Expression of the subtracted bovine cDNAs was analysed in bovine retinal, RPE, kidney/muscle, heart/liver and brain tissues by Northern blot analysis. The table shows the various expression patterns observed and the number of clones exhibiting each expression pattern.

Table 2. Subtracted bovine cDNAs homologous to known mammalian genes

Clone name	Expression in this study	Homology to known gene	Species	Reference
Homology to bovine genes:				
S779	Retina	Guanine nucleotide binding protein, gamma subunit in cones	Bovine	[18]
S810, S1932	RPE	SFRP5	Bovine	[20]
S774	RPE	RGR	Bovine	[21]
S1934, S2066	Retina, RPE	RGR	Bovine	[21]
S2084	Retina, RPE	Retinal cyclic GMP phosphodiesterase gamma-subunit	Bovine	[19]
Homology to retinal disease genes:				
S709	Retina, RPE	PHR1	Human, mouse	[24]
S1926	Retina, RPE	NRL	Human	[6]
S2077	Retina, RPE	HRG4, RRG4	Human, rat	[7]
Homology to other genes				
S727	RPE	Stra6	Mouse	[12]
S743	Retina, RPE	WDR6	Human	[41]
S751	Retina, brain	TTYH1	Human	[30]
S766	Retina, brain	40S ribosomal protein S13	Human	
S905	All tissues tested	Glyoxalase 1	Human	[26]
S1631	All tissues tested	SH3PX1	Human	
S1929	Retina, RPE	ACP33	Human	[28]
S1944	Retina, RPE, kidney/muscle	Cytochrome b5 reductase 1	Human, bovine	[42,43]
S1947	RPE	Melastatin	Mouse, human	[44]

BLAST analysis was performed for each cDNA query sequence against the non-redundant nucleotide, protein, human EST and human genomic sequence databases. The Northern blot and BLAST analyses results are tabulated.

ern blot analysis of poly-A^+ RNA may reveal the transcripts and tissue distribution of these cDNAs. Out of the 45 bovine cDNAs, whose expression was detected on Northern blots, 29 were expressed in the RPE (Table 1). Of these 29 cDNAs, 11 were specifically expressed in the bovine RPE and another 11 were expressed in both RPE and retina. Two cDNAs were expressed in the retina and brain in addition to RPE, and five cDNAs had expression in one or more non-ocular tissues besides RPE. The RPE expression of approximately two thirds of the cDNA clones demonstrates that the subtractive hybridization method employed in this study successfully enabled the enrichment of genes expressed in the bovine RPE. Only three out of 45 clones were expressed in heart/liver, the driver tissues used for subtraction, further supporting the validity of the subtraction method. Sixteen of the 45 cDNAs had no RPE expression but were instead expressed in the retina. The expression of thirteen of these 16 clones was restricted to the retina and three clones were expressed in the retina as well as brain. The identification of these cDNAs indicated that the starting "RPE" RNA population was contaminated with a small proportion of retinal RNA. As bovine retina is strongly adherent to the RPE, it is difficult to completely dissect the retina away from the underlying RPE. Hence, while removing the retina from the eye-cup, some photoreceptor outer segments and probably cells remain attached to the eye-cup and thus are inadvertently collected along with the RPE cells. Obtaining retinal genes during this study in fact worked to our advantage. In addition to identifying RPE expressed genes, we also identified some potentially interesting genes that are predominantly expressed in the bovine retina.

Sequence analysis: Additional sequence data were obtained for the 45 clones whose tissue distribution was detected by Northern analysis. The BLAST analyses for these cDNAs were repeated against the nucleotide, protein, human EST and human genome sequence databases at NCBI in 2000 and 2001. The additional sequence information of these clones revealed that two clones were present in duplicate. Nineteen of the remaining 43 clones had significant homology to known mammalian genes whereas 24 subtracted clones did not match to any known genes in the database.

Out of the nineteen clones with homology to known mammalian genes, seven corresponded to known bovine RPE/retinal genes (Table 2). The cDNAs S779 and S2084 represented bovine genes involved in the phototransduction process [18,19]. The clones S810 and S1932 corresponded to different regions of the RPE-specific gene *SFRP5* that was previ-

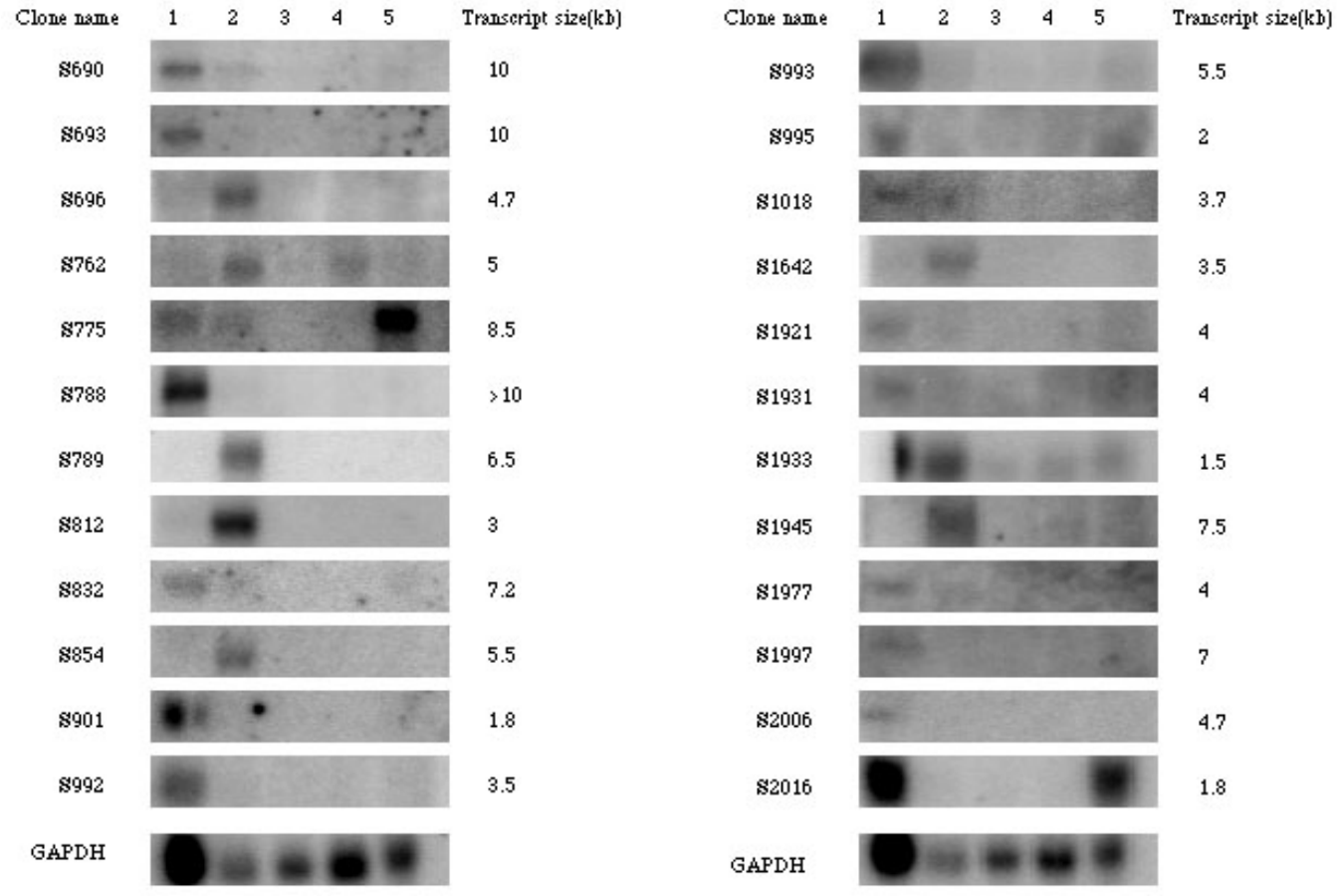

Figure 1. Northern blot analysis of total RNA from ocular and non-ocular bovine tissues. Seven μg each of retinal (lane 1), RPE (lane 2), heart/liver (lane 3), kidney/muscle (lane 4), and brain (lane 5) RNA were probed with each radiolabelled subtracted bovine cDNA. The cDNAs used as probes and the detected transcript sizes are shown. GAPDH=Glyceraldehyde-phosphate dehydrogenase.

ously isolated from this subtracted library [20]. S774, S1934 and S2066 were homologues of the bovine RPE-retinal G protein coupled receptor RGR [21]. In the present study, S774 detected a 3.4 kb transcript in the RPE, whereas S1934 and S2066 hybridized to a 1.5 kb transcript in both the RPE and retina. These results are consistent with the reported RGR expression in the bovine RPE and retina [21]. RGR binds to all-*trans*-retinal and is involved in the formation of 11-*cis*-retinal in mice [22]. Mutations in the human gene encoding RGR have been associated with retinitis pigmentosa [23]. Identification of RGR from the subtracted bovine library is encouraging as it means that the subtracted clones can be a useful resource for the identification of novel genes important for retinal function and in retinal disease. Three bovine cDNAs were orthologues of the human retinal genes, *PHR1* [24], *NRL* [6] and HRG4 [7], respectively. Mutations in the photoreceptor-specific genes *NRL* and HRG4 lead to autosomal dominant retinitis pigmentosa and dominant cone-rod dystrophy, respectively [25,26]. Their homology to known human retinal disease genes reiterates that the subtracted bovine cDNAs can be a valuable starting material for the identification of novel retinal disease genes, found as orthologues of the bovine RPE/retinal cDNAs. The transcript sizes of the bovine orthologues of *PHR1*, *NRL* and HRG4 corresponded to the transcripts encoded by these genes in the human retina (data not shown). However, as opposed to the retinal expression reported for the human genes [6,7,24], the bovine orthologues were expressed in both the bovine retina and RPE. "Expression" of these genes in the bovine RPE is most likely due to a retinal contamination in the RPE, although the possibility of species-specific difference in RPE expression cannot be excluded.

Table 3. Novel bovine cDNAs expressed in the bovine RPE/retina

Bovine clone	GenBank Accession number	Database homology	Human UniGene identifier
S690	AF451165		
S693	AF451166		
S696	AF451167	DKFZp586B0621	Hs.157211
S762	AF451168	KIAA1522	Hs.322735
S775	AF451169		
S788	AF451170	RP4-791K14*	
S789	AF451171		
S812	AF451172	AdRab-G, FJ30107	Hs.343553
S832	AF451173	KIAA1157	Hs.21894
S854	AF451174		
S901	AF451175		
S992	AF451176		
S993	AF451177		
S995	AF451178		
S1018	AF451179		
S1642	AF451180		
S1921	AF451181		
S1931	AF451183		
S1933	AF451184		
S1945	AF451185	RP1 163G9*	
S1977	AF451186		
S1997	AF451187	KIAA0562	Hs.200595
S2006	AF451188	FLJ10018	Hs.322045
S2016	AF451189		

*Human BAC (Bacterial artificial chromosome) clone

For each cDNA the GenBank accession number, any homology to uncharacterised human sequences and the corresponding Unigene identification are listed.

Nine of the nineteen clones were orthologues of known mammalian genes originally cloned from non-ocular tissues (Table 2). The knowledge about the function of these genes in non-ocular tissues combined with the present finding of their expression in the RPE/retina can be extremely useful in elucidating the biochemical pathways involved in retinal function and dysfunction. For example, the bovine cDNA S905, expressed in the retina and RPE in this study was an orthologue of the human Glyoxalase 1 (*Glo 1*) [27], a gene ubiquitously expressed in human tissues. We have detected *Glo 1* expression in the human retina and RPE (S Sharma, unpublished data). *Glo 1* is involved in detoxification of methylglyoxal, a by-product of the cellular glycolytic pathway [28]. This may imply that dysfunction of *Glo 1* in the human retina/RPE might lead to accumulation of methylglyoxal in these tissues and compromise retinal function. The bovine S1929 was an orthologue of ACP33, a CD4 interacting protein that inhibits CD4 function in T cells [29]. ACP33 is widely expressed in human tissues [29], and its bovine orthologue was expressed in the bovine RPE and retina in the present study. The RPE cells do not express CD4 [30], however, the expression of ACP33 in bovine RPE suggests that it may interact with other proteins in the RPE. Another cDNA expressed in the bovine retina and brain in our study was an orthologue of *TTYH1* cloned from human brain [31]. TTYH1 is a transmembrane protein and has structural similarity to yeast iron-transporter proteins [31]. The putative function of TTYH1 as a transporter and retinal expression of its bovine orthologue warrants investigation of its expression in the human retina.

Novel genes expressed in bovine RPE/retina: Twenty-four bovine clones expressed in the RPE and/or retina (Figure 1) did not have significant homology with known gene sequences in the databases and therefore appear to be novel. The sequences of these novel cDNAs have been submitted to the GenBank (NCBI) and their GenBank accession numbers are listed in Table 3. Although these cDNAs had no homology to "known genes," some bovine cDNAs exhibited significant homology to uncharacterised human cDNAs or to human genomic sequences in the database (Table 3) suggesting that latter are the human orthologues of the bovine clones. A number of bovine genes expressed in the retina or RPE have led to the identification of important human retinal and RPE genes such as rhodopsin and RPE65 [32,33]. Mutations in the genes encoding the human rhodopsin and RPE65 lead to degenerative retinal disease [34]. We anticipate that the human orthologues of the subtracted bovine cDNAs would be expressed in the human RPE and/or retina similar to their bovine counterparts and represent novel RPE/retinal genes and potential candidates for retinal disease.

S696, expressed in the bovine RPE (Figure 1), showed significant sequence identity to a human cDNA present in the UniGene cluster Hs.157211 that is mapped at 11q23.3. The human cDNA exhibits some similarity to human complement-C1q tumor necrosis factor related protein 5 [35]. Several inherited retinal dystrophies have been mapped to chromosome

11, however, the human orthologue of S696 does not map to a disease locus on this chromosome and is therefore unlikely to be a retinal disease candidate. The Hs.157211 is constituted by ESTs from various human tissues including RPE and brain, indicating that besides its expression in non-ocular tissues, the gene represented by this cluster is also expressed in neural tissues. Bovine cDNAs S788 and S1945 expressed in the retina and RPE, respectively (Figure 1), had significant sequence similarity with regions of human bacterial chromosome (BAC) clones (Table 3). This nucleotide homology suggests the existence of human orthologues of S788 and S1945, which are expected to express in the human retina and RPE, respectively. S812 expressed in the bovine RPE (Figure 1) had significant nucleotide and amino acid similarity to an uncharacterised hypothetical protein AdRab-G identified from rabbit intestine [36]. It was also homologous to human ESTs from neuroglioma and retinoblastoma cell lines. Likewise, S762, S832, S1997, and S2006 exhibited homologies to uncharacterized cDNAs from human brain. These homologies indicate that the human orthologues of these bovine clones are expressed in neuronal tissues and are likely to be expressed in human RPE/retina. However, the Unigene clusters consisting of these uncharacterized human cDNAs also include ESTs from non-ocular tissues. Though we observed predominant expression of S762, S812, S832, S1997, and S2006 in the bovine RPE/retinal, their human orthologues may be more widely expressed in human tissues. This does not preclude these genes from being potentially important in retinal function, as widely expressed genes can be vital for normal retinal function. For example, mutations in TIMP-3, a widely expressed human gene, lead to Sorsby's fundus dystrophy that is characterised by accumulation of lipid deposits in Bruch's membrane beneath RPE and sub-retinal neovascularisation [37,38]. Thus, investigation of the expression and function of the human orthologues of bovine RPE/retinal genes identified in this study is likely to yield potentially important genes in these tissues.

Sixteen bovine cDNAs did not have any significant match in the databases at NCBI. The reason for not identifying the human orthologues of these bovine cDNAs can be absence of ESTs from human orthologues of bovine cDNAs in the database. Furthermore, some subtracted bovine cDNAs may include the 3'-untranslated region of the gene and not the coding region that is more likely to be conserved between bovine and human and thus likely to reveal the human orthologue. Additional sequence information of these bovine cDNAs will facilitate the identification of their respective potentially novel human orthologues.

In conclusion, all the subtracted bovine cDNAs whose expression was detectable by Northern blotting were expressed in the RPE and/or retina, demonstrating the efficacy of the subtraction method. The homology searches revealed the human orthologues of about twenty percent of these bovine clones, present as uncharacterised cDNAs in the database. Approximately forty percent of bovine cDNAs represent novel genes. These bovine cDNAs predominantly expressed in the RPE/retina will serve as a valuable resource for the identification of their human counterparts, likely to be expressed in the RPE or retina. Earlier, the RPE-specific genes *SFRP5*, *BMP-4* and *Kir7.1* were cloned from this subtracted library [19,39,40]. The possibility of detecting human RPE/retinal genes as orthologues of subtracted bovine RPE/retinal cDNAs extends a useful strategy for identifying novel genes as well as candidates for retinal disease without using human RPE/retina as the starting material.

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