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Expression of transthyretin and retinol binding protein mRNAs and secretion of transthyretin by cultured monkey retinal pigment epithelium

Bruce A. Pfeffer,[1] S. Patricia Becerra,[2] Diane E. Borst,[3] Paul Wong[4]

[1]Research, Bausch and Lomb, Rochester, NY; [2]Laboratory of Retinal Cell and Molecular Biology, National Eye Institute, NIH, Bethesda, MD; [3]Department of Anatomy, Physiology and Genetics, Uniformed Services University of the Health Sciences, Bethesda, MD; [4]Department Biological Sciences, University of Alberta, Edmonton, Alberta, Canada

Purpose: To document the expression of mRNA for transthyretin (TTR) and retinol binding protein (RBP) in native and cultured Rhesus monkey retinal pigmented epithelium (RPE); to compare mRNA transcripts for these two proteins expressed in RPE with those found in whole monkey liver and brain; to demonstrate the secretion of TTR by RPE during short-term maintenance in a protein-free, defined medium, as a manifestation of the differentiated state of these cells in vitro.

Methods: Total RNA was isolated from cultured RPE in first passage, after incubation for eight days in defined, protein-free medium. Conditioned medium was collected for western analysis at this time. Total RNA was also extracted from RPE/choroid freshly dissected from monkey eyes. Using cDNA probes for human TTR and RBP, northern analysis was performed on the total RNA from fresh and cultured RPE samples, together with poly(A^+) mRNA purified from monkey liver and brain.

Results: Conditioned medium from RPE yielded TTR protein of the expected monomer subunit molecular size. The TTR secreted de novo from the cultured cells was detectable in the absence of biosynthetic labeling. With the exception of some extremely low abundance transcripts expressed in cultured RPE, all samples contained a single 900 bp transcript for TTR. Based on relative amounts of actual message, RPE ranks higher than liver in abundance of TTR mRNA. In contrast, both native monkey RPE and cultured RPE cells expressed comparatively low levels of mRNA for RBP. All samples displayed a single RBP mRNA transcript at 1100 bp.

Conclusions: Our results indicate that TTR is a significant gene product of the RPE, and may be considered as a marker for a differentiated phenotype for these cells in culture. There is increased recognition of various forms of ocular pathology associated with mutations or other malfunctions involving TTR and RBP, warranting a greater understanding of mechanisms of transcriptional and translational control for these two proteins.

Previous investigations have established that cultured mammalian retinal pigmented epithelial (RPE) cells can be utilized as a model system for studying the visual cycle of vitamin A (retinoids) [1-4]. Many of the binding proteins and enzymes (or enzyme activities) that are dedicated to the uptake, storage, chemical transformations, and release of retinoid compounds within the native neural retina-RPE complex [5,6] are expressed in pure cultures of RPE from various vertebrate sources, including human. One exception would be interphotoreceptor retinoid binding protein (IRBP), which is a product instead of photoreceptor cells, and which fulfills functions associated with translocation and stabilization of retinoids in the extracellular space between the apical RPE and the photoreceptor outer segments [7,8]. In addition, vitamin A, originating from its dietary source and supplied from the circulation in the form of all-trans-retinol, is required, for its entry into the visual cycle, to cross the blood-retina barrier at the level of the RPE [9,10]. A systemic carrier, retinol-binding protein (RBP), synthesized by the liver, is responsible for transport of all-trans-retinol to its target tissues, including the RPE, via receptor-mediated uptake [11]. The functions of the binding proteins that subserve these extracellular translocations are to increase the aqueous solubility of vitamin A, to regulate its delivery to target cells, to prolong the stable half-life of the retinoid ligand in extracellular compartments, and to protect tissues from cytotoxicity engendered by high local concentrations of retinoids [12-15]. Within the eye, vitamin A may also perform roles not directly related to photoreceptor-based visual transduction. In fact, RBP also can be detected intraocularly, in both intracellular and fluid compartments, despite its inability to penetrate the blood-retinal barrier [16]. Therefore, RBP is one of three extracellular proteins with the potential to bind vitamin A and transport it within the eye, along with IRBP and serum albumin [17,18] (also Liao and Gonzalez-Fernandez, unpublished data, cited in [8]). Recent investigations suggest that RBP of RPE origin does have more than a vestigial role in maintaining homeostasis at the level of the visual cycle. Since IRBP “knockout” mice retain high rates of recovery of visual function after bleaching [19], RBP fits a

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Correspondence to: Bruce A. Pfeffer, Ph.D., Bausch and Lomb, Inc., 1400 North Goodman Street, Rochester, NY, 14609; Phone: (585) 338-5006; FAX: (585) 338-6223; email: Bruce_Pfeffer@bausch.com

profile of a putative auxiliary carrier of retinoids, including 11-cis-retinal, in the interphotoreceptor space [20,21]. In addition, there is evidence of constitutive basal efflux of all-trans-retinol from RPE, such efflux being promoted by elevated 11-cis retinoids and impaired in RPE-65 “knockout” mice [22]. RBP is the most likely, of any retinoid-binding proteins that are known to originate in the RPE, to possibly undergo basal release [23].

As evidenced by in situ hybridization to localize message for RBP in native ocular tissues, the RPE is the intraocular source of this protein [24]. Messenger RNA for transthyretin (TTR; formerly known as prealbumin) has also been localized exclusively to the RPE by means of in situ hybridization [25]. TTR is normally found as a homotetrameric protein released by the liver to the circulation, predominantly in a 1:1 complex with RBP. [26]. TTR, both free and bound to RBP, independently functions as a transport protein for thyroxine (T4) and for triiodothyronine (T3). In addition to being a plasma protein of hepatic origin, TTR under normal conditions also attains significant concentration levels in the brain [27]. Meanwhile, immunocytochemical methods have shown that, in addition to RPE, TTR protein is prominent in eye tissues such as ciliary and iris epithelium, corneal endothelium, and the optic nerve fiber layer of the retina [28]. Consequently, expression of TTR and RBP mRNA, and the secretion of TTR and/or RBP, may be considered markers for RPE in its differentiated state. It has now been established that under cell culture conditions, rat, fetal bovine, and fetal human RPE do indeed secrete these two proteins [23,29]. The only additional ocular cell type that may display this expression phenotype is iris pigment epithelium, a tissue that shares optic vesicle origins with the RPE; RBP message was recently demonstrated via reverse transcription-polymerase chain reaction (RT-PCR) analysis of cultured iris pigment epithelial cells [30]. In the present study we investigated the expression of TTR and RBP by adult monkey RPE, both native and in vitro. We extended previous findings by demonstrating that monolayers of RPE cells cultivated in a protein-free medium express mRNA for these two genes, and that the accumulation of RPE-derived TTR may be detected without biosynthetic labeling.

METHODS

Cell culture and collection of conditioned media: First passage cultures derived from freshly harvested, normal Rhesus monkey (*Macaca mulatta*) RPE were employed for these experiments. Animal care and experimental procedures were in accordance with “Principles of Laboratory Animal Care” (NIH Publication No. 86-23, revised 1985), as well as with the guidelines set forth in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Detailed methodologies for obtaining RPE sheets for primary culture, for subculture without the use of proteolytic enzymes, and for formulating media designed to promote either proliferation or differentiation of RPE, as well as selective criteria for evaluating the latter state in these cells have been described [31]. Briefly, incubation of posterior segments of monkey eyes, from which sclera was partially removed, with 2.4% (w/v) crude Dispase (Grade II, Roche Applied Science, Indianapolis, IN) allowed harvesting of intact RPE sheets free from contamination by either neural retinal or choroidal cells. Attachment, spreading, and proliferation to confluence commenced in 35 mm dishes using a low-calcium growth medium based on a 1:1 mixture of Medium 199 and Dulbecco’s modified Eagle’s medium (formulated by the Media Unit, NIH, Bethesda, MD), with bovine calf serum (“defined CS”; HyClone, Logan, UT; $[Ca^{2+}]$=3.5 mmol/L) at 1% (v/v), and total calcium ion concentration reduced to approximately 0.1 mmol/L (remainder from a 200X stock of $CaCl_2$). Among the relevant supplements added (see [31] for a complete list) were: 0.5% of a saline extract of bovine retinas (v/v; final dilution to approximately 7.5 μg protein/ml from an original 200X stock); 200 mg/L of Albumax, a fatty acid supplement (GibcoBRL Invitrogen Life Technologies, Rockville, MD); 10 μg/L “Long R^3IGF-1” (LRIGF; Sigma Chemical Co., St. Louis, MO); 15 mg/L human transferrin (30% iron-saturated, Roche); 0.43 μmol/L retinol acetate, in a noncovalent complex with methyl-β-cyclodextrin (Sigma); and 10 nmol/L triiodothyronine. Other non-protein hormones and trace nutrients were added to enrich the reduced serum formulation. Cells were maintained as stable monolayers in 12 well Costar clusters (Corning Life Sciences, Oneonta, NY) for at least two weeks in medium containing 0.45 mmol/L Ca^{2+}, designed to optimize the expression of a variety of biochemical and physiological markers characteristic of the differentiated tissue in vivo [31,32] (a representative micrograph has been published previously [32]). These markers include de novo melanization, morphological apical-basal polarization, formation of domes (emblematic of vectorial fluid transport), and expression of cellular retinaldehyde binding protein. Confluent wells contained $6x10^5$ cells each, as calculated from hemacytometer counting of parallel cultures.

For production of conditioned media (RPE-CM), RPE cells were maintained in a protein-free defined medium that was a modification of the one described above in the following respects: Serum, bovine retinal extract, LRIGF and transferrin were omitted; Albumax was replaced by Chemically Defined Lipid Concentrate (Gibco) at 0.1% (v/v); (apo-) dimethyl-β-cyclodextrin and hydroxypropyl-β-cyclodextrin (Cyclodextrin Technologies Development, High Springs, FL) were added, each at 0.1% (w/v). Several quick rinses of the monolayers with the protein-free medium ensured that there was virtually no carryover of serum proteins that could confound the results obtained with the RPE-CM. The initial medium volume per well was 1.0 ml, and after five days of the eight-day conditioning period fluid levels in the wells were restored to 1.0 ml by the addition to each well of from 0.1 to 0.3 ml of balanced salt solution supplemented with glucose, vitamins, and amino acids. After the eight-day conditioning period the pH of the medium remained within the physiological range, as indicated by the color of the phenol red, and cell morphology was unchanged, based on the appearance of the cultures in the inverted microscope. RPE-CM from six wells were pooled and stored frozen until use. The RPE monolayers were then processed for isolation of RNA (see below).

Preparation of RNA from cells and tissues: Freshly enucleated Rhesus monkey eyes were placed on ice and rapidly microdissected to obtain native RPE samples, which also included Bruch's membrane plus adhering choroidal tissue. Tissue samples were then snap frozen on dry ice and stored at -70 °C until further processing.

For northern analysis, total RNA was isolated from RPE-choroid tissue following the method of Chomczynski and Sacchi [33], using RNAzol (Tel-Test, Friendswood, TX); RNA was dissolved in DEPC-treated water, and stored frozen at -80 °C. Total RNA from cultured cells was purified using the modification of Peppel and Baglioni [34], and was stored until further use under absolute ethanol at -70 °C. Poly(A^+) RNA samples from Rhesus monkey liver and whole brain were purchased from Clontech Labs (Palo Alto, CA).

Northern blotting and preparation of probes: A partial-length human TTR cDNA clone containing 540 base pairs (bp) [35] was kindly provided by Dr. J. Herbert, New York University, and a human RBP cDNA clone of approximately 1000 bp [36] was kindly supplied by Dr. D.R. Soprano, Temple University, Philadelphia, PA. The cloned cDNA insert from each plasmid clone (either for TTR or RBP plasmids) was cut out of the construct using specific restriction endonucleases, isolated, and purified using standard protocols [37]. In each case, the purified insert cDNA was radiolabeled using a random primer labeling protocol that incorporated [α^{32}P]dCTP (Perkin-Elmer, Shelton, CT) into the DNA probe synthesized. The specific activity of each probe was not less than 10^7 cpm/µg.

RNA samples for electrophoresis were applied to 1% agarose gels in a formaldehyde running buffer system [37]. Sample sizes were 1 µg for monkey liver and brain poly(A^+) RNA, and 3 µg for total RNA from native RPE-choroid and cultured RPE. After separation of RNA species, northern transfers were made onto Genescreen Plus nylon membranes (Perkin-Elmer) by means of passive blotting in 10X SSC (final concentrations: 1.5 mol/L NaCl; 0.15 mol/L Na citrate, pH 7.0). Blots were UV cross-linked and heat baked for 2 h at 80° C under vacuum before use. Prehybridization, hybridization, and blot washes were carried out in Hybrisol II (Serologicals Corp., Gaithersburg, MD) according to standard protocols. Probes were hybridized to the blot at 65° C and washed stringently in 0.1X SSC at 65° C. Autoradiography was carried out at -70 °C using X-omat film (Eastman Kodak, Rochester, NY) between two intensifying screens. The profile of expression was verified by the analysis of duplicate northern blots. To account for differences in band intensities caused by differences in RNA loading, the relative intensity of the ribosomal RNA bands in each total RNA sample, stained with ethidium bromide, was used to quantitatively normalize values of corresponding mRNA for the proteins in question [38].

Densitometry: For densitometric analysis of northern blots, autoradiographs and gel photographs were scanned. From these saved image files, calculations of band and background intensity and volume in pixels were done using the histogram function in Adobe Photoshop version 7. Analysis of these densitometry results was performed using Microsoft Excel version 8 for Windows.

Polyacrylamide gel electrophoresis and western blotting: Standard methods of polyacrylamide gel electrophoresis (PAGE) were used to separate proteins from the RPE-CM. The sample volume of RPE-CM was 20 µL, containing 8.8 µg total protein. Authentic TTR from human was obtained from The Binding Site (San Diego, CA), to be used as a positive control. Proteins were subsequently transferred onto nitrocellulose paper (0.45µm, BioRad, Hercules, CA) by the method of Towbin et al. [39]. The blot was pretreated with 1% BSA in TBST (20 mmol/L Tris, 150 mmol/L NaCl, 0.05% (v/v) Tween-20, pH 7.5) for 1 to 2 h at room temperature and then reacted in the same buffer, also containing 1% (w/v) BSA, with polyclonal ovine antiserum against human TTR (The Binding Site), at 1:100 dilution for 16 h at 4° C. The blot was subsequently treated with biotinylated rabbit anti-sheep IgG (Vector Laboratories, Burlingame, CA) at a 1:400 dilution in TBST with 1% BSA for 1 to 2 h at room temperature, followed by avidin-biotinylated horseradish peroxidase (HRP) complex (Vectastain ABC Kit, Vector) for 1 h. Biotinylated molecular weight standards were obtained from BioRad. Color development to visualize TTR ensued by means of a HRP color development kit (BioRad), as previously described [40].

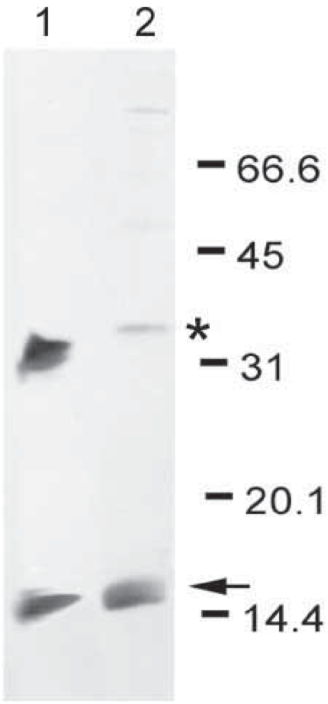

Figure 1. Immunochemical detection of transthyretin in RPE-conditioned medium. Immunochemical detection of transthyretin in conditioned medium from cultured monkey RPE (retinal pigmented epithelium). Western immunoblot analysis was performed on purified human TTR (lane 1), and on non-concentrated RPE-conditioned medium (lane 2). Immunoreactive bands are displayed at an apparent molecular size of 15 kDa (arrow), representing TTR monomer, in both samples. In addition, both samples reveal a higher molecular mass band, most likely corresponding to TTR dimer, at approximately 31 kDa (lane 1) and 35 kDa (lane 2, asterisk). The molecular size scale is based on biotinylated standards.

RESULTS

Western blot for transthyretin: The western blot of RPE-CM for TTR shows an immunopositive band at the expected molecular size for the monomer of 15 kDa (Figure 1, lane 2 (arrow)), as previously demonstrated for fetal bovine and human RPE in culture [23]. As an experimental control, 4 ml of culture medium that had been incubated in parallel without cells was concentrated 1:200, before loading 20 μl for PAGE and western blotting for TTR; this sample displayed no bands labeled by the antiserum for TTR [results not shown]. The identification of the band at 15 kDa as TTR was confirmed by comparison with the identically migrating labeled band from a sample of authentic TTR (Figure 1, lane 1). There was apparently sufficient cross-reactivity of antibody against human TTR with macaque TTR for detection in the non-concentrated RPE-conditioned medium. During the conditioning period enough TTR was released by the RPE, when cultured in totally defined medium, for detection without the use of biosynthetic radioactive labeling. Significant intracellular stores of TTR should be detectable in RPE as well [41], but we did not assay cell homogenates.

An additional diffuse band was observed in the western blot for authentic TTR, that corresponded to the apparent molecular mass of approximately 31 kDa for TTR dimer (Figure 1, lane 1), as well as a faint auxiliary band for the RPE-CM sample, migrating at approximately 35 kDa (Figure 1, lane 2 (asterisk)). In fact, the latter mobility value result is characteristic of how TTR tetramer would run, that is, as a dimer, in a native gel [42], and this might have resulted from incomplete denaturation of TTR in our SDS-PAGE system. Other faint higher molecular size immunopositive bands from the RPE-CM might indicate the formation of TTR aggregates during the conditioning period. TTR is known, even as the wild type, to undergo irreversible conformational changes to form ag-

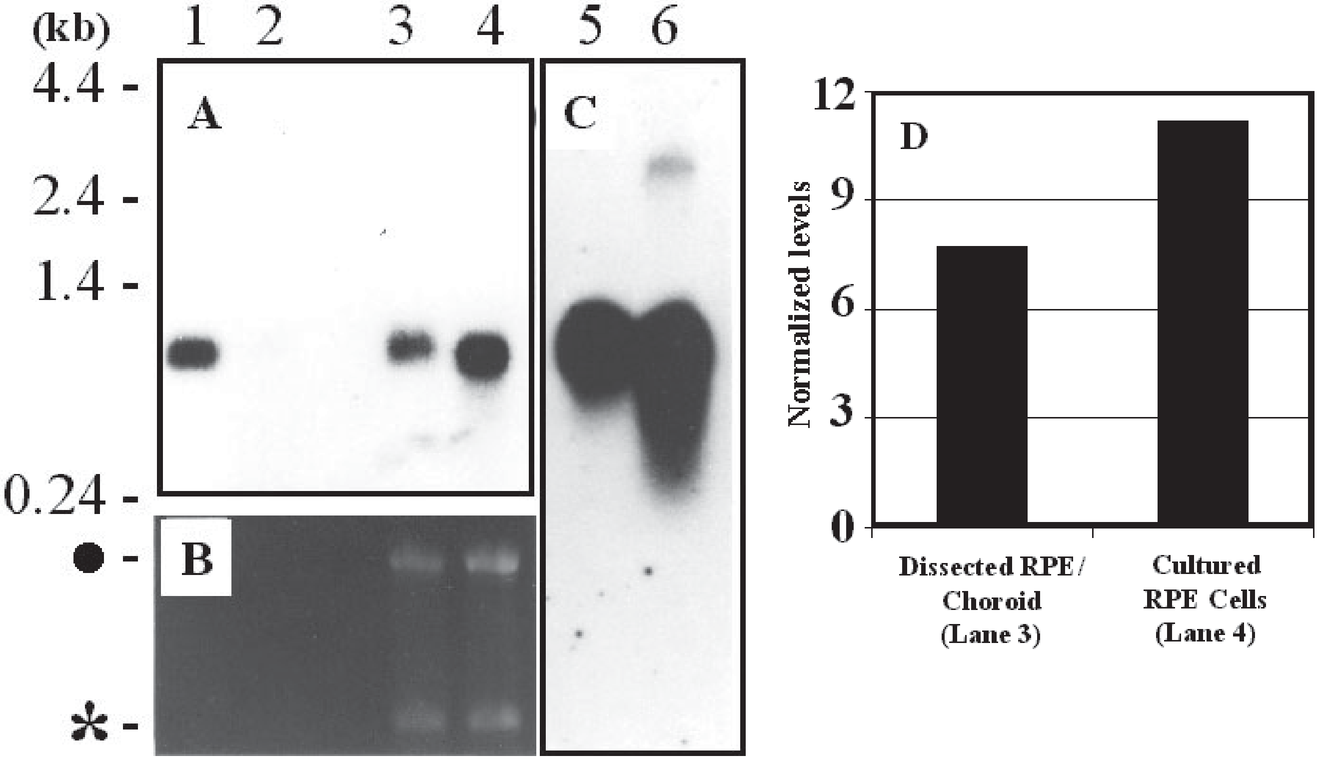

Figure 2. Northern analysis for transthyretin in monkey tissues. **A**: Two hour autoradiographic exposures are displayed in the upper part of lanes 1-4. For lanes 1 and 2, 1 μg poly(A^+) mRNA from monkey liver and brain, respectively, were loaded. For lanes 3 and 4, 3 μg of total RNA from freshly dissected monkey RPE/choroid complex and from cultured monkey RPE (retinal pigmented epithelium) cells, respectively, were loaded. Monkey liver expresses one major transcript at ca. 900 bp (lane 1). No signal is evident from the monkey brain sample at this short exposure time (lane 2). One 900 bp transcript is seen from the fresh monkey RPE/choroid sample (lane 3), while for cultured monkey RPE, the single 900 bp transcript band in lane 4 is denser than that for native RPE/choroid. **B**: The RNA electrophoresed on the agarose gel was stained with ethidium bromide and visualized with ultraviolet illumination before transfer to nylon membrane. The lower part of lanes 1-4 corresponds to the samples described in **A**, above. The density of the 28 s (denoted by black dot) and 18 s (asterisk) ribosomal RNA bands for fresh RPE/choroid (lane 3) and cultured RPE (lane 4) are a function of the relative loading with total RNA. **C**: Four day autoradiographic exposures for total RNA from monkey RPE/choroid and cultured RPE are displayed in lanes 5 and 6, respectively. No additional bands are evident in the native tissue sample, while overexposure elicits a faint, larger mRNA species, ca. 3 kbp for cultured RPE. Molecular size scale in kilobase pairs (kb). **D**: Densitometric analysis of the 900 bp bands from lanes 3 and 4 of the northern blot in A, above, using the 18 s ethidium bromide stained band in lane 4 of **B**, for normalization. By this measurement, the TTR mRNA in cultured RPE is 1.4 times as abundant as that for freshly harvested RPE/choroid.

gregates at high concentrations and when RBP is not available in stoichiometric amounts to stabilize the tetramer [43].

Northern blots: As can be seen from the northern blots depicted in Figure 2A,C, the probe based on the human TTR cDNA sequence was sufficiently complementary to monkey TTR mRNA to evince specific binding under our hybridization conditions. The predominant molecular size of monkey TTR mRNA is approximately 900 bp in brain, liver, native RPE, and cultured RPE, compared with human at 700 bp, as determined by Soprano et al. [35]. On the basis of previous findings [24,25,44], we have ruled out a significant contribution of message for TTR or RBP by the choroidal elements of our freshly dissected RPE-containing monkey tissue. It is not surprising that the signal is quite strong from the enriched poly(A$^+$) mRNA sample obtained commercially from monkey liver, the major source of circulating TTR in vertebrates (Figure 2A). Furthermore, given that the choroid plexus and meninges would be expected to be the major contributor of specific TTR mRNA expression in the brain [35,45], considerable dilution of the message must have taken place in the whole brain sample we utilized, as no signal is seen in the 2 h exposure (Figure 2A). A much longer exposure did reveal a faint band representing a TTR transcript of the expected size, 900 bp, for monkey brain (unpublished data). Bearing in mind, first, that the two RPE samples were composed of total RNA, of which only a small percentage (possibly as low as 2% by weight) would be expected to be the poly(A$^+$) mRNA fraction, and second, the relative density, as revealed by visual inspection, of the bands from the RPE samples vs. liver poly(A$^+$) mRNA, it can be deduced that TTR mRNA levels are dramatically higher in native RPE than in liver. Quantitative densitometry was utilized to demonstrate that TTR mRNA in RPE may be further upregulated as a result of culture conditions (Figure 2A,D). Cultured RPE may also be expressing some higher molecular size splice variants, at 2.4 kbp (Figure 2C) and 7.5 kbp (unpublished data) in low copy number, observed in longer exposures. These auxiliary bands do not represent ribosomal RNA, since they are not present in the native RPE/choroid sample. Their appearance may indicate transcription of heterogeneous nuclear RNA originating with open reading frames within introns; however, these large species are not believed to be productively expressed in other tissue sources of TTR [46].

The northern blot for RBP (Figure 3A), in like manner, indicates that the human probe we employed provided sufficient specific binding to detect message for RBP in all the monkey tissues examined, at a transcript size of 1100 bp, which corresponds well with the accepted size of the main human transcript [36]. As opposed to the result for TTR, both native and cultured monkey RPE express this mRNA species in relatively low abundance, especially when compared with monkey liver. Nevertheless, to an even greater extent than for TTR, densitometric analysis of the 1100 bp bands revealed that cultured RPE expressed RBP message in higher copy number than freshly dissected RPE/choroid (Figure 3A,C).

DISCUSSION

This is the first characterization of expression of message for TTR and RBP in cultured RPE, and it is notable that this was possible utilizing total RNA extracted from the equivalent of one 12-cluster well containing fewer than 10^6 cells, and by means of northern analysis without amplification by RT-PCR. Furthermore, the ability to detect message for monkey TTR and RBP using human probes of full or partial length, and the high stringency conditions utilized for the analysis, are indicative of high homology between the human and monkey RNA transcripts for these two proteins. Therefore, the design of specific probes for further investigations of TTR and RBP expression in monkey tissues would be straightforward.

Although it cannot always be assumed that expression levels of a particular protein and the copy number(s) of its corresponding mRNA transcript(s) are directly and functionally correlated, our results agree qualitatively with the findings of previous studies with respect to the abundance of both TTR and RBP mRNA and protein in the rat eye [24,25,28].

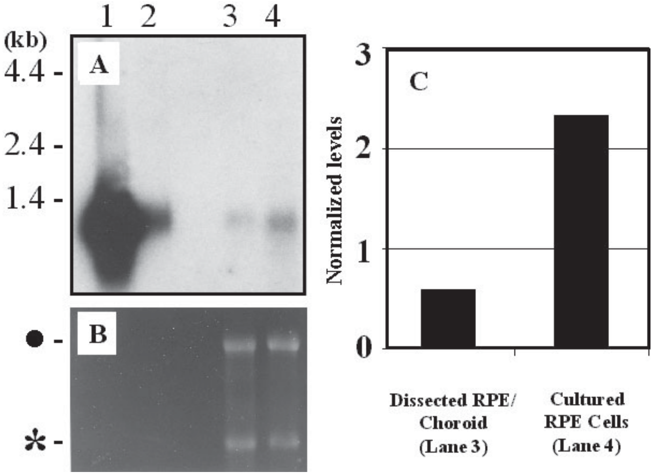

Figure 3. Northern analysis for retinol binding protein in monkey tissues. **A**: 18-day autoradiographic exposures are displayed in the upper part of lanes 1-4. For lanes 1 and 2, 1 μg poly(A$^+$) mRNA from monkey liver and brain, respectively, were loaded. For lanes 3 and 4, 3 μg of total RNA from freshly dissected monkey RPE/choroid complex and from cultured monkey RPE cells, respectively, were loaded. For monkey liver, a single dense band centered around ca. 1100 bp is evident (lane 1). Monkey brain also expresses one 1100 bp transcript (lane 2). Both monkey RPE/choroid (lane 3) and cultured RPE (lane 4) exhibit 1100 bp bands. **B**: The RNA electrophoresed on the agarose gel was stained with ethidium bromide and visualized with ultraviolet illumination before transfer to nylon membrane. The lower part of lanes 1-4 corresponds to the samples described in **A**, above. The density of the 28s (denoted by black dot) and 18s (asterisk) ribosomal RNA bands for fresh RPE/choroid (lane 3) and cultured RPE (lane 4) are a function of the relative loading with total RNA. **C**: Densitometric analysis of the 1100 bp bands from lanes 3 and 4 of the northern blot in **A**, above, using the 18 s ethidium bromide stained band in lane 4 of **B**, above, for normalization. The RBP mRNA in cultured RPE is 3.9 times as abundant as that for freshly harvested RPE/choroid.

Essentially, rat RPE in situ exhibits a strong signal representing TTR message, in contrast to that for RBP, which is at a relatively lower level. For protein expression, TTR immunoreactivity in rat RPE is intense, while this cell layer is not considered a prominent site of RBP intracellular localization, compared to other eye tissues. Accordingly, we find that TTR message occurs in high abundance in both native and cultured monkey RPE cells, while RBP mRNA transcripts are maintained at a much lower, but still detectable level. This disparate relationship—for two proteins that normally show close physiological association in the systemic circulation—is not inconsistent with the apparently efficient biosynthesis and release of TTR, and our ease of detection for this protein, in contrast to the lack of same for RBP, in the RPE-CM. TTR was plentiful enough, after 10 days of accumulation in initially protein-free culture medium, to be demonstrated by western blotting; however, RBP was below our level of detection with a commercially available antibody [unpublished data]. For bovine and fetal human RPE in culture, Ong, et al. [23] calculated the TTR:RBP ratio in conditioned media to be approximately 50:1 and 30:1, respectively. Their purification methods suggest that, at least in isolated cell culture, there is some expected physicochemical association of the two molecules either during, or after, their secretion into conditioned medium, but it is clear that an excess of TTR is produced by RPE cultures.

Our findings may well indicate that, save for the choroid plexus [47,48], the abundance of TTR message in RPE is unmatched by any other tissue of the body. This possibility further raises questions concerning the normal function of TTR in the eye, and whether such a prominently secreted protein is implicated in ocular pathology. For example, the demonstration that TTR is a component of Drusen, although not at the frequency or intensity of detection as other more prominent Drusen constituents, suggests the involvement of TTR in age-related macular degeneration [49]. Whether TTR in this case originates from the affected RPE cells or from plasma has not yet been ascertained. Both the senile and familial forms of amyloidotic polyneuropathy, characterized by fibrillar accumulations of TTR, may have ocular involvement [50]. White and Kelly [43] have demonstrated that holo-RBP stabilizes the native tetramer of TTR and prevents amyloid fibril formation. In this regard, their findings suggest that, first, the availability of retinol as a potential inducer of RBP release (e.g., [51]; see below) and, consequently, the levels of transcription and of translation for RBP mRNA would both modulate the ocular penetrance of TTR mutations predisposing individuals for this type of amyloidosis. Ultimately, therefore, the proportionate activities of the TTR and the RBP genes in the RPE may be among the critical factors maintaining homeostasis within the eye.

Tissue-specific regulation of levels of mRNA for TTR and RBP by RPE cells is expected to be influenced by interactions between certain consensus sequences in the individual promoter regions of the genes for these two proteins, and transcription factors differentially expressed or activated in RPE. In experiments utilizing a human hepatoma cell line, both retinol and retinoic acid stimulated RBP gene expression at the mRNA level [52]. This latter result may reflect the elucidation of a retinoid-responsive element in the human RBP gene [53]. Whether this route of induction is operative for cultured RPE, and what concentrations of retinoids in the medium would be required to up-regulate RBP gene and/or protein expression above the levels seen in the present study, remain to be determined. The precise mode of delivery of vitamin A to the cultured RPE may prove to have a bearing on its effects [4]. Other factors that may modulate RBP expression in vitro are the presence of serum [54], and the cyclic AMP status of the cells [55].

Since in our study the size of the major transcript for TTR in monkey was identical in RPE, liver, and brain, the implication is that identical initiation sites exist in the TTR gene for all three tissues, regardless of the relative position of any regulatory DNA sequences. Our finding of low abundance, additional transcript sizes for TTR in cultured RPE could be indicative of regulatory elements governing a transcription start site further upstream from the preeminent one utilized in hepatocytes. We did not detect any variations in the size of the RBP mRNA, among the tissues we tested, that would have indicated variations in splicing or in initiation of transcription.

A more complete understanding of the transcriptional control of the two genes investigated here could yield practical information with clinical significance. Much as the predominant expression of RPE-65 in RPE has been explored as a platform for gene therapy [56], so the in vitro expression of TTR by RPE, including secretion and extracellular accumulation of the protein, may prove amenable to therapeutic exploitation. In this regard, the possibilities include applications in gene therapy, such as transplantation of transfected RPE, as well as gene manipulations to drive in vitro protein production. It is therefore pertinent that we have demonstrated here robust expression of the TTR gene, with respect to transcript abundance, in primate RPE cultured in a totally defined, protein-free medium.

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