



Regional expression of disease-related genes in human and monkey retina

Steven L. Bernstein,¹ Paul Wong²

¹Laboratory of Molecular Research, Department of Ophthalmology, University of Maryland, Baltimore, MD; ²Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada

Purpose: Although specific genes play a role in regional retinal disease, the correlation of regional gene expression in the disease-affected site has not been previously ascertained. Non-human primates are widely used in models of human retinal function and are theorized to have identical (to human) patterns of expression, but no correlation between primate and human regional retinal gene expression has ever been performed. We wanted to evaluate the pattern of regional gene expression for a number of genes whose dysfunctions are known to selectively affect specific regions of the human retina, and to determine whether patterns of regional gene expression in nonhuman primates correlate with the human.

Methods: Human and rhesus monkey eyes were dissected into retina, retinal pigment epithelium (RPE)/choroid and isolated RPE. Retinal regions were dissected, total RNA was isolated and northern analysis performed. Complementary DNA (cDNA) probes were prepared from genes associated with regional retinal disease. These genes are: rod opsin, the α -subunit of rod phosphodiesterase, RDS-peripherin, rod outer membrane (ROM) protein, ornithine aminotransferase (OAT), choroideremia gene product (CHM), tissue specific inhibitor of metalloproteinases-3 (TIMP-3), and red/green photoreceptor pigment protein. We also compared expression of Norrie disease product (NDP), a gene whose mutation is known to globally affect the retina.

Results: Rod-specific mRNA expression is highest in the retinal midperiphery, and cone-specific mRNA levels were highest in total RNA from the cone-dominant fovea. mRNA levels for genes coding for proteins expressed in both rod- and cone photoreceptors (RDS-peripherin and ROM-1) are also highest in total RNA from the retinal midperiphery. Regional mRNA levels of CHM and OAT do not directly correlate with their patterns of disease expression. NDP mRNA expression was equivalent in both fovea and midperipheral retina total RNA. Patterns of gene expression were qualitatively similar for both human and rhesus monkey retina.

Conclusions: Regional retinal gene expression is an important factor in regional disease. However, for genes not solely expressed by a single photoreceptor subtype, other factors, such as regional metabolic differences, intra- and intercellular interactions, are also likely to be important in predisposing a single retinal region to disease. The pattern of neural retina OAT mRNA expression may have important implications in determining the appropriate tissue approach in gene therapy for gyrate atrophy. Regional retinal gene expression likely plays a significant, but nonexclusive role in the development of regional retinal disease.

The primate retina is regionally specialized for distinct functions. The retinal foveomacula, enriched in cone photoreceptor cells, is responsible for daylight and acute (20/20) vision, while the retinal periphery consists largely of rod photoreceptor cells, and is responsible for peripheral and night (scotopic) vision [1,2]. Many retinal diseases in man preferentially affect, or have their greatest debilitating effect on one retinal region. These include retinitis pigmentosa (RP), choroideremia (CHM), gyrate atrophy (GA) and cone-rod dystrophies. The initial affected area can involve either fovea or peripheral retina, and may gradually progress to involve the entire retina [3].

The variable clinical presentation and location of regional retinal diseases suggests that a number of factors are responsible for regional disease. Despite our knowledge of causative gene mutations however, the reasons for relative regional involvement in the development of these diseases remains ob-

scure. Since fovea and peripheral retina have relative differences in the ratio of specific photoreceptor types, one cause of regional retinal disease may be relative regional differences in the number of cells expressing a given gene. Relative cell number differences however, cannot be the only variable. Mutations in a number of universally transcribed genes (genes expressed in all tissues) can also result in specific retinal regional disease [4-6]. This suggests that other factors, such as relative differences in regional metabolism, may also predispose specific retinal regions to disease [6-8]. The latter hypothesis suggests that genes whose activity is selectively required at increased levels for region-intensive function (differential gene expression), are therefore candidates for region-specific disease [9]. Strategies based on this hypothesis have been used in a number of studies, to identify such gene candidates for region-specific disease [9-12].

Supporting the latter hypothesis are genes associated with region specific disorders and known to be differentially expressed in preferentially affected retinal regions or associated tissues. These include the mitochondrially encoded gene NADH4, which is more highly expressed in the fovea than in the midperipheral retina [13], and the gene for tissue inhibitor

Correspondence to: Steven L. Bernstein, Laboratory of Molecular Research, Department of Ophthalmology, University of Maryland, MSTF Building, Room 5-65, 10 South Pine Street, Baltimore, MD, 21201; Phone: (410) 706-4482; FAX: (410) 706-4483; email: slbernst@umabnet.ab.umd.edu

of metalloproteinases-3 which is highly expressed in retinal pigment epithelium (RPE) cells [14]. Mutations in the NADH4 gene underlie the majority of cases of the foveomacular disease Lebers Hereditary Optic Neuropathy (LHON) [6]. LHON is a primary disorder of retinal ganglion cells, which are found in greatest concentration in the foveomacular region, and whose axons comprise the optic nerve [2]. Mutations in TIMP-3 are known to be responsible for Sorsby's fundus dystrophy, a disorder that preferentially affects the RPE [5]. These findings suggest that differential gene expression may help to explain, in some cases, the presentation of region specific retinal disease. However, except for NADH4 and TIMP-3, the regional tissue expression patterns of other genes known to be associated with regional retinal disease are unknown. We undertook the present study to determine the regional retinal expression profiles of genes known to underlie different forms of genetic retinal degeneration, and to correlate regional levels of specific mRNAs in specific regions of the normal retina which are known to be affected by the respective disorder. We evaluated for differential expression of photoreceptor cell-specific genes directly involved in the phototransduction cascade (opsin, red/green cone photoreceptor pigment, and α -PDE). Mutations in opsin and α -PDE underlie specific forms of retinitis pigmentosa (RP), a group of diseases with a similar clinical

phenotype, which preferentially affects the midperipheral retina [15-17]. Mutations in red cone photoreceptor pigment are associated with progressive cone degeneration (PCD) [18]. We also evaluated photoreceptor-specific structural genes common to cones and rod photoreceptors, rod outer membrane (ROM-1) protein and retinal degeneration slow (RDS/peripherin). Mutations in these have been implicated in forms of RP [19,20], as well as in foveomacular dystrophies [21,22]. The regional retina- and associated tissue expression of an additional group of non-retina specific genes, in which mutations are known to underlie various forms of regional (peripheral) retinopathy, were also compared. These are ornithine aminotransferase (OAT), involved in gyrate atrophy (GA) and CHM, which is associated with choroideremia. Northern analysis of TIMP-3 mRNA levels in retinal regions, the RPE-choroid complex and isolated RPE, was also performed. The expression of the NDP gene was also compared. Mutations in NDP are associated with generalized retinopathy (Norrie disease), as well as severe retinopathy of prematurity [4,5,23-25]. We also compared the regional retinal tissue gene expression patterns of human and another primate, the rhesus monkey. Monkey retina is often used as a model of human retinal function, because human tissue is difficult to obtain, and in most cases there is a significant length of time (in humans) between death and isolation of individual tissues. However, a direct correlation between human and monkey retinal gene expression has never been previously performed, thus little empiric evidence exists to provide a comparative baseline. Our study provides a comparison of regional retinal expression patterns of the two primate species. This will augment future work in the role of human regional gene expression in regional retinal disease.

METHODS

Human donor eyes were obtained through the National Disease Research Interchange (NDRI, Philadelphia, PA). Eyes were obtained from donors ranging from 12-80 yr of age. A total of ten pairs of donor eyes were utilized in this study with the average donor age 50.3 yr. The average time from death until dissection was 43.5 hr. Prospective donor tissue was rejected if the donors had any ocular or systemic disease or therapy known to potentially directly affect retinal function. Enucleated eyes were kept on ice and retinal tissue was dissected on ice. Dissected tissues included a 1.5 mm diameter central macular region centered over the foveolar umbo (Figure 1, the fovea); and a 3 x 5 mm midperipheral retinal region, 2 mm nasal to the optic nerve (Figure 1, midperipheral retina). Dissected tissues were quick frozen on dry ice, and stored at -70 °C until use. Following retinal dissection, combined retinal pigment epithelium-choroidal (R/CH) tissue was isolated and immediately frozen on dry ice (flash frozen). Washed human choroidal tissue, and isolated retinal pigment epithelium (RPE) cells were prepared by washing the RPE-choroid complex with Dulbeccos phosphate buffered saline to separate the two tissues. RPE cells were isolated by low speed centrifugation of the wash supernatant. Both the choroid and isolated RPE cells were flash frozen separately and stored at -70 °C.

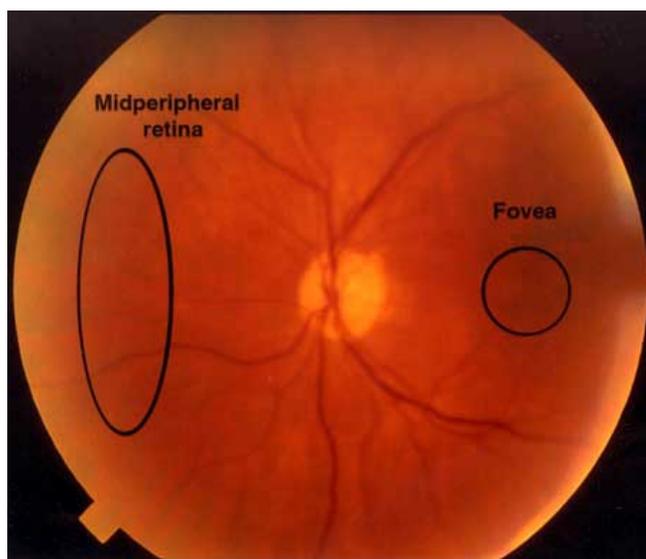


Figure 1. Human and rhesus monkey retinal regions used in the study. Demarcated areas indicate retinal regions used for the study. Human donor eyes were dissected on ice; the central 1.5 mm diameter (circle) surrounding the foveolar umbo, as well as a strip of midperipheral retina (oval area) were freed from underlying RPE and choroidal tissue and flash frozen on dry ice. Dissection of rhesus monkey retina was similar, except that the diameter of the dissected foveal tissue was 1.0 mm, due to the smaller size of the monkey eye. Pooled tissue from five individuals was used for total RNA isolation. Human RPE cells were isolated by washing the anterior surface of the choroid/RPE complex with iced phosphate buffered saline, centrifuged at low speed, and stored as a pellet prior to RNA extraction. Pooled RPE/choroid from monkey was flash frozen without further dissection. Total RNA was isolated as described in methods.

Rhesus monkey eyes were kindly provided by J. Cogan (Bureau of Biologics, Bethesda, MD). Animals were humanely sacrificed at 24-36 months of age. All animals used in this experiment were cared for and sacrificed humanely, following the guidelines outlined in the United States Public Health Service policy on the humane care and use of laboratory animals. Following sacrifice and enucleation, rhesus monkey ocular tissues were regionally dissected on ice, as described above, except that the fovea tissue region was restricted to 1.0 mm in order to account for the relatively smaller size of the monkey eye. Total RNA was extracted from all tissues using RNAzol B (Tel-test, Friendswood, TX). Total RNA was reprecipitated and resuspended in DEPC treated water, and stored at -70 °C until use.

cDNA probe preparation: cDNA fragments corresponding to the mRNA sequences of individual genes (opsin, red/green cone photoreceptor pigment, the α -subunit of rod phosphodiesterase (α -PDE), RDS-peripherin, TIMP-3, OAT, CHM, and NDP) were synthesized using sequence-specific primers generated from information obtained from Genbank (Table 1). Oligonucleotide primers were synthesized on a model 373 DNA synthesizer (Perkin-Elmer/ABI, Foster City, CA). cDNA inserts were generated by the polymerase chain reaction (PCR), using first-strand cDNA reverse-transcribed from either human retina or brain poly(A+) RNA, and the appropriate primers. PCR products used as probe substrates were analyzed for size and sequenced to insure fidelity. A cDNA corresponding to mRNA for human ROM-1 protein was identified from the human fovea library [10]. cDNA fragments were labelled us-

ing a prime-it II kit (Stratagene systems, La Jolla, CA) and ³²P-labelled dCTP (Dupont-NEN, Boston, MA), to a specific activity of approximately 1 x 10⁹ CPM/g.

Northern analysis: Five μ g of total RNA per lane from either human (HF) or monkey fovea (MF), midperipheral retina (human: HPR, monkey: MPR), total retina (HR), RPE/choroid, isolated human choroid, and isolated human retinal pigment epithelial cells (RPE) were denatured, electrophoresed, transferred and immobilized as previously described [10]. When rhesus monkey retina RNA was analyzed (MF or MPR), five μ g of human total retina RNA was included in the northern analysis as a positive control (HR). In all cases northern blots were prehybridized in Hybrisol II (Oncor, Gaithersburg, MD) for 4 hr, followed by hybridization with the specific radiolabelled cDNA probe at 63 °C for 18 hr. After hybridization, membranes were stringently washed in 0.2X SSC at 63 °C and exposed to Kodax XAR or BMR photographic film for varying lengths of time at -70 °C. Band intensities on autoradiographs and photographic negatives of ethidium bromide-stained gels were quantitated using an LKB XL enhanced laser densitometer and gel-scan XL (version 2.1) software (Pharmacia, Piscataway, NJ). To control for slight differences in total RNA loading, the relative intensity of the 18S ribosomal band in each sample was used to normalize the DNA probe value per band in question [26].

RESULTS

Expression of rod and cone specific genes in human and rhesus monkey retinal regions: An essential feature of fovea histol-

Table 1. cDNA probes used in the study. The sequence of the oligonucleotide primer pairs used to generate the cDNA probes are shown, in the 5'-3' orientation. Predicted cDNA gene product size is shown, in base pair (bp). cDNA product identities were confirmed by dideoxynucleotide sequencing. The predicted sizes of known mRNA species corresponding to published genes are shown (expected). Accession numbers for the gene sequences used to design the oligonucleotide primer pairs are shown. PCR conditions used to generate the cDNA fragments are described in methods. Opsin: human rod opsin. α -PDE: the α -subunit of rod phosphodiesterase. ROM-1: human ROM-1. RDS: Human RDS/peripherin. CHM: Choroideremia gene product. OAT: Ornithine aminotransferase. TIMP-3: Human tissue of metalloproteinases-3. NDP: Norrie disease gene product.

Name	5' Primer Sequence	cDNA size	mRNA (exp)	Genbank Accession Number
Red Cone	GCT ACC TCC AAG TGT GGC TG ATG CAG GCG ATA CCG AGG ACA	400 bp	1.5 kb	M13300, M13301, M13302, M13303, M13304, M13305
Opsin	GCC GGG GCC ACC TGG CTC GTC TC CGT CTT GGA CAC GGT AGC AGA G	156 bp	1.75 kb	K02281
α -PDE	AGA CGA GAG CCT GAA TAT CT CAG TGG TGT TAC TGG ATG CA	600 bp	3.0 kb	M26061
ROM-1	hfv100327; fovea EST (library)	800 bp	1.4 kb	M96759
RDS	CAA GTA TGC CAG ATG GAA GC TGA GGC TGC TGT AGT AGC TCA	530 bp	3.0, 5.5 kb	M62958
CHM	AGC CTC TGG CTG TAA ACT GTC T TGT GCT CAC GTG TGC AAT ACA	548 bp	3.5 kb	X78121
OAT	ACG TTG TTC TAT TAT GTA TCA ACT CAT GAA GCT ACC TCC TGA	424 bp	2.2 kb	M14963
TIMP-3	CAT GTG CAG TAC ATC CAT ACG GTC TGT GGC ATT GAT GAT GCT	400 bp	5.5, 2.8, 2.4 kb	U02571
NDP	TGC GAG GGG CAC TGC AGC CAG TGG CTG GTC GAA CTG CCT CTA	270 bp	1.86 kb	X65882

ogy is a high density of cone photoreceptor cells compared to other regions of the retina [1]. Northern analysis using total RNA from retinal tissues taken from both human and rhesus monkey eyes demonstrate that the hybridization signal of a red/green cone photoreceptor pigment probe is strongest in total RNA from fovea, as opposed to midperipheral retinal tissue (Figure 2A, HF and HPR). In contrast, the hybridization signal following northern analysis using rod photoreceptor cell specific probes (opsin and α -PDE) is stronger using total RNA from human and rhesus monkey midperipheral retina (Figure 2B,C, MPR and HPR), than from fovea (Figure 2B,C; MF and HF). The differential hybridization patterns of the cone and rod photoreceptor specific gene probes (markers

of the foveomacular and midperipheral retina respectively) thus correlate well with the relative cell densities of cone and rod photoreceptors in the two retinal regions. The northern signal generated by the opsin cDNA probe reveals three distinct bands in both human (Figure 2B, HPR) and monkey retinal tissues (Figure 2B, MPR), similar to that reported by other investigators [27]. Variation in opsin mRNA size is apparently due to the presence of cryptic polyadenylation sites in the untranslated region in the primate opsin gene [27]. The relative difference in signal intensity between fovea and midperipheral retina for rod and cone-specific genes studied are similar in human and monkey retina, both for relative regional concentration differences, as well as for the number of apparent mRNA species. The relative ratios of the opsin band signal intensities between the midperipheral retina and fovea lanes are also similar for both human (3.9:1) and monkey (2.7:1). The signal differences give a relative range of expression for identical genes between primate species. Like opsin levels, the mRNA signal for the rod photoreceptor specific gene α -PDE is higher in total RNA

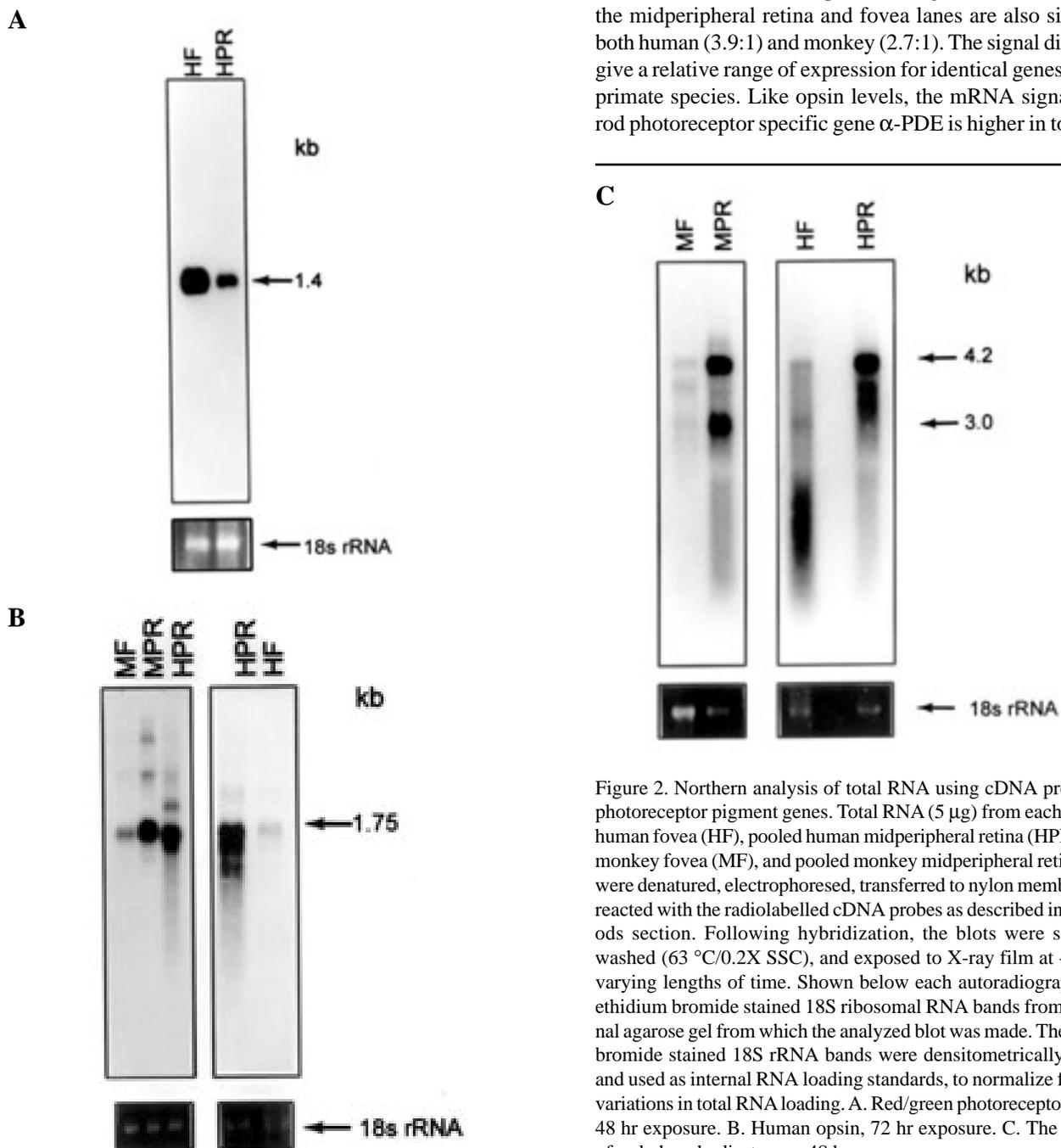


Figure 2. Northern analysis of total RNA using cDNA probes from photoreceptor pigment genes. Total RNA (5 μ g) from each of pooled human fovea (HF), pooled human midperipheral retina (HPR), pooled monkey fovea (MF), and pooled monkey midperipheral retina (MPR) were denatured, electrophoresed, transferred to nylon membranes and reacted with the radiolabelled cDNA probes as described in the methods section. Following hybridization, the blots were stringently washed (63 $^{\circ}$ C/0.2X SSC), and exposed to X-ray film at -70 $^{\circ}$ C for varying lengths of time. Shown below each autoradiograph are the ethidium bromide stained 18S ribosomal RNA bands from the original agarose gel from which the analyzed blot was made. The ethidium bromide stained 18S rRNA bands were densitometrically analyzed and used as internal RNA loading standards, to normalize for sample variations in total RNA loading. A. Red/green photoreceptor pigment, 48 hr exposure. B. Human opsin, 72 hr exposure. C. The α -subunit of rod phosphodiesterase, 48 hr exposure.

from retinal midperiphery than from the fovea (Figure 2C). Increased background is seen in the human tissue α -PDE northern blot, suggesting slight degradation; a common problem when using human tissue (Figure 2C, compare MPR to HPR).

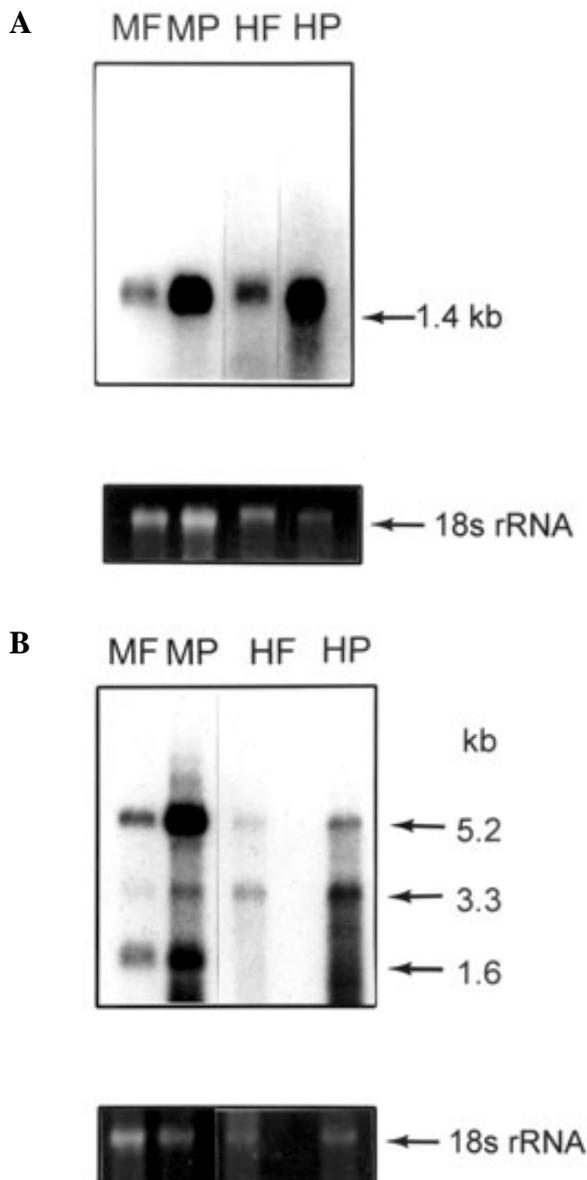


Figure 3. Northern analysis of total RNA using cDNA probes from photoreceptor structural genes. Total RNA (5 μ g) from each of pooled human fovea (HF), pooled human midperipheral retina (HP), pooled monkey fovea (MF), and pooled monkey midperipheral retina (MP) were denatured, electrophoresed, transferred to nylon membranes and reacted with the radiolabelled cDNA probes as described in methods. Following hybridization, the blots were stringently washed (63 $^{\circ}$ C/0.2X SSC), and exposed to X-ray film at -70 $^{\circ}$ C for varying lengths of time. Shown below each autoradiograph are the ethidium bromide stained 18S ribosomal RNA bands from the original agarose gel from which the analyzed blot was made. The ethidium bromide stained 18S rRNA band was analyzed densitometrically and used as an internal RNA loading standard. A. RDS/peripherin, 5 day exposure, B. ROM-1, 5 day exposure.

However, there is consistency of relative band intensities between human and monkey tissue (compare all bands in Figure 2C). This again demonstrates the value of direct comparison between human and monkey tissue. Northern analysis using the α -PDE probe recognizes a single abundant mRNA species at 4.2 kb in both primate types (Figure 2C, MPR and HPR). However, faint bands at 0.6, 1.0 and 2.8 kb are also detected in both primate species (Figure 2C, MPR and HPR). The corrected signal intensity ratio for the major transcript in total RNA from rhesus monkey midperiphery and fovea is 3.5:1.

Northern analysis of RDS-peripherin and ROM-1 in retina and RPE/choroid: RDS-Peripherin and ROM-1 are photoreceptor-specific genes that are proposed to play a major structural role in maintaining photoreceptor integrity [28-31]. It has been suggested that distinct rod and cone-specific ROM-1 genes may exist [28]. Rds/peripherin is believed to be a single gene locus that is expressed in both cone and rod photoreceptors [29]. Mutations in the rds-peripherin gene have been implicated in both peripheral [19,20], as well as foveomacular genetic disorders [21,22]. Mutations in ROM-1 have only been shown to be associated with peripheral retinopathy [20]. In the current study, a single ROM-1 mRNA transcript was found in total RNA from fovea and midperipheral retina, in humans and monkey (Figure 3A). The ROM-1 message concentration is higher in midperipheral retina total RNA, than in fovea total RNA, for both primate species (ratio of 3.0:1 for human: Figure 3A, HP and HF; ratio of 1.7:1 for monkey: Figure 3A, MF and MP). Northern analysis using the rds/peripherin probe detects two (5.2 and 3.3 kb) mRNA species in human retina (Figure 3B), consistent with previous observations [29]. However, the rds/peripherin cDNA probe, reacted with monkey retinal total RNA from fovea and midperipheral retina, detects three messenger transcripts; two major bands at 5.2 and 1.6 kb, and a minor species at 3.3 kb (Figure 3B, MF and MP). The signal is strongest for total RNA from midperipheral retina for both human and monkey (compare MF to MP and HF to HP in Figure 3B). The midperiphery:fovea signal ratio of the 5.2 kb mRNA species in monkey retinal RNA is 2.1:1.

Northern hybridization analysis of TIMP-3 mRNA: Mutations in TIMP-3 underlie the retina-specific disorder, Sorsby's fundus dystrophy [5], which has its greatest visible effect on the foveomacular region. Northern analysis using both human and monkey midperipheral and fovea total RNA generated a low but detectable signal, after long exposures (Figure 4, HF, HPR, MF and MPR). TIMP-3 activity is apparently intrinsic to the retina, and not an artifact of residual RPE contamination of the retinal RNA preparation. This is because hybridization of a short (100 bp) radiolabelled RPE65 cDNA probe, specific to RPE cells [32], did not generate a detectable signal in total RNA from monkey retinal regions (data not shown). TIMP-3 signal is considerably higher in total RNA from monkey mixed choroid/RPE tissue than retinal tissue (Figure 4, R/CH). To identify the tissue that expresses the highest level of TIMP-3 mRNA in the human fundus, northern analysis was performed on total RNA from isolated human RPE cells, and compared with total RNA from washed choroid (Figure 4, RPE). Significantly higher levels TIMP-3 mRNA signal is

detected in total RNA from isolated RPE cells (Figure 4, RPE), than in washed choroid (Figure 4, choroid). This suggests that, in the ocular fundus, TIMP-3 activity is expressed in a variety of primate ocular tissues, but at highest level in the RPE.

Northern hybridization analysis of CHM: CHM gene mutations underlie choroideremia, a disease initially affecting the midperipheral retina with sparing of the foveomacular region [23,33,34]. Northern analysis was performed using a cDNA probe for CHM mRNA, against total RNA from human and monkey retinal regions and underlying choroid/RPE. These results are seen in Figure 5. For this study, the CHM probe was also reacted with total RNA from human brain (HB), ciliary body (HCB) and liver (HL); these are shown in Figure 5. A signal corresponding to a 4.3 kb messenger in total monkey RNA from both retinal regions is seen (Figure 5A, MF and MP). The signal is weaker in total RNA from human, but faintly detectable after long exposure (Figure 5A, HF and HP). The strongest CHM signal is detected in total RNA from human brain (Figure 5A). The apparently enhanced signal in brain total RNA, compared to retinal tissues, is an artifact of RNA loading, since densitometric correction using the 18S rRNA band indicates similar levels for both brain and retina. There is no difference in corrected CHM mRNA signal intensity in total RNA from either fovea or midperipheral retina in either primate species; this is best seen in total RNA from rhesus monkey (Figure 5A, compare MF to MP). A very faint signal is detected using the CHM probe against total RNA from RPE/choroid in rhesus monkey (Figure 5A). Little CHM signal is detected in total RNA from human ciliary body (HCB) or human liver (HL). These results suggest that the majority of CHM gene activity in the posterior pole of the primate eye is found in the neuroretinal compartment.

Northern analysis of OAT mRNA: Mutations in the OAT gene underlie the retinal disease gyrate atrophy (GA) [4]. Affected individuals exhibit myopia, night blindness and peripheral vision loss early in the course of the disease, with blindness typically by the fourth decade of life [35]. Typically, there is sparing of central vision until late in the disease [35]. Northern analysis was performed by reacting a radiolabelled OAT cDNA probe against total RNA from fovea and midperipheral retinal regions, from human retina and RPE/choroid (Figure 5B, HF, HP, and HR/CH) and monkey retina and RPE/choroid (Figure 5B, MF, MP and MR/CH). For both primate species, a single band is observed, corresponding to a 2.2 kb mRNA transcript. This result is consistent with previous observations in total RNA [33]. In both primate species, the OAT mRNA signal is strongest from total RNA from fovea (Figure 5B, HF and MF) compared to total RNA from midperipheral retina (Figure 5B, HP and MP). A weak OAT mRNA signal is generated from total RNA from RPE/choroid from both primate species (Figure 5B, MR/CH and HR/CH). A strong signal is seen from human brain total RNA (Figure 5B, HB), and a reduced signal from human liver and ciliary body total RNA (Figure 5B, HL and HCB).

Northern analysis of NDP mRNA: Norrie disease presents clinically as a generalized, retinal dysplasia [24,36]. A radiolabelled cDNA probe corresponding to a 247 bp fragment of

the NDP mRNA generates an equivalent signal, when reacted against total RNA from monkey fovea and midperipheral retina (Figure 5C, compare MF to MP). An equivalent or stronger

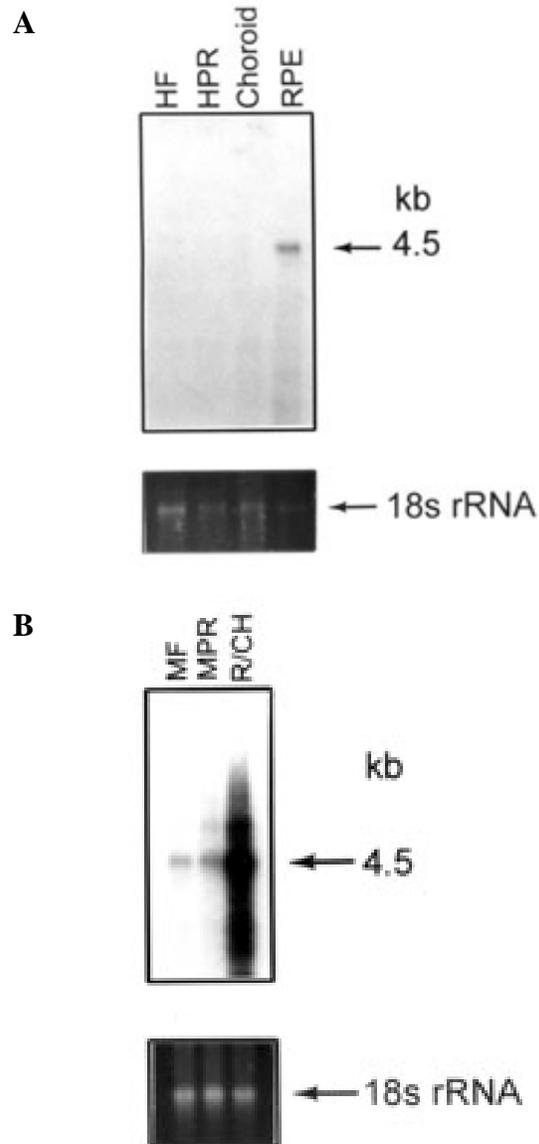


Figure 4. Northern analysis of total RNA using a cDNA probe from TIMP-3. Total RNA (5 μ g) from each of pooled human fovea (HF); pooled human midperipheral retina (HPR); washed (RPE free) human choroidal tissue (Choroid); isolated, washed human RPE (RPE); pooled monkey fovea (MF); pooled monkey midperipheral retina (MPR); and monkey mixed choroid/RPE tissue (R/CH) were denatured, electrophoresed, transferred to nylon membranes and reacted with the radiolabelled cDNA probes as described in methods. Following hybridization, blots were stringently washed (63 $^{\circ}$ C/0.2X SSC), and exposed to X-ray film at -70 $^{\circ}$ C for varying lengths of time. Shown below each autoradiograph are the ethidium bromide stained 18S ribosomal RNA bands from the original agarose gel from which the analyzed blot was made. The ethidium bromide stained 18S rRNA bands were analyzed densitometrically and used as internal RNA loading standards. RDS to normalize variation in individual RNA loading [26]. A. Analysis of expression from human fundus tissues, 14 day exposure. B. TIMP-3 expression in rhesus monkey tissue, 5 day exposure.

signal is generated from human total (undissected) retina total RNA from an 80 yr old individual (Figure 5C, HR). A single band corresponding to a 1.4 kb mRNA species is generated from total RNA in all tissues and species (Figure 5C). The NDP mRNA signal was strong even after short exposures (Figure 5C), suggesting that NDP mRNA is expressed at high levels in both the retina and choroid/RPE of juvenile (24-36 month old) rhesus monkey individuals, and may continue to be expressed at high levels later in life (compare MF and MP with HR).

DISCUSSION

This is the first study to evaluate regional retinal expression of genes known to cause regional retinal disease, and to correlate human and monkey retinal gene expression for these markers. Previous reports have indicated that retinal gene expression is not homogeneous across the retinal surface, but may be partially related to regional retinal cell composition [9,10] and specific metabolic requirements that vary in the different retinal regions [7,8]. Of the genes examined in this communi-

cation, there is an absolute conservation of qualitative expression between human and monkey eye tissues, in the different retinal regions.

In total, we have analyzed 9 genes for differential expression within different regions of the primate retina (see Table 1). Mutations within these genes are responsible for 10 distinct retinal disorders. Three of the disorders manifest primarily in the foveomacular region (Red cone photoreceptor pigment: progressive cone dystrophy, RDS-Peripherin: butterfly and pattern macular dystrophy, TIMP-3: Sorsby's fundus dystrophy). Six of the disorders are characterized by primary clinical affects in the peripheral retina. A single case, Norrie disease, affects the entire retina uniformly. Our analysis shows that in 6 of the 10 clinical pictures, the topographic levels of the mRNAs correlate with the initially affected retinal region. This suggests that, assuming only that a gene primarily associated with a retinal disease is expressed in the affected tissue, there is a 60% probability that the normal allele of a disease gene may be expressed in a region-specific profile which mirrors the region initially affected by the disorder.

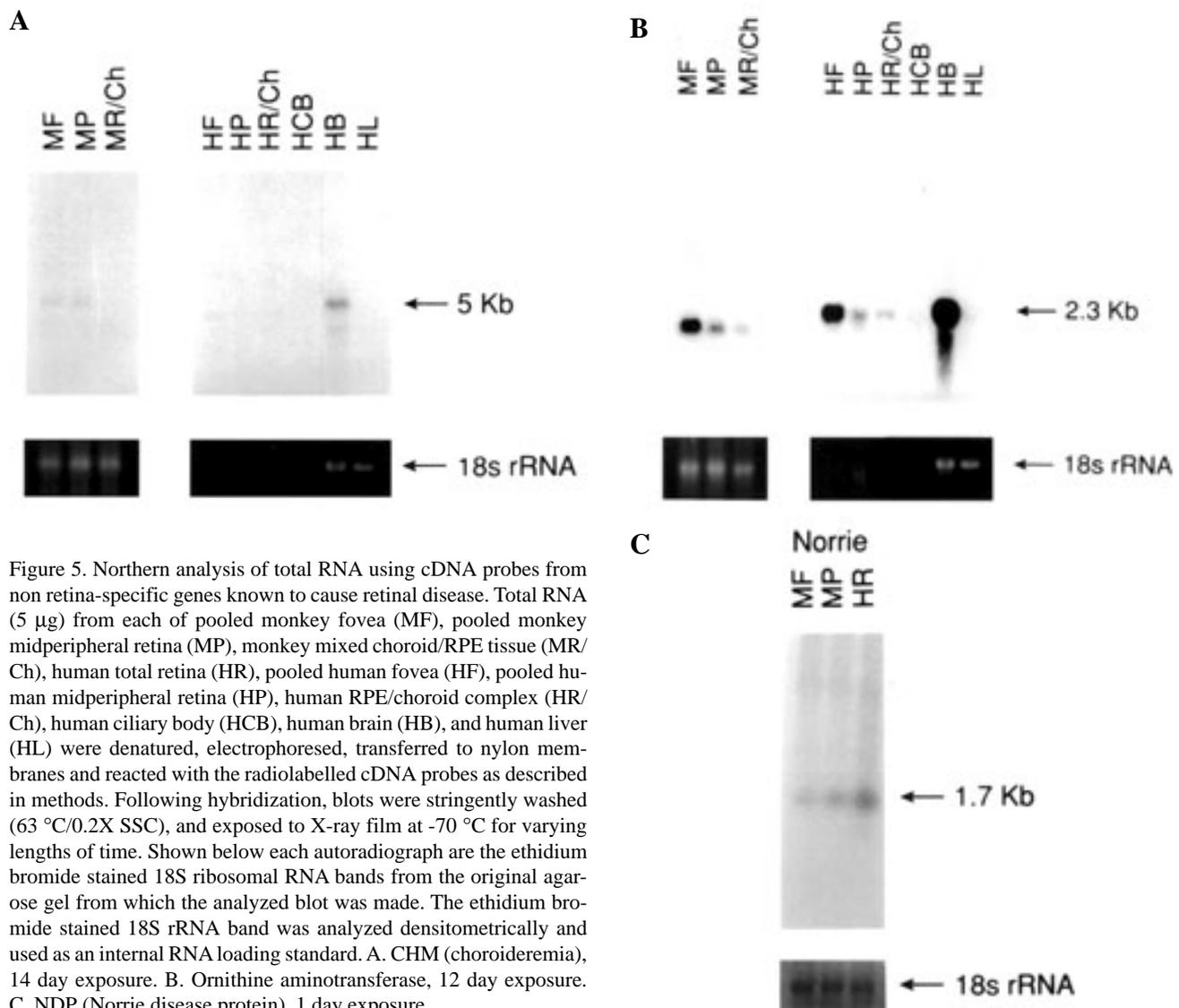


Figure 5. Northern analysis of total RNA using cDNA probes from non retina-specific genes known to cause retinal disease. Total RNA (5 μ g) from each of pooled monkey fovea (MF), pooled monkey midperipheral retina (MP), monkey mixed choroid/RPE tissue (MR/Ch), human total retina (HR), pooled human fovea (HF), pooled human midperipheral retina (HP), human RPE/choroid complex (HR/Ch), human ciliary body (HCB), human brain (HB), and human liver (HL) were denatured, electrophoresed, transferred to nylon membranes and reacted with the radiolabelled cDNA probes as described in methods. Following hybridization, blots were stringently washed (63 $^{\circ}$ C/0.2X SSC), and exposed to X-ray film at -70 $^{\circ}$ C for varying lengths of time. Shown below each autoradiograph are the ethidium bromide stained 18S ribosomal RNA bands from the original agarose gel from which the analyzed blot was made. The ethidium bromide stained 18S rRNA band was analyzed densitometrically and used as an internal RNA loading standard. A. CHM (choroideremia), 14 day exposure. B. Ornithine aminotransferase, 12 day exposure. C. NDP (Norrie disease protein), 1 day exposure.

The 40% dis-concordancy in regional tissue expression indicates that there is no absolute association between regional expression and the manifestation of the diseased state. The four discordant cases include the RDS-Peripherin gene with respect to macular degeneration, TIMP-3 with Sorsby's fundus dystrophy, OAT with gyrate atrophy, and CHM with choroideremia. Mutations in a rod photoreceptor-specific gene have also been recently identified as causative for a recessive form of Stargardt's macular dystrophy [37]. Consideration of these exceptions is important in order to obtain a more complete perspective on the compounding factors, which may influence the manifestation of the disease phenotype.

Mutations in the ornithine aminotransferase gene are known to underlie gyrate atrophy (GA). OAT is a nuclear-transcribed gene, whose protein localizes to the mitochondrial matrix [35]. OAT catalyzes the transfer of ammonia from ornithine and α -ketoglutarate to pyrroline 5-carboxylate and glutamate, allowing the degradation of ornithine to citrulline in the mitochondrial ammonia cycle [35]. OAT mRNA was initially reported to be expressed at highest levels in the retinal pigment epithelium [38]. Primate tissue, however, was not utilized in the earlier study. In contrast, we find that OAT mRNA levels are highest in neuroretina, with OAT mRNA levels highest in total RNA from the fovea, compared to the signal generated from midperipheral retina total RNA, in both primate species (Figure 5B). Interestingly, the lowest relative level of OAT activity in the fundus is seen in RPE/Choroid (Figure 5A). The neuroretina apparently has the highest level of oxidative phosphorylation (OX-PHOS) activity in the body [7,10]. Total RNA from the retina has a greater concentration of mitochondrial genome-transcribed mRNAs than total RNA from RPE/Choroid (data not shown). While no comparative ultrastructural studies exist, this suggests that neuroretina may have a higher relative intracellular concentration of mitochondria, than the underlying RPE/choroid complex. Thus, a high level of retinal expression of OAT, a mitochondrially associated protein, is not entirely unexpected. In general terms, knowledge of whether the primary defect is RPE- or retina-based is important to consider when attempting to develop appropriate local therapies for the treatment of this disease. Therapy strategies focusing on RPE-based OAT gene replacement may not provide adequate function to overcome OAT deficiency; suggesting that photoreceptor-based transfer may be more appropriate. Importantly, the relative OAT mRNA levels in the two retinal regions apparently do not correlate with the temporal appearance of the disease. In GA, the retinal midperiphery is involved first, with foveomacular symptoms a late manifestation of the disease [35]. This again suggests that, in addition to simple OAT gene activity deficiency, additional factors may be involved in the development of the regionally associated disease, gyrate atrophy.

The mRNA for red/green cone photoreceptor pigment is highest in total RNA from the cone-dominant fovea region (Figure 2A). In contrast, rod-cell specific gene expression (Figure 2B,C, opsin and α -PDE), is highest in total RNA from the rod photoreceptor cell-dominant retinal midperiphery. Expression of these photoreceptor-type specific genes thus correlate

with their known involvement with forms of RP [3,4,15,17], an otherwise heterogeneous group of diseases involving rod photoreceptors, which have a common presentation of night blindness and a progressive loss of the midperipheral retina visual field [3], and PCD [18]. This suggests that mutations in genes expressed specifically in primarily affected cells indeed correlate with their clinical presentation.

The regional gene expression patterns of both ROM-1 and RDS/peripherin suggest complex retinal functions. Northern analysis indicates that these genes are expressed at highest levels in total RNA from the retinal midperiphery; region of rod-photoreceptor dominance (Figure 3A,B). ROM-1 and RDS/peripherin proteins are hypothesized to play a major role in maintenance of the photoreceptor discal rim region, where they may form heteromeric complexes [28,29]. ROM-1 and RDS/peripherin are apparently expressed by both rod and cone photoreceptors [28,29]. Because both genes are expressed by all photoreceptors, one might predict that ROM-1 and RDS/peripherin mRNA levels should be similar in total RNA from both fovea and periphery. However, the relative levels of ROM-1 and RDS/peripherin message in rods and cones are unknown. An increased activity requirement for these proteins in rod photoreceptors, compared with cones, would account for a difference in the rod-enriched environment of the midperipheral retina. Other cellular requirements, such as differential messenger stabilization, may also play a role in regulating ROM-1 and RDS/peripherin mRNA levels in the two photoreceptor cell species. Our analysis predicts that levels of these proteins are higher in rod than in cone photoreceptor cells, which would be consistent with the morphological structure of the outer segment regions of each cell type as well as with the clinical profile of RP. RDS/peripherin gene mutations have been implicated in cases of autosomal dominant RP [19]. ROM-1 is associated with a form of digenic RP [20]. The expression of RDS/peripherin and ROM-1 thus correlate with the clinical presentation of RP.

Interestingly, mutations in RDS/peripherin have also been implicated in pattern macular dystrophies [39-41]. The precise mutations in RDS/peripherin underlying cases of RP and macular dystrophy are different, suggesting that the functional significance of certain amino acids in RDS/peripherin may be different in cone and rod photoreceptor cells [39]. Thus, RDS/peripherin may have cone-photoreceptor specific, and rod photoreceptor cell specific functions.

Alternatively, subtle alterations in RDS/peripherin structure may result in differences in foveal photoreceptor packing densities or foveal rod photoreceptor dysfunction. Our northern analysis of RDS/peripherin (and ROM-1) mRNA levels cannot differentiate between cone and rod photoreceptor derived RDS/peripherin and ROM-1 messages. Much work needs to be done to resolve this apparent discrepancy. Until we can differentiate between cone- and rod-derived RDS/peripherin, we can only conclude that the net profile of RDS-Peripherin mRNA expression is inconsistent with the clinical manifestation profile of a foveomacular pattern dystrophy.

TIMP-3 gene defects are associated with Sorsby's fundus dystrophy (SFD), a disease localized largely to the retinal

pigment epithelium [5]. TIMP-3 protein is a secreted inhibitor of metalloproteinase 3; a protein which plays an important role in extracellular matrix function [42]. In the mouse, *in situ* localization studies have suggested that TIMP-3 mRNA is transcribed solely by mouse RPE [14]. Our northern analysis of TIMP-3 mRNA in both human and rhesus monkey fundus indicate that while the majority of TIMP-3 mRNA activity resides in the RPE, low levels are also found in the normal primate neuroretina in a nonregional pattern (Figure 4A,B). Other investigators have found neuroretina expression of TIMP-3 only in eyes with retinitis pigmentosa [43]. In the present study, the majority of TIMP-3 mRNA activity (in the fundus) evidently resides in the RPE alone, with little signal found in total RNA from washed choroid (Figure 4A). This finding is significant and consistent with a previous observation [14]. Mutations in TIMP-3 most likely primarily affect the RPE. It is the secondary compromise of retinal function that results in scarring of the foveomacular region, and subretinal neovascularization [5].

CHM gene mutations underlie the disease Choroideremia, which clinically presents with visible choroid:RPE alterations, and subsequent impairment of normal retinal activity and retinal degeneration [34,44,45]. The CHM gene codes for REP-1, a geranyl-geranyl transferase, which activates RAB transferase, and functions by presenting newly synthesized RAB membrane proteins for prenylation and membrane delivery [33,45]. In the primate fundus, we find that CHM is expressed in the neuroretina (Figure 5A, MF and MP) rather than by the RPE/choroid (Figure 5B, RPE/choroid), where the disease is clinically apparent. We did not find any apparent regional concentration differences of CHM mRNA in fovea or midperipheral total RNA, in either human or monkey (Figure 5A). Clinically, however, affected individuals typically show sparing of central vision until late in the course of the disease. An issue to consider in the pathology of CHM (and OAT) is how non-specifically expressed genes can give rise to tissue- or region-specific disease. One attractive proposal is that other region specific factors may influence the manifestation of these disease forms [46]. Thus, regional expression of currently unidentified proteins that interact with the disease gene in question may bias tissue or region-specific disease susceptibility.

The global expression of the Norrie (NDP) gene correlates well with the clinical appearance of Norrie disease. In this condition, there is generalized fundus dysfunction and retinal hypoplasia [24]. The NDP gene apparently codes for a growth factor similar to glial derived nerve factor, which is critical for normal retinal development and function [47]. Northern analysis shows that expression of NDP mRNA levels are equivalent in total RNA from monkey fovea and midperiphery (Figure 5C, MF and MP), and similar to that from total RNA from older human retina (Figure 5C, HR). The signal strength generated by the NDP cDNA probe suggests a relatively high concentration of NDP mRNA in both primate species. These findings imply that NDP dysfunction is likely to cause widespread, non-regional changes in retina, and that the requirement for the NDP gene product is not age-

dependent.

Our study suggests that, in addition to regional distribution of rod and cone photoreceptors, a number of factors are likely to be involved in the pathophysiology of regional retinal disease. These include differences in the intragenic site of mutation, regional differential metabolic requirements, intercellular and tissue-tissue interactions, as well as other currently unknown factors [15,48]. With the caveat that closely related primate species can have significantly different-sized messenger species, there appears to be a general agreement with the retinal region transcription pattern of both humans and rhesus monkeys, at least in the mRNAs tested. In summary, we find that in 6 of 10 known cases, the retinal region expression pattern of the respective gene locus correlates with the retinal region affected in the disease state (NADH4/LHON, Opsin/RP, α -PDE/RP, RDS-Peripherin/RP, ROM-1/RP, NDP/Norrie disease). These findings suggest that analyzing the regional retinal gene expression of specific candidate genes can provide important clues for dissecting the pathophysiological basis for regional retinal disease.

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