Technical Brief

Stability of housekeeping gene expression in the rat retina during exposure to cyclic hyperoxia

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Recent evidence suggests a genetic component to oxygen-induced retinopathy (OIR), a robust experimental model of human retinopathy of prematurity. OIR lends itself well to quantitative analysis of gene expression in rodents with well-defined genetic backgrounds. Such analysis by real-time reverse transcription polymerase chain reaction (RT-PCR) requires the use of reference genes as internal standards for purposes of normalization. We sought to identify housekeeping genes showing stable retinal expression across different rat strains and developmental stages, that were not regulated by oxygen tension. Real-time RT-PCR was used to examine in normal (control) neonatal rat retina the expression of five candidate reference genes: acidic ribosomal phosphoprotein (ARBP), cyclophilin A (CYCA), gamma 2 actin (ACTG2), hypoxanthine guanine phosphoribosyltransferase (HPRT), and RNA polymerase 2 (RNAP2). ACTG2 was poorly expressed, whereas quantification of CYCA was confounded by putative amplification of pseudogenes. Expression of ARBP, HPRT, and RNAP2 was then quantified in dissected retinas from neonatal rats of three inbred strains (Fischer 344, Sprague Dawley, and Dark Agouti) under two different conditions of exposure to inspired oxygen (exposure to room air for 14 days from birth; exposure to cyclic hyperoxia for 14 days from birth). The average variation in relative expression between each pair of these three genes within each of the six cDNA test samples was used to assess stability of gene expression, relative to a standard retinal cDNA pool. The relative expression values for ARBP and HPRT were more closely correlated (r^2=0.80) than were those for either gene with RNAP2 (ARBP and RNAP2: r^2=0.31; HPRT and RNAP2: r^2=0.25). There was little variation among the six experimental groups for the normalized expression of ARBP or HPRT (p>0.05). In contrast, the normalized expression of RNAP2 varied significantly amongst experimental groups: Within each strain, expression was higher in the oxygen-exposed group than in the room air-exposed group (p<0.05). We conclude that ARBP and HPRT exhibit expression that is sufficiently stable under conditions of varying oxygen tension, to permit their use as housekeeping genes in at least one model of OIR in the neonatal rat.

The use of reference genes is indispensable in the quantification of real-time reverse transcription polymerase chain reactions (RT-PCR) [1,2]. The characteristics of an ideal reference gene include stable expression in samples from different subjects and under different experimental conditions, and expression to a similar extent as the gene of interest [1,3-5]. Constitutively-expressed genes subserving housekeeping or structural functions are commonly used as reference genes. However, any gene that is stably expressed in the tissue of interest may serve as a reference gene for the normalization of samples derived from the same tissue. Housekeeping genes are usually regulated to some extent [5-8], and the transcription of even stably-expressed genes varies with fluctuations in the cell cycle, and with oxygen and nutrient status [5].

The human neovascular retinopathies include diabetic retinopathy, sickle cell anemia, the wet form of age-related macular degeneration, retinal vein occlusion, and retinopathy of prematurity (ROP), a potentially blinding condition of premature infants. These disparate conditions converge on a common pathological pathway characterized by the proliferation of hyper-permeable, aberrant retinal blood vessels that are prone to hemorrhage and that predispose to retinal detachment [9]. The emergence of ROP as a clinical entity in the 1940’s and its inexorable rise in the ensuing decade triggered a multitude of studies aimed at determining its cause. Inspired supplemental oxygen therapy was soon identified as a major etiological factor [10], and a well-controlled multicenter clinical trial provided strong evidence of a causal association between early oxygen treatment and ROP [11]. Oxygen treatment does not account for all instances of this multifactorial disease [12-14], but it is widely accepted as a key agent in the pathogenesis of this condition.

Oxygen-induced retinopathy (OIR) is a useful experimental model of retinopathy of prematurity [15-21], this is increasingly being used to identify the pathways and genes involved in retinal neovascularization [22-26]. The under-vascularized state of the premature human retina closely resembles that of various newborn animals, for which retinal vascularization ordinarily occurs ex-utero. In OIR, neonatal animals (mice,
rats, cats or dogs) are exposed to supplemental oxygen for a period of days to weeks [15]. An initial hyperoxic exposure causes attenuation of the normal process of postnatal vascular development, and a subsequent period of relative hypoxia, typically involving exposure to the normoxia of room air, induces aberrant retinal vascular proliferation. The animals used in models of ROP are not premature and therefore lack many of the comorbidities of premature infants, such as problems resulting from pulmonary and gastrointestinal immaturity. Many of these comorbid ailments have the potential to modulate the ROP risk and its rate of progression. Despite these shortcomings, animal OIR models are still of value in the study of human ROP [17].

Retinopathy can be induced in rats, the model of OIR used in this study, by alternating cycles of hyperoxia and hypoxia [17-19]. The typical pathology affecting the retinal microvasculature is shown in Figure 1. Several experimental protocols have been devised to achieve arterial oxygen tensions in rats that closely approximate those observed in premature human neonates. However it appears that irrespective of the oxygen exposure protocol used, the various OIR models show similar changes in retinal vascular architecture and may show similar alterations in the expression of key angiogenic growth factors. Indeed, the pattern of retinopathy induced by cyclic hyperoxia in the rat is similar to that seen in premature human infants: Neovascularization typically occurs just posterior to the vascularizing front, and adjacent neovascular tufts occasionally unite to form a pre-retinal ridge [15]. However, the model also differs from human ROP in several respects. The concomitant proliferation of connective tissue with neovascularization is limited, such that the formation of a true fibrovascular ridge, a pathognomonic feature of human ROP, is rare, as is retinal detachment [15,17,18].

Recent evidence suggests a genetic component to OIR [27-29]. Appropriate reference genes must be identified for comparisons of gene expression among different inbred strains of rodent and among treatment protocols. As fluctuations in oxygen tension are central to OIR induction, a key criterion for the selection of candidate reference genes is stability of expression under varying oxygen tension. A search of the literature was conducted to identify potential candidate reference genes for use in studies of retinal gene expression during development and under conditions of hyperoxia as well as hypoxia [5,30-41]; pertinent studies are summarized in Table 1. Following this evaluation, acidic ribosomal phosphoprotein (ARBP; ribosomal protein), cyclophilin A (CYCA; protein folding and intracellular transport), hypoxanthine guanine phosphoribosyltransferase (HPRT; purine biosynthesis), RNA polymerase 2 (RNAP2; mRNA transcription), and gamma-2 actin (ACTG2; cytoskeletal protein) were chosen for further investigation. These genes play roles in a diverse range of cellular processes and are generally considered to be developmentally stable.

**METHODS**

**Experimental animals:** Inbred Sprague Dawley (SPD; albino coat, red eyes) and Fischer 344 (F344; albino coat, red eyes) rats were bred within the institutional animal facility. Inbred
Dark Agouti (DA; agouti coat, pigmented eyes) rats were obtained from the Institute of Medical and Veterinary Science (Adelaide, SA, Australia). Rats were allowed unlimited access to water and rat chow and were exposed to a 12 h light-dark cycle and a room temperature of 24 °C with 60% humidity. Animals were euthanized with an inhaled overdose of halothane anesthesia. All experiments were approved by the institutional Animal Welfare Committee and animal care guidelines comparable to those published by the Institute for Laboratory Animal Research (Guide for the Care and Use of Laboratory Animals) were followed.

Exposure of neonatal rats to cyclic hyperoxia and normoxia: The OIR model had, as its initial exposure phase, 14 alternating 24-hour cycles of hyperoxia (80% oxygen in air) and relative hypoxia (21% oxygen in air), hereafter referred to as cyclic hyperoxia. Female rats and their newborn litters were exposed within 12 h of birth to hyperoxia (80% O2 in air) for 24 h periods, alternating with 24 h periods of normoxia (21% O2 in air) in a humidified chamber for 14 days using a procedure described in reference [28]. An anesthetic blender and high-flow oxygen regulator were used to deliver oxygen to the chamber at 25 l/min. An oxygen concentration of 80±1% was maintained for the 24-hour duration of hyperoxic cycles. Age and strain-matched rats reared in room air were used as controls.

Preparation of RNA and cDNA samples: Rat eyes were enucleated immediately post-mortem into chilled diethylpyrocarbonate-(DEPC)-treated normal saline. Retinas were dissected, snap-frozen in liquid nitrogen, and stored at -80 °C. Total RNA was isolated from each retina using an RNeasy mini-kit (Qiagen, Valencia, CA). Contaminating genomic DNA was removed with DNaseI (DNA-free, Ambion, Austin, TX). Samples free of visible DNA contamination on a 1% agarose gel and with a ratio of 28S:18S rRNA approximating 2:1 were quantified by spectrophotometry (BioPhotometer, Eppendorf, Hamburg, Germany). RNA yields typically ranged from 4-16 mg per retina (mean 7.8 mg). Samples with an absorbance ratio at 260:280 nm of 1.9 were used for reverse transcription. Test cDNA samples were comprised of the cDNA products of pooled RNA samples made from equal quantities of RNA from one retina of three rats of one strain from at least two different litters, which had been exposed to identical experimental conditions. One mg of each RNA pool was reverse-transcribed using a first-strand cDNA synthesis kit (SuperScript III First-Strand Synthesis System, Invitrogen, Carlsbad, CA). A reverse transcriptase-free control sample was synthesized in parallel with each cDNA sample. In addition, retinal RNA derived from 10 rats, comprised of retiniae from 4 rats (F344, SPD and DA) exposed to room air and collected at postnatal days 5, 10, 14 and 18, 3 rats (one of each strain) exposed to cyclic hyperoxia for 14 days from birth and 3 rats (one of each strain) exposed to cyclic hyperoxia for 14 days and normoxia for a further 4 days.

Primer sequences for quantitative real-time reverse-transcription polymerase chain reaction: Primers for rat ARBP, ACTG2, CYCA, RNA2P, and HPRT were designed to flank an intron or exonic region with an absolute size of at least 60 bp.

<table>
<thead>
<tr>
<th>Reference gene</th>
<th>Study context</th>
<th>Stability of gene expression</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARBP; CYCA; GAPDH; 28S rRNA</td>
<td>Hypoxia in rats</td>
<td>ARBP expression stability not assessed.</td>
<td>30</td>
</tr>
<tr>
<td>ARBP</td>
<td>Normal murine post-natal retinal development.</td>
<td>ARBP expression stable during development, minor variation in expression between earliest time-points (day 1 and day 3).</td>
<td>32</td>
</tr>
<tr>
<td>ARBP</td>
<td>Mouse retina, brain, liver: RNA from mice of mixed ages and genotypes.</td>
<td>ARBP expression stability not specifically addressed.</td>
<td>33</td>
</tr>
<tr>
<td>ARBP; 28S rRNA; cyclophilin; GAPDH</td>
<td>Murine OIR: Measurement of gene expression after hyperoxia and during relative hypoxia.</td>
<td>ARBP expression stable at all time-points; 28S rRNA expression decreased slightly after 12 h of relative hypoxia; expression stability of other genes not stated.</td>
<td>34</td>
</tr>
<tr>
<td>Beta-actin; GAPDH; HPRT; SDHA</td>
<td>Rat cultured cortical neurons: response to anoxia with or without growth factors.</td>
<td>Expression stability of individual reference genes not stated; geometric mean of all four genes used for normalization.</td>
<td>35</td>
</tr>
<tr>
<td>Beta-actin; HPRT; GAPDH</td>
<td>Adult rat retina: Specific episodes of elevated intracerebral pressure.</td>
<td>HPRT and GAPDH showed stable expression.</td>
<td>36</td>
</tr>
<tr>
<td>Beta-actin; CYCA; GAPDH</td>
<td>Adult rat focal cerebral ischemia model.</td>
<td>beta-actin was upregulated in ischemia compared with controls.</td>
<td>37</td>
</tr>
<tr>
<td>CYCA</td>
<td>Murine OIR: Gene expression measured at 10 time-points after birth.</td>
<td>Significant down-regulation of CYCA and GAPDH with ischemia; beta-actin expression increased.</td>
<td>38</td>
</tr>
<tr>
<td>GAPDH; 18S rRNA</td>
<td>Neonatal rats exposed to hypoxia and reoxygenation; brain, other tissues examined.</td>
<td>No mention of reference gene expression stability but similar results attained after normalization with either gene.</td>
<td>39</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Adult rat retina; Ischemia/reperfusion injury. Development of rat brain from embryonic day 11 to adult; expression assessed at protein level.</td>
<td>GAPDH expression stability not assessed.</td>
<td>40</td>
</tr>
<tr>
<td>Beta-actin; gamma-actin</td>
<td>13 reference genes including RNAS2</td>
<td>Beta-actin significantly downregulated in neurons during normal development; gamma-actin developmentally stable.</td>
<td>41</td>
</tr>
</tbody>
</table>

The following abbreviations were used: acidic ribosomal phosphoprotein (ARBP), cyclophilin A (CYCA), glyceraldehyde-3-phosphate dehydrogenase, (GAPDH), hypoxanthine guanine phosphoribosyltransferase (HPRT), RNA polymerase II (RNAS2), succinate dehydrogenase complex, subunit A (SDHA).
Lytic genomic amplicon was always more than twice the length of the target cDNA amplicon.

Quantitative real-time reverse transcription-polymerase chain reaction: RT-PCR was performed with a RotorGene 2000 Thermal Cycler (Corbett Research, Mortlake, New South Wales, Australia). Each 20 µl reaction mixture contained 10 µl of SYBR Green master-mix (QuantiTect SYBR Green PCR Master Mix, Qiagen, Valencia, CA) containing hot-start Taq DNA polymerase, SYBR Green I, dNTPs and PCR buffer (5 mM MgCl₂, Tris-Cl, KCl, (NH₄)₂SO₄, pH 8.7). 2 µl each forward and reverse primers (0.5 µM each final concentration), and 6 µl cDNA sample diluted 1/100 v/v with Ultra Pure water (Fisher Biotech, West Perth, Western Australia, Australia). Each 20 µl reaction mixture contained 10 µl of SYBR Green master-mix (QuantiTect SYBR Green PCR Master Mix, Qiagen, Valencia, CA) containing hot-start Taq DNA polymerase, SYBR Green I, dNTPs and PCR buffer (5 mM MgCl₂, Tris-Cl, KCl, (NH₄)₂SO₄, pH 8.7). 2 µl each forward and reverse primers (0.5 µM each final concentration), and 6 µl cDNA sample diluted 1/100 v/v with Ultra Pure water (Fisher Biotech, West Perth, Western Australia, Australia). Reaction conditions were as follows: initial denaturation (95 °C, 15 min), 50 cycles of denaturation (94 °C, 20 s), annealing (55 °C, 30 s), extension (72 °C, 30 s), final extension (72 °C, 4 min, followed by 25 °C, 5 min).

Test cDNA samples were generated from each of three rat strains (F344, SD, and DA) for each of two exposure conditions: (room air for 14 days from birth; cyclic hyperoxia 14 days from birth). Every sample was tested in triplicate, and every gene of interest was tested in duplicate experiments, giving a total of six replicates for each test sample, per gene. The standard cDNA pool was included in triplicate in each PCR run. A single RT negative control for each sample and two water controls (no-template control) were included in each experiment. A negative control sample was considered to be negative if the threshold cycle (Ct) for amplification was >5 cycles greater than the corresponding test sample.

Real-time RT-PCR products of all genes were separated on agarose gels, purified, sequenced, and compared with the predicted amplicon sequence to confirm identity. DNA was extracted from agarose gels using the Qiaquick column purification system (Qiagen, Hilden, Germany) and eluted in 10 mM TrisCl pH 8.5 for quantification and sequencing. Purified DNA was labeled using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and resolved using the ABI 3100 Genetic Analyser (Applied Biosystems, Foster City, CA). Melt-curve analysis was used to confirm amplicon specificity for each test sample for each gene of interest. Melting of PCR products was performed with 0.5 °C steps at 5 s intervals from 60-99 °C. The melt-curve of each real-time PCR product was compared with that of the corresponding sequenced product [43].

Analysis of gene expression data with adjustment relative to a standard sample: Relative quantification of gene expression was performed using the delta Ct method, with adjustment for amplification efficiency [44]. Amplification efficiencies ranged 56-94%. The expression of each candidate reference gene in each of the nine samples was determined relative to the geometric mean expression of the same gene in triplicates of the standard retinal cDNA pool [45] and subjected to expression stability analysis using GeNorm software v1.01 (Ghent University Hospital, Ghent, Belgium) [7]. The software program SPSS v11.0.2 (SPSS Inc, Chicago, IL) was used for further statistical analysis of gene expression among experimental groups. In all cases normalized expression data were normally distributed and were compared by two-way analysis of variance (ANOVA). The significance level (α) was set at 0.05.

RESULTS

Primer pairs for the five candidate reference genes (ARBP, ACTG2, CYCA, RNA2P, and HPRT) were tested in real-time RT-PCR on a 1:10 dilution in water of cDNA derived from five day-old DA retinal RNA. With the exception of CYCA, the melt-curve analysis on the amplicons was consistent with

### Table 2: Primer Sequences of Candidate Reference Genes for Quantitative Real-time Reverse Transcription Polymerase Chain Reactions on Rat Retina

<table>
<thead>
<tr>
<th>Gene (accession number)</th>
<th>Primer sequence (5’-3’)</th>
<th>Nucleotide position</th>
<th>Tm (°C)</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acidic ribosomal phosphoprotein (ARBP) NM_022402.1</td>
<td>F: AAGGGGTCTTGCTTTTGTGC R: GCAAATGAGATGGATCG</td>
<td>766-856</td>
<td>60-59</td>
<td>91</td>
</tr>
<tr>
<td>Actin gamma 2 (ACTG2) NM_012893.1</td>
<td>F: GGGAAGAGATCGGACCACCT R: CCCCCTGCTTTTGGGTTT</td>
<td>264-369</td>
<td>59-60</td>
<td>104</td>
</tr>
<tr>
<td>Cyclophilin A (CYCA) NM_017101.1</td>
<td>F: GTCTGCTTCTCGACTTGGTGTC R: AATCCCTTTCCTCCCAAGGTGTC</td>
<td>100-179</td>
<td>61-60</td>
<td>80</td>
</tr>
<tr>
<td>RNA polymerase 2 (RNAP2) XM_343922.2</td>
<td>F: GTCCAAATGATCATGGGAGA R: CCAATAGGAGATGACATGG</td>
<td>4011-4106</td>
<td>59-60</td>
<td>96</td>
</tr>
<tr>
<td>Hypoxanthine guanine phosphoribosyl-transferase (HPRT) NM_012583.2</td>
<td>F: TTGTGTGTATATGCCCTTGACT R: CGCGTGTTTTTTAGGCTTTG</td>
<td>629-733</td>
<td>59-60</td>
<td>105</td>
</tr>
</tbody>
</table>

The following abbreviations were used: melting temperature (Tm), base pairs (bp), forward primer (F), and reverse primer (R).
a single reaction product. Amplicons for ARBP, RNA2P, HPRT, ACTG2 and CYCA were separated by agarose gel electrophoresis (Figure 2) and with the exception of CYCA, a single amplified product matching the predicted size was observed. Given the apparent amplification of pseudogenes, CYCA was abandoned as a candidate reference gene. When real-time RT-PCR was repeated at a working dilution of cDNA of 1:100, the expression of ACTG2 was so low, consistent with known expression, as to preclude further investigation. The PCR products of ARBP, RNA2P and HPRT were purified and sequenced (data not shown). In each case, sequencing confirmed the amplification of a single product identical to the predicted sequence.

The primer pairs for ARBP, HPRT, and RNAP2 were then used in real-time RT-PCR experiments with test cDNA samples and the standard cDNA pool. Expression values of each reference gene relative to the standard cDNA pool were determined for each of three rat strains (F344, SPD and DA) at each of two experimental end-points room air controls at day 14, and cyclic hyperoxia-exposed tests at day 14 (Figure 3). The expression data for each of the three candidate reference genes in each of the retinal cDNA pools were subjected to expression stability analysis by pairwise comparisons amongst all samples [7]. RNAP2 was identified as the least stably expressed of the three genes. There was greater correlation for the expression of ARBP and HPRT (r²=0.80) than for the other gene pairs, ARBP and RNA2P (r²=0.31), or HPRT and RNAP2 (r²=0.25). The exclusion of RNAP2 from the GeNorm analysis significantly improved the average gene expression stability value.

We then normalized the data against ARBP and HPRT. A comparison of the normalized expression of each of the reference genes in the six different cDNA pools demonstrated that ARBP and HPRT were stably expressed in all of the test cDNA samples (Figure 4A,B). In contrast, the expression of RNAP2 differed across strains and across different oxygen exposure protocols to a significant extent (p<0.05) (Figure 4C). Expression of the former two genes did not differ significantly among strains, nor did they vary with oxygen exposure (p>0.05). ARBP and HPRT were therefore validated as reference genes for one particular model of experimental OIR in the rat.

DISCUSSION

Several methods have been proposed to allow accurate normalization of gene expression using quantitative real-time RT-PCR. An approach that is gaining increasing acceptance is the use of several reference genes for normalization [1,2,7,37]. In a study of 10 housekeeping genes, Vandesompele et al. demonstrated that reliance on a single gene may lead to three fold and 6.4 fold differences in target gene quantification in 25% and 10% of cases, respectively [7]. In sporadic cases, variation was greater than 20 fold. They proposed a method of assessing the expression stability of candidate reference genes based on comparisons between gene pairs. The method assumes that the expression ratio of two ideal control genes will be the same in different samples [6]. The mean pair-wise variation between the expression of a given reference gene and all other candidate reference genes is used as a marker of expression stability. GeNorm, a public-domain software application, provides a convenient means for testing gene expression stability.

OIR can be induced in neonates of a number of different experimental species [15], and irrespective of differences in the protocol for oxygen exposure, the available evidence suggests that the pathophysiology underlying the changes in retinal vascular phenotype may be similar in all. Vascular endothelial growth factor is a central mediator of retinal angiogenesis [9], and its downregulation during periods of hyperoxia leads to retinal vaso-obliteration [22]. However, whether similar phenotypic differences in different experimental models correlate with identical changes at the molecular level remains uncertain. For studies of genetic influences on neovascular retinopathy, small rodents (mice and rats) are particularly useful because of the availability of genetically-inbred strains, their ease of handling, and the generally large litter sizes. The rat may offer a slight advantage over the mouse in such experi-
Figure 3. Candidate reference gene expression in neonatal rat retinal cDNA pools. Expression of each reference gene was determined relative to the expression of the same gene in the pooled standard sample. Non-normalized expression values are shown for acidic ribosomal phosphoprotein (ARBP; A), hypoxanthine guanine phosphoribosyltransferase (HPRT; B), and RNA polymerase 2 (RNAP2; C). The average variation in relative expression between each pair of genes within each of the six cDNA test samples was used to assess stability of gene expression. The relative expression values for ARBP and HPRT were more closely correlated than were those for either gene with RNAP2. RNAP2 showed the lowest expression stability. In the figure n=6 replicates per experimental group of three pooled rat retinas and error bars represent ±SD. The following abbreviations were used: Sprague Dawley (S), Fischer 344 (F), Dark Agouti (D), room air raised, day 14 (R14), and cyclic hyperoxia exposed, day 14 (O14).

Figure 4. Normalized expression of reference genes in rat retinal cDNA pools. Gene expression in each pooled sample was normalized by dividing the relative expression value by the geometric mean of the expression levels of the two selected reference genes, acidic ribosomal phosphoprotein (ARBP) and hypoxanthine guanine phosphoribosyltransferase (HPRT), in the same sample. There was little variation between groups for the normalized expression of ARBP (A) or HPRT (B). In contrast, the expression of RNA polymerase 2 (RNAP2; C) varied considerably between groups: Within each strain, expression was higher in the oxygen exposed group (O14), than in the room air group (RA). In the figure, n=6 replicates per experimental group of three pooled rat retinas and error bars equal ±SD. The following abbreviations were used: Sprague Dawley (S) Fischer 344 (F), Dark Agouti (D), room air raised, day 14 (R14), and cyclic hyperoxia exposed, day 14 (O14).
ments, because the larger size of the former means that more retinal tissue is available for examination.

To determine appropriate reference genes for use in one particular model of OIR, we examined the expression of five candidate reference genes in the neonatal rat retina: ARBP, CYCA, ACTG2, HPRT, and RNASP2. We showed that ARBP, RNASP2, and HPRT were all expressed with moderate abundance in the neonatal rat retina and were considered suitable for further analysis. ACTG2 was expressed at a low level. CYCA has numerous processed pseudogenes and pseudogene amplification may persist even when rigorous efforts are undertaken to minimize genomic DNA contamination [46-48], as was found in this study. We did not consider 18S and 28S ribosomal RNA as candidate reference genes in this study, as the expression ratios of rRNA and mRNA vary considerably [7]. Moreover, 18S and 28S rRNA are expressed to a far greater extent than are most other genes, making them inappropriate as reference genes for some applications [5,7]. Oxygen tension has a profound influence on gene expression. With the exception of a relatively small number of hypoxia-induced genes, transcription is significantly reduced under conditions of hypoxia. Interestingly, many studies examining the effects of hypoxia on gene expression have utilized glyceraldehyde-3-phosphate dehydrogenase as a reference gene, even though its expression is upregulated by hypoxia inducible factor-1 (HIF-1) in hypoxia [49,50]. We found that both ARBP and HPRT were stably expressed in the retina of three different rat strains (F344, SPG, and DA) under specific conditions of normoxia and cyclic hyperoxia. In contrast, RNASP2 was regulated to a significant extent by oxygen tension: Gene expression was higher in oxygen-exposed rat retinas of all three strains examined than in retinas from neonates raised in room air. Further, the level of expression differed among the different rat strains under conditions of normoxia and under cyclic hyperoxia. In conclusion, we demonstrate that ARBP and HPRT are suitable reference genes for quantitative gene expression studies in at least one model of OIR in the rat.

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