Rat messenger RNA for the retinal pigment epithelium-specific protein RPE65 gradually accumulates in two weeks from late embryonic days

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Received 2 December 1997; revised version received 18 January 1998

Abstract The RPE65 protein appears late during the retinal development. To study the basis for this regulation, the rat RPE65 cDNA was sequenced and the mRNA subsequently quantitated at various stages by competitive RT-PCR. RPE65 mRNA was detected as early as E18 (36 copies/ng of whole eye total RNA). It gradually accumulates up to P12 (27 000 copies/ng) at which point it reaches a steady state level. This increase is interrupted for 3 days (P2–P4) during which the levels of mRNA remain stable. This timing and rate of accumulation parallels that of rat and mouse opsin mRNA and suggests that common factors may control the activation of genes in photoreceptors and retinal pigment epithelium cells.

Key words: RPE65; Retinal pigment epithelium; Retina; mRNA; Competitive reverse transcription polymerase chain reaction

1. Introduction

The retinal pigment epithelium (RPE) is a pigmented single cell-layered tissue interposed between the vascular-rich choroid and the neural retina. This ocular tissue is in close contact with the photoreceptor rod and cone outer segments, extending its microvilli through the interphotoreceptor matrix around the outer segments. Among the many roles of the RPE cell which have been characterized, the periodic phagocytosis of the outer segment tips [1] and retinol metabolism [2] are essential to the visual process. In fact, it is in the RPE that critical steps of the visual cycle of retinoids [3] are performed, including the esterification of the all-*trans*-retinol that results from the bleaching of the photopigment, rhodopsin, and the isomerization of the retinyl ester to 11-*cis*-retinol and oxidation to 11-*cis*-retinal [4–6].

RPE65 is an abundant 65-kDa microsomal RPE-specific protein conserved in vertebrates [7–10]. Although the function of RPE65 remains unknown, several observations indicate that this protein is an enzyme involved in the RPE-specific metabolism of 11-*cis*-retinol [11]. Recent findings of mutations in the *RPE65* gene causing severe, childhood-onset retinal dystrophy indicate that RPE65 defects dramatically affect photoreceptor survival and further point to the critical role of RPE65 in RPE physiology [12,13].

We previously showed that RPE65 expression is developmentally regulated [14]. Using monoclonal antibody RPE9 on tissue sections of rat retina [15], we found that RPE65, which is constantly expressed in adult animals, appears in RPE cells only at postnatal days 4–5, whereas earlier expression could be expected since many RPE cells exhibit a differentiated phenotype by embryonic day 18 [16]. This late expression raised the question of a delayed transcriptional activation of the *RPE65* gene. In addition, because this timing coincides with the appearance of the first photoreceptor outer segment membranes [17,18], a coordinated developmental expression of RPE65 with certain photoreceptor-specific proteins was possible. In order to examine when the *RPE65* gene is turned on, we quantified RPE65 mRNA during retinal development by competitive RT-PCR. As a prerequisite to this study, the rat RPE65 cDNA was sequenced.

2. Materials and methods

2.1. Materials

Restriction and modification enzymes, oligonucleotides and agarose were from Eurogentec (Seraing, Belgium). The guanidine thiocyanate solution (RNAXEL) and glass beads (RNABIND) were from Eurobio (Les Ulis, France). Proteinase K was obtained from Merck (Darmstadt, Germany). RNase blocker and sequencing primers for pUC-derived plasmids were from Promega (Madison, WI, USA).

2.2. RNA extractions

Eyes from adult Wistar rats were homogenized with a motor-driven homogenizer at 1000 rpm for 1 min in a guanidine thiocyanate solution [19]. Total RNA was then extracted following the APCG single-step method [19] and purified on glass beads. For the construction of the retina+RPE library, whole eyes from 12-day-old Wistar rats were incubated in a 0.1% proteinase K solution in Hanks' balanced salt solution at 37°C for 15 min and the retina+RPE dissected out according to Sakagami et al. [20]. RNA from this tissue was then extracted as described above.

For the quantitation of the RPE65 mRNA during the development, whole eyes from Wistar rats at embryonic days (E) 17, 19 and 20 and from postnatal days (P) 1–8 as well as from P10, P12, P15, P20 and P25 were enucleated, weighed, and the total RNA extracted as above. All RNAs were quantified by UV spectrophotometry. Only those with a $\lambda_{260}/\lambda_{280}$ ratio higher than 1.80 were retained and stored at −70°C in DEPC-treated H_2O for a maximum of 6 months. Quality of RNAs from different developmental stages was verified by Northern blot hybridized with a digoxigenin-labeled glyceraldehyde 3-phosphate dehydrogenase riboprobe.

2.3. cDNA cloning and sequencing

To obtain the open reading frame of the rat RPE65 cDNA, 2 μg of total RNA from whole eyes was reverse transcribed. Because of the high level of identity between the rat and bovine RPE65 cDNA coding sequences, couples of 20-mer primers matching the bovine RPE65 cDNA sequence [7] could be used to amplify most of the rat RPE65

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Abbreviations: DEPC, diethylpyrocarbonate; RPE, retinal pigment epithelium; RRP, recombinant reverse primer; RT-PCR, reverse transcription coupled to polymerase chain reaction

The nucleotide sequence of the rat RPE65 cDNA is available in GenBank under accession number AF035673.

PII S0014-5793(98)00081-7

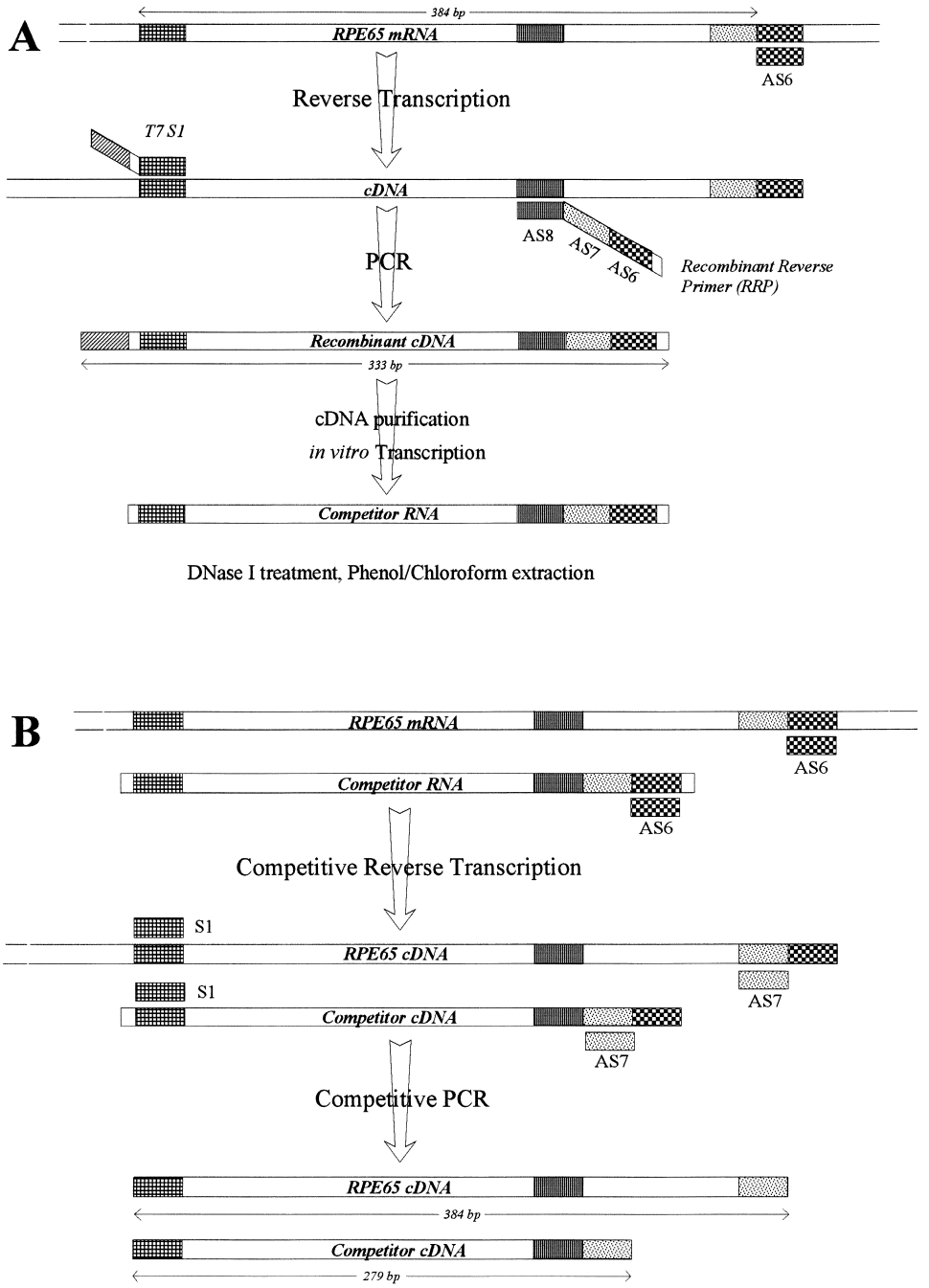

Fig. 1. Summary of the procedures followed for the synthesis of competitor RNA (A) and for the competitive RT-PCR (B). Sequences of T7S1 and recombinant reverse primer are given in Section 2.

open reading frame. Amplified cDNA fragments were purified and directly sequenced in both directions with the PCR primers. To obtain the 5′ and 3′ ends of the RPE65 cDNA, we generated a rat retina+RPE library in λgt11 with the CapFinder PCR cDNA library construction kit (Clontech, CA, USA) and screened it by PCR using cDNA-specific and plasmid primers. The cDNA fragments were purified on agarose gels, cloned into pCR-Script (Stratagene) and sequenced in both directions using vector and internal primers.

2.4. Competitive RT-PCR

To quantitate the mRNA for RPE65 during development, we used the competitive RT-PCR technique [21] in which the competitor was a truncated mRNA fragment of RPE65 obtained by transcription of a recombinant cDNA [22]. The strategy used to synthesize the competitor RNA and the process of the competitive RT-PCR are illustrated in Fig. 1.

To synthesize the competitor RNA, 2 μg of whole eye total RNA was reverse transcribed in a 10-μl reaction volume containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 500 μM dNTPs, 0.5 μM of AS6 (5′-CTTTTGTCCTGCCCCAGG-3′), 12.5 units of Moloney murine reverse transcriptase and incubated at 37°C for 1 h. Following inactivation of the reverse transcriptase at 68°C for 20 min, a truncated recombinant cDNA was obtained by PCR amplification of 1 μl of cDNA diluted into a 20-μl PCR reaction mixture containing 75 mM Tris-HCl (pH 9.0), 20 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 1.5 mM $MgCl_2$, 200 μM dNTPs, 1 unit of Taq DNA polymerase and 25 pmol each of the forward primer (T7S1, Fig. 1) bearing the T7 polymerase promoter sequence in 5′ (5′-GCGCGTAATACGACTCACTATAGGGCGAATGTCAGGAGATACGTT-

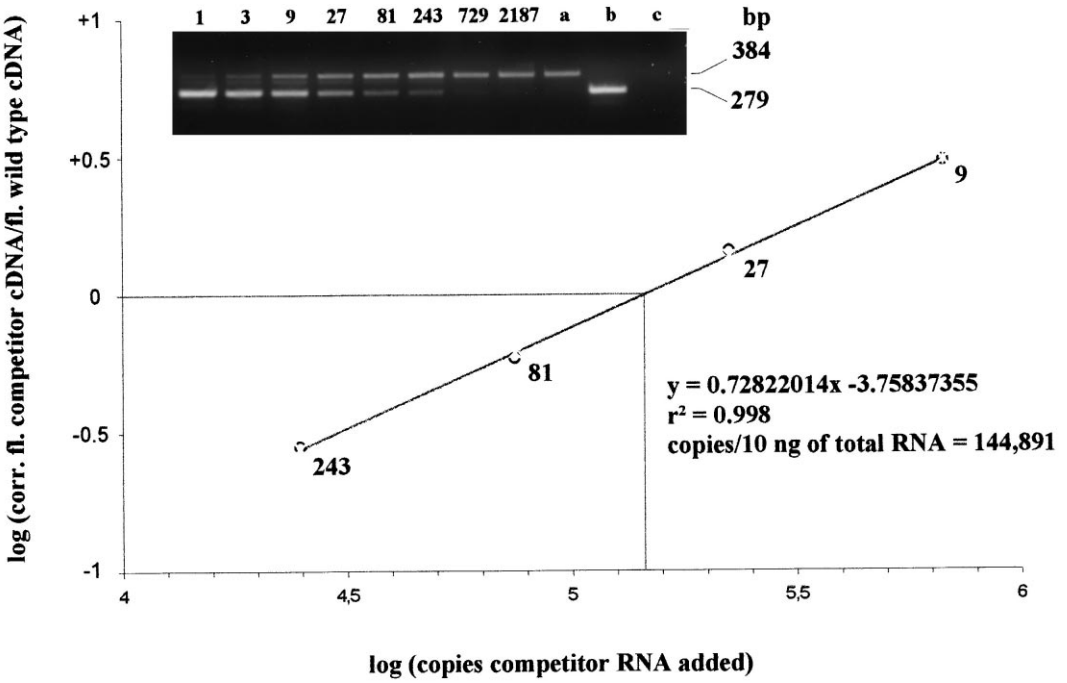

Fig. 2. Quantitation of RPE65 mRNA at postnatal day 25. Competitive RT-PCR was performed as described in Fig. 1B. 10 ng of whole eye total RNA were distributed in each of eight tubes along with serial threefold dilutions of competitor RNA starting from $60\,498\times10^2$ copies (1 pg of RNA, dilution coefficient 1) to 2766 copies (dilution coefficient 2187). Ethidium bromide-stained agarose gel shows the cDNA fragments obtained from wild type RPE65 mRNA (384 bp) and from the shorter, competitor RNA (279 bp). The dilution coefficient of the competitor RNA (1–2187) is indicated in each lane. Control amplifications without competitor RNA (lane a), without whole eye total RNA (lane b) or without any RNA (lane c) were included. Densitometry of cDNA fragments for dilutions 9, 27, 81 and 243 were measured and the $\log_{10}$ of the ratio of the corrected (multiplied by 384/279) fluorescence of competitor cDNA over the fluorescence of the wild type cDNA was calculated. The graph shows these data plotted as a function of the $\log_{10}$ of the number of copies of RNA added in each tube. The function of the linear regression depicted in the graph ($y=ax+b$) and the determination coefficient (r^2) are indicated. Interpolation for the $\log_{10}$ of the ratio of fluorescence intensities = 0 (equivalence point) gives a value of 144 891 copies/10 ng of total RNA.

CTTCC-3′) and the recombinant reverse primer (RRP, Fig. 1) composed of the juxtaposed AS6, AS7 and AS8 sequences (5′-AGCAGGCTTTTGTCCTGCCCCAGGCTCACCACCACACTCAG-AACTCAGCTTACACAACTTGTCTGG-3′). AS7 and AS8 are separated in the wild type mRNA by 105 nucleotides. In both T7S1 and RRP, the insertion of additional nucleotides (underlined in the sequences above and white boxes in Fig. 1) was necessary to obtain proper in vitro transcription of the recombinant cDNA and efficient reverse transcription of the competitor RNA in the subsequent steps. The program used was: 95°C/3 min, (95°C/30 s, 57°C/30 s, 72°C/1 min)×2 cycles, (95°C/30 s, 70°C/30 s, 72°C/1 min)×33 cycles. The recombinant cDNA was purified on agarose gel and 100 ng was in vitro transcribed at 37°C for 30 min in a 20-μl volume containing 40 mM Tris pH 8.0, 8 mM $MgCl_2$, 2 mM spermidine, 50 mM NaCl, 0.4 mM rNTPs, 30 mM DTT, 40 units of RNasin and 10 units of T7 RNA polymerase. DNA was subsequently digested with 0.5 units of RNase free DNase at 37°C for 15 min; the synthesized competitor RNA was extracted with water-saturated phenol/chloroform and precipitated in the presence of ammonium acetate. The competitor RNA was finally dissolved in water and quantified by UV spectrophotometry with a $\lambda_{260}/\lambda_{280}$ ratio between 1.90 and 2.10. The correct size of the competitor RNA was verified on agarose formaldehyde gels. It was stored in aliquots at −70°C.

For each stage of development, eight reaction tubes containing the same amount of total RNA (1500 ng for the embryonic stages, 1000 ng from P1 to P5, 100 ng from P6 to P10 and 10 ng from P12 to P25) and eight serial threefold dilutions (1 pg to 3×10^{-4} pg) of the competitor RNA were prepared as well as three control reactions containing either total RNA only, competitor RNA only, or no RNA. These RNAs were reverse transcribed in a 2-μl volume containing 0.1 μM of the AS6 primer and 1.25 units of reverse transcriptase in the conditions described above. Specific recombinant and wild type RPE65 cDNAs, annealed with the same primers, were then amplified (Fig. 1) by adding to each reverse transcription tube 18 μl of a PCR mixture (see above for ionic and dNTP components) containing 0.5 μM each of the forward S1 (5′-GTCAGGAGATACGTTCTTCC-3′) and reverse AS7 (5′-CTCACCACCACACTCAGAACT-3′) primers using the following program: 95°C/3 min, (95°C/30 s, 57°C/30 s, 72°C/1 min)×35 cycles, 72°C/5 min. Amplicons from wild type RPE65 mRNA (384 bp) and competitor RNA (279 bp) were finally separated on 4% ethidium bromide-stained agarose gels in 1×TAE.

2.5. Quantitation of the RPE65 mRNA

The fluorescence intensity of the PCR products was determined by measuring the densitometry of the DNA bands from negative photographs of gels using the TITN Answare software program (Alcatel, France). Since comparisons and equivalence point determination in competitive RT-PCR are based on molar amounts, the fluorescence associated with the shorter product (279 bp) was corrected by multiplication by the ratio 384/279 to enable direct comparison of corrected fluorescence intensity of the 279-bp band with the measured fluorescence intensity of the 384-bp band. The $\log_{10}$ of the ratio of the corrected fluorescence of the competitor RNA to the fluorescence intensity of the mRNA was then plotted as a function of the $\log_{10}$ of the number of copies of competitor RNA ($60\,498\times10^2$/pg) added in each tube and the linear regression of this function calculated using the Excel program (Microsoft Inc., USA). The equivalence point at which the number of copies of messenger RNA equals that of competitor RNA was finally determined from the linear regression plot by interpolation for a value of the $\log_{10}$ of the ratio of fluorescence intensities of 0.

3. Results

3.1. Nucleotide and amino acid sequences of rat RPE65

The rat RPE65 cDNA is 2123 nt long and encodes, as in human and bovine species, 533 amino acids (cDNA and amino acid sequences are available in GenBank under accession

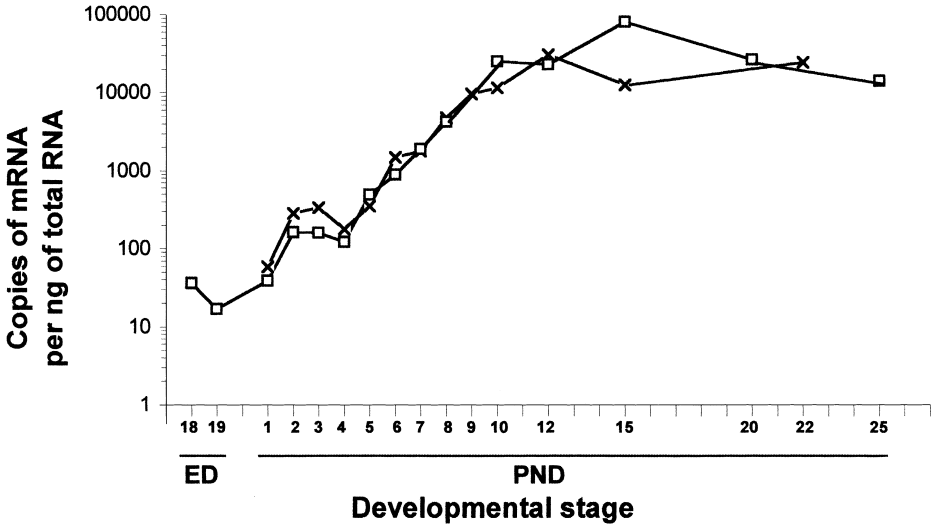

Fig. 3. Quantitation of RPE65 mRNA as a function of age. The graph shows the $\log_{10}$ of the number of copies of RPE65 mRNA per ng of whole eye total RNA plotted against the age in days at embryonic (ED) and postnatal (PND) stages. Curves from first (open squares) and second (crosses) series of RNA extraction and quantitation are indicated.

number AF035673). Rat and bovine, and rat and human amino acid sequences are 94.0% and 94.5% identical, respectively. Interestingly, rat and bovine, and rat and human sequences are 73.5% and 67.3% identical in the 5′-UTR, and 70.8% and 74.3% identical in the 3′-UTR stretch, respectively. This is particularly relevant in the 3′-UTR as a possible regulation of translation exerted on this region has been suggested [7].

3.2. Quantification of RPE65 mRNA

Efficient amplification by RT-PCR from whole eye total RNA using S1 and AS7 was first verified. Subsequently, a 105 nt-deleted recombinant cDNA fragment was obtained using T7S1 and RRP primers (Fig. 1). Efficient amplification of this latter fragment with S1 and AS7, the primers used in competition, was also verified. Northern blot analysis showed that total RNA samples from various developmental stages were not degraded (not shown).

Two independent series of total eye RNA extraction and competitive RT-PCR were performed. For each series and each developmental stage from E18 to P25, the fluorescence of cDNA fragments resulting from amplification of wild type RPE65 mRNA and of competitor RNA was measured and the number of copies of mRNA per ng of total RNA calculated (Fig. 2). Copy numbers for each series and at each developmental stage were then plotted against the development time course in a semi-logarithmic scale (Fig. 3).

RPE65 mRNA was detected as early as E18 with 36 copies/ng of total RNA. From late embryonic stages (E18 and E19), mRNA levels increased about 10-fold to reach a mean value of 226 ± 62 copies at P2. Subsequently, the levels of mRNA were stable from P2 to P4 with even a slight decrease to 150 ± 29 copies at P4. Starting from P5, a second, 200-fold increase in the accumulation of mRNA gradually brought the level to $27\,282 \pm 1675$ copies/ng at P12. From P10–P12 to later stages, the levels in the mRNA remained stable with variations being less than one log magnitude.

4. Discussion

Using competitive RT-PCR, RPE65 mRNA was quantified during the development of the retina in rat. RPE65 mRNA was detectable as early as E18 and, from this stage, it was found to gradually accumulate up to P10–P12. At this point, it plateaued at about 1000-fold the level of the late embryonic stages. This increase was interrupted for 3 days (P2–P4) during which the levels of mRNA remained approximately stable.

In rat, the development of the RPE starts soon after optic vesicle formation as evidenced by the presence of pigmented cells in the outer layer of the retina at E13.5 [16]. These pigmented, differentiating RPE cells divide actively. By E18, most RPE cells have achieved a morphologically differentiated aspect with apical microvilli and numerous melanosomes at all stages of maturity [16]. In the same time, mitotic activity drops but does not cease completely [23]. After birth, the rat RPE undergoes a second wave of division characterized by a burst of acytokinetic mitosis starting at P2, peaking at P2–P3, decreasing at P5 and virtually ending by P11 [23]. This second wave leads to binucleation of most of RPE cells in the posterior retina.

RPE65 mRNA accumulation parallels steps of RPE development. Indeed, amounts of mRNA were found to be quantifiable first by E18 (RPE65 mRNA was detected at E17 but the level was too low to be reliably quantified), indicating that the RPE65 gene is already turned on when the first wave of cell division is ending, the RPE displaying several of its specific features. Starting from this timepoint, a 1000-fold increase in the levels of mRNA that lasts about 2 weeks is observable. This increase is unlikely to result from cell multiplication as mitotic activity from E18 to P1 is low and since the second wave of division, from P2 to P11, only doubles the number of nuclei, the uninucleated RPE cells entering the mitotic cycle only once [23]. Rather, it suggests a sustained transcriptional activity of the RPE65 gene. There is, however, a transient arrest in the growing accumulation of mRNA from P2 to P4 that coincides with the peak of the second wave of RPE cell division. This probably reflects a temporary release of specific transcriptional activity when RPE cells enter the mitotic cycle. After P4, as many binucleated cells are formed and cease mitotic activity, the increase resumes.

Although the RPE65 mRNA was found at prenatal stages,

the protein itself could not be detected before P4–P5 in few RPE cells of the posterior retina [14]. This apparent delay might simply be due to the sensitivity limit of protein detection with monoclonal antibodies on tissue sections, a certain amount of RPE65 protein being necessary, which itself depends on sufficient amounts of RPE65 mRNA. However, other factors may restrain the finding of the RPE65 protein at early stages. In fact it is at P5 that the endoplasmic reticulum, the organelle in which the RPE65 protein is found, extensively develops in the RPE cells [24]. Another possibility could be a translational inhibition of the RPE65 mRNA before P4–P5. Previous results have shown that RPE65 mRNA, although present in RPE cultures, is not translated [7]. An interpretation of this result is that a factor which might control translation in vivo is lacking in culture. The fact that 3′-UTRs from human, bovine and rat RPE65 mRNAs ([10] and our results) are conserved suggest potential sites for regulation in these mRNAs. This awaits precise description of such a control and the identification of factors effectively regulating the translation.

RPE65 mRNA accumulation seems to follow a timing shared by certain genes in the outer retina during its development. Indeed, the rat [25] and mouse [26] opsin mRNAs undergo a rapid increase in their accumulation during the first postnatal week and also reach a steady state level at P10–P12. The rates of mRNA accumulation for RPE65 and opsin are also quite similar since the rat mRNA for opsin increases from 1 to 100% of the adult level from P2–P3 to P12 [25] while that for RPE65 occurs from P4–P5 to P12. It is during this 1–100% increase that the photoreceptor outer segments appear and grow in the subretinal space, coinciding with the extension of the RPE microvilli [17] and a greater capacity of the RPE cells to phagocytize the outer segment tips [27]. In bovine retina, it is also during the appearance and growth of outer segments that mRNAs for several rod-specific genes increase from 1 to 100% of their adult value [28,29]. Moreover, this increase seems to be switched on at the same time for cone and rod opsins [30], even though cones are born earlier than rods [31].

Taken together, these observations suggest that common elements control the activation of genes from different cell types including rods, cones and RPE cells. The fact that, in monkey retina, cell births in RPE and neuroretina demonstrate a remarkable spatial and temporal correspondence [32] is another indication of common factors controlling proliferation and/or differentiation in play in the outer retina. Given that conserved *cis*-acting elements are found in the promoter region of various photoreceptor genes [33] and that parts of a single promoter can confer transcriptional specificity to several cell types such as blue cones and cone bipolar cells [34], it is plausible that elements are shared by photoreceptor and RPE genes. In that respect, examination of promoter sequences and specificities of newly available RPE genes will be of great interest.

Acknowledgements: This work was supported by SOS Rétinite, Montpellier, France. We thanks Jean-Louis Pasquier for photographic work, and Robert Assié and Guy Rebillard for their help and advice.

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