The Upstream Region of the **Rpe65** Gene Confers Retinal Pigment Epithelium-specific Expression *in Vivo* and *in Vitro* and Contains Critical Octamer and E-box Binding Sites*

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RPE65 is essential for *all-trans*- to *11-cis*-retinoid isomerization, the hallmark reaction of the retinal pigment epithelium (RPE). Here, we identify regulatory elements in the **Rpe65** gene and demonstrate their functional relevance to **Rpe65** gene expression. We show that the 5' flanking region of the mouse **Rpe65** gene, like the human gene, lacks a canonical TATA box and consensus GC and CAA/T boxes. The mouse and human genes do share several cis-acting elements, including an octamer, a nuclear factor one (NFI) site, and two E-box sites, suggesting a conserved mode of regulation. A mouse Rpe65 promoter/β-galactosidase transgene containing bases −655 to +52 (TR4) of the mouse 5' flanking region was sufficient to direct high RPE-specific expression in transgenic mice, whereas shorter fragments (−297 to +52 or −188 to +52) generated only background activity. Furthermore, transient transfection of analogous TR4/luciferase constructs also directed high reporter activity in the human RPE cell line D407 but weak activity in the non-RPE cell lines HeLa, HepG2, and HS27. Functional binding of potential transcription factors to the octamer sequence, AP-4, and NFI sites was demonstrated by directed mutagenesis, electrophoretic mobility shift assay, and cross-linking. Mutations of these sites abolished binding and corresponding transcriptional activity and indicated that octamer and E-box transcription factors synergistically regulate the RPE65 promoter function. Thus, we have identified the regulatory region in the **Rpe65** gene that accounts for tissue-specific expression in the RPE and found that octamer and E-box transcription factors play a critical role in the transcriptional regulation of the **Rpe65** gene.

All-*trans*- to *11-cis*-isomerization of vitamin A is an obligate and tissue-specific enzymatic step in the renewal of *11-cis*-retinal, the universal chromophore of rhodopsin and other visual pigment proteins, in the visual cycle (1) of the retinal pigment epithelium (RPE). Several components, including *cis*-retinol dehydrogenase (2), cellular *11-cis*-retinaldehyde-binding protein (CRALBP) (3, 4) and lecithin:retinol acyltransferase (5, 6), all essential to the visual cycle activity, are found highly expressed, but not exclusively expressed, in the RPE. However, the retinol isomerase activity (7–9), central to *11-cis*-chromophore synthesis, is expected, mechanistically, to be highly tissue-specific. A tissue-specific component of the RPE, RPE65 (10–12), which copurifies with *11-cis*-retinol dehydrogenase (2), appears to play a crucial role in retinoid isomerization. Thus, in the RPE65-deficient mouse (13), rod photoreceptor function is abolished due to lack of the *11-cis*-retinal chromophore. Furthermore, mutations in the human **RPE65** gene cause several forms of severe early onset blindness (14–17). Clearly, RPE65 is essential to the visual cycle in general and to all-*trans*- to *11-cis*-retinoid isomerization in particular.

RPE65 is the major protein of the RPE microsomal membrane fraction. The bovine (10), human (18), dog (19), rat (20), and salamander (21) cDNAs have been cloned, as have the human (18) and mouse genes. RPE65 is specific to the vertebrate RPE and is also highly conserved at the level of protein sequence. Previous data suggest a complex transcriptional and translational regulation of RPE65. At the transcriptional level, our knowledge is limited (22), and we lack functional evidence concerning the transcriptional elements involved in the activation of the gene and in its specific expression in the RPE. Transcription of **Rpe65** appears to be developmentally regulated, with the protein first appearing at about postnatal day 4 in the rat (11), coincident with the first appearance of the photoreceptor outer segments. Reverse transcription-polymerase chain reaction (reverse transcription-PCR) analysis of RPE65 in embryonic and newborn rat suggests a biphasic induction of RPE65 mRNA expression (20). At the level of translation, we have found that a 170-nucleotide region of the **RPE65** 3' untranslated region acts as a translational inhibition element (23). Also, when RPE cells are explanted into culture, they lose expression of RPE65 protein within 2 weeks, although the expression of RPE65 mRNA can continue (10, 11).

Here, we present the sequence of the 5' flanking region of the mouse **Rpe65** gene and indicate its similarity to the corresponding human gene region. We have generated transgenic mice containing Rpe65 promoter-reporter constructs and show that the **Rpe65** 5' flanking region −655 to +52 can drive lacZ reporter gene expression specifically in the RPE. In addition, we
show that this fragment also displays a high transcriptional activity in D407 RPE cells in vitro. Furthermore, by directed mutagenesis, electrophoretic mobility shift assay (EMSA), and cross-linking, we demonstrate functional binding of transcription factors to an octamer sequence and AP-4 and NFI sites and show their importance to the transcriptional regulation of the mouse Rpe65 gene.

**EXPERIMENTAL PROCEDURES**

**DNA Cloning and Sequence Analysis—**A P1 clone containing the complete mouse Rpe65 gene was isolated (Genomic Systems, St. Louis, MO). Restriction fragments containing perfect 5′-flanking region of the human Rpe65 gene were identified by Southern blot hybridization to a random-primed 32P-labeled bovine CDNA 5′ end probe (10). cDNA subclones containing the 5′ region of the Rpe65 gene were sequenced. One such clone, E1–12, was found to contain the first three exons of mouse Rpe65, as well as 2.8 kilobase pairs of 5′ flanking region. This was compared with the sequence of the 5′ flanking region of the human Rpe65 gene, obtained in the same way (15), using the GeneWorks 2.5 and MacVector 6.5 sequence analysis programs (Oxford Molecular, Beaverton, OR).

**Reporter Constructs—**For transgenic mice, three constructs, TR2, TR3, and TR4, containing sequences included in the mouse 5′ flanking region, were amplified. For amplification, oligonucleotide primer pairs containing HindIII restriction sites at their 5′ ends were used. The forward primers were used to insert the restriction sites underlined in each primer (Table II) as follows: TR4, 5′-CCCAAGCTTGAATGGTAGAAGCATA-3′; TR3, 5′-GCAACCTTCCATGAGTAGAGCA-3′; and TR2, 5′-CCACACCTGATTCAAGTCTGGAAAATA-3′. The common reverse primer used was 5′-CCCCAGCTTCTCCATGAGTAGAGCA-3′ and simian virus 40 polyadenylation signal sequences.

For transient transfection luciferase assay, constructs TR3 and TR4 were inserted into the plasmid pGL3-Basic (Promega, Madison, WI). The forward primers were the same as those used to amplify TR3 and TR4 fragments during the production of transgenic mice. The common reverse primer also overlapped with the primer used before, but it lacked four bases at the 3′ end.

**Site-directed Mutagenesis of the Mouse Rpe65 Promotor—**Mutations were introduced by DNA amplification using QuickChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA). A total of four site-directed mutants using the pTRlac plasmid as a template were generated. These were named m1AP4, m2NF1, m2AP4, and mOct. 11 additional constructs were generated (Table II), containing combinatorial mutations in two, three, or all of the cited elements were created using single, double, or triple mutants as a template, respectively. Mutated oligonucleotides used for DNA amplification are shown in Table II. The introduction of mutations was verified by DNA sequencing.

**Production and Analysis of Transgenic Mice—**DNA constructs were microinjected into the male pronuclei of single cell FVB/N mouse embryos. The transgenic founder was crossed into pseudo-pregnant CD1 foster mothers, using standard techniques. Transgenic founder mice and their progeny were identified by PCR of a region common to all of the transgenes. For some founders, copy number was estimated by Southern blot analysis of PSV1-digested genomic DNA hybridized with a lacZ gene probe. Transgenic founders were bred to CD1 mice to generate F1 progeny. β-Galactosidase (β-gal) reporter gene activity was assayed using the chemiluminescent Galacto-Light Plus assay (Tropix/PI Applied Biosystems, Bedford, MA). Eyes were dissected into three parts: the anterior segment, comprising the cornea, iris, and ciliary body; the posterior segment, comprising the retina, RPE, choroid, and sclera; and the lens. Noneye tissues assayed were brain, liver, lung, heart, kidney, and spleen. For histochemistry, tissues were fixed for 1 h in 4% paraformaldehyde in phosphate-buffered saline and washed three times in 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) rinse buffer (100 mM sodium phosphate, pH 7.3, 2 mM MgCl2, 0.01% sodium deoxycholate, 0.02% Nonidet P-40). The eyes were stained overnight in a solution of 2 mg/ml X-gal in X-gal rinse buffer containing 5 mM each potassium ferrocyanide and potassium ferricyanide. After staining, tissues were postfixed in 4% paraformaldehyde and embedded in methacylate. Sections were cut at a thickness of 4 μm, counterstained with neutral red, and evaluated for presence of blue product. In addition, stained eyes were postfixed in 4% paraformaldehyde and dissected to remove anterior segment, lens, and retina. The resultant eyecup was quartered and flat-mounted in 50% glycerol for an en face preparation of the RPE/choroid/sclera complex.

**Cell Culture and Transient Transfections—**The human retinal pigment epithelium cell line D407 was obtained from Richard C. Hunt (25) and grown in high glucose Dulbecco’s modified Eagle's medium (Life Technologies, Inc.) supplemented with 3% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. HeLa, HepG2, and HB27 cells were obtained from American Type Culture Collection (Manassas, VA) and grown in the same media as used for D407 except that the concentration of fetal bovine serum was 10%.

Approximately 2.5 × 106 cells were plated onto six-well tissue culture dishes and allowed to grow for 48–72 h (until 80–90% confluent). To correct for differences in transfection efficiency, 2 μg of each luciferase plasmid and 90 ng of pBSV40/β-gal were added to the cells in a solution of 20% Matrigel (Collaborative Research, Bedford, MA), 10% FBS, and 10% dimethyl sulfoxide. The ratio of luciferase activity to β-gal activity in each sample served as a measure of normalized luciferase activity. Experiments were performed in triplicate at least four times.

**EMSA—**Nuclear extracts from D407 and freshly dissected RPE bovine cells were prepared by the method of Dignam et al. (26). For EMSA, double-stranded oligonucleotides (Table II) were labeled with polynucleotide kinase and [γ-32P]ATP (6000 Ci/mmol). Approximately 15 μg of nuclear extract were added to binding buffer (33 mM Tris·HCl, pH 7.5, 1.6 mM NaCl, 1.6 mM dithiothreitol), 4 μg of poly(dI-dC), 0.04% Nonidet P-40, 8% glycerol, and [32P]-labeled probe (30,000–50,000 cpm) and incubated at room temperature for 30 min. For supershift assay, a 50–2000-fold molar excess of unlabelled wild type, mutant, or nonspecific competitor oligonucleotide was used along with the labeled probe. The DNA-protein complexes were resolved on 5% polyacrylamide gels in 0.5× Tris borate-EDTA buffer and visualized by autoradiography. For antibody supershifts, nuclear extracts were incubated with 1 μl of CTE/NFI polyclonal antibody (provided by Naoko Tanese) or 4 μl of α-Oct-1 monoclonal antibody (provided by Winship Herr, isotype IgG1, κ) for 1 h at room temperature prior to addition of labeled probe.

**RESULTS**

**Sequence Conservation of the Mouse and Human 5′ Flanking Regions—**We have sequenced approximately 2.8 kilobase pairs of 5′ flanking region upstream of the putative transcription start site of the mouse Rpe65 gene (Fig. 1A; numbered +1 based on homology with the human gene (22)) and searched for sequences evolutionarily conserved between the mouse and human Rpe65/RPE65 genes 5′ flanking regions. When 2.8 kilobase pairs of the 5′ flanking region of the mouse and human Rpe65/RPE65 genes were compared by dot matrix analysis, a diagonal of similarity was seen distally to approximately –1200 with a short region of similarity closer to the 5′ end of each segment (data not shown). The proximal 628 and 581 nucleotides of the human and mouse 5′ flanking regions, respectively, and 5′ untranslated regions were compared by ClustalW alignment (Fig. 1B). Many conserved blocks of sequence were noted between the two, including NFI (at –178 to –165), octamer (at –498 to –491), and two E-box consensus (at –84 to –79 and –257 to –252) binding sites. The overall homology is over 70%. A possible site homologous to the human CRALBP gene (27) element, noted by Nicoletti et al. (22) in the human RPE65 5′ flanking region is present at –72 to –63 in the mouse Rpe65 5′ flanking region. This element, however, is not represented in the mouse CRALBP gene promoter. The NFI site in both genes has a similar intervening nonconsensus sequence (AATACT/CAG) only seen previously in the human RPE65 gene (22), but the
**Fig. 1.** The 5′ flanking and 5′ untranslated region of the mouse *Rpe65* gene. *A,* the 5′ flanking region of the mouse *Rpe65* gene. Based on homology with the human gene (22), the mouse *Rpe65* transcriptional start site is numbered 1. A nonconsensus possible TATA box is underlined and labeled (TATA box). Various consensus binding sites (AP-4, NFI, and octamer) are underlined and labeled. The 5′ ends of fragments used to generate transgene constructs (TR2, TR3, and TR4) are indicated by asterisks. Each fragment is indicated by an underlined boldface designation (TR2–TR4). This sequence has been submitted to GenBankTM (accession number AF271297). This sequence has been scanned against the GenBank data base (July 1999), and the only sequence with significant relatedness identified was the 5′ untranslated region of the rat RPE65 cDNA (AF035673).

**B,** optimal alignment of mouse and human *Rpe65/RPE65* proximal promoter sequences. Conserved nucleotides are boxed and shaded, and consensus-binding elements for transcription factors are indicated. Putative Ret1/PCE1, TATA box, and human CRALBP gene elements are also indicated.
consensus binding half-sites (TGGA-N5-GCCA) match perfectly with the CTF/NF1 family consensus. The two E-box sites are consensus basic helix-loop-helix (HLH) protein binding sites, although Nicoletti et al. (22) specifically ascribed them to AP-4. An octamer sequence was also identified in both promoters; they differed by only one nucleotide from the consensus octamer sequence ATGCAAAT. Both human and mouse genes lack consensus GC and CAAT boxes. A possible TATA box was found in both promot-ers; they differed by only one nucleotide from the consensus sequence necessary of the assay reagents but with no tissue. A, pTR4lacZ (−297/+52); B, pTR4lacZ (−655/+52). The means and S.E. are shown (n ≥ 3).

Analysis of Rpe65 Promoter-driven LacZ Activity in Transgenic Mouse Tissues—To identify sequence elements and transcriptional factors responsible of Rpe65 expression, we first determined the minimal Rpe65 promoter sequence necessary for the in vivo specific expression of the Rpe65 gene in the RPE. We made three constructs containing the upstream sequence of the Rpe65 gene coupled to the lacZ gene/SV40 poly(A) signal sequence, and we analyzed their corresponding β-gal activity in transgenic mice. These contained the following sequence positions (construct name in parentheses): −188 to +52 (TR2), −297 to +52 (TR3), and −655 to +52 (TR4). Microinjection of these constructs into fertilized oocytes resulted in the generation of several founder lines for each construct. Copy number was estimated by comparing the hybridization signal of probe with genomic DNA from transgenic mice to serial dilutions of a known quantity of the relevant linearized construct. The number of founders analyzed and the number of copies and β-gal expression levels of each construct are presented in Table I.

Although RPE65 has been shown to be expressed specifically in the RPE of the eye and is not found in nonocular tissues (11), we surveyed expression of RPE65 promoter-lacZ gene constructs in a variety of tissues. We assayed eye tissues (the anterior segment, comprising the cornea, iris and ciliary body; the posterior segment, comprising the retina, RPE, choroid, and sclera; and the lens) and non-eye tissues (brain, heart, lung, liver, kidney, and spleen) from transgenic and nontransgenic (control) F1 or F2 littermates were assayed for β-gal activity. The blank (Bl) contained all of the assay reagents but with no tissue. A, pTR4lacZ (−297/+52); B, pTR4lacZ (−655/+52). The means and S.E. are shown (n ≥ 3).

Table I

| Construct | Founder | Copy no. | PS-βgal | Ectopic |
|----------|---------|----------|---------|---------|
| TR2      | 4       | 6        | ND      | –       |
| TR3      | 8       | 12       | 1       | 1       |
|          | 12      | 10       | 1       | Retina  |
| TR4      | 9       | 4        | ND      | –       |
|          | 3       | 6        | 2       | +++     |
|          | 6       | 30       | 2       | +++     |
|          | 184     | 3        | ND      | +++     |
|          | 190     | 1        | ND      | +       |

* Number of mice analyzed.
* Number of transgene copies integrated.
+ β-Gal expression in the posterior segment (PS) of the eye. ++++, +, and – represent comparison of β-gal activity. ++++, high expression; +, low expression; –, no expression.
+ Ectopic β-gal expression in non-RPE tissues.
* ND, value not determined.

A, pTR4lacZ (−297/+52); B, pTR4lacZ (−655/+52). The means and S.E. are shown (n ≥ 3).

Concerning TR4, very similar values were obtained for F1 progeny of founder 6 and 184, but founder 190 had values 75% lower than these.

Discernible staining was seen only in the eyes of pTR4lacZ (−655/+52) transgenic animals (founders 3, 6, and 184). At the gross level, staining of the whole eyes revealed a punctate pattern (Fig. 3A). In sections of these eyes examined by light microscopy, the blue X-gal product was seen to be restricted in its distribution to the RPE cells of transgenic mice (Fig. 3C), whereas none was present in nontransgenic littermates (Fig. 3, B and D). No staining of lens, anterior segment, or neural retina was observed in transgenic and nontransgenic animals (data not shown). Staining of the RPE was patchy, however, and this was best appreciated by en face light microscopy of transgenic RPE/choroid/sclera flat mount (Fig. 3E). Again, no staining was present in nontransgenic littermate controls (Fig. 3F). Although most, if not all, cells of the RPE demonstrated some level of X-gal staining in the cytoplasm, about 15% of cells were much more highly stained and filled with blue product.

Analyses of fixed TR4 noneye tissues stained with X-gal did not reveal any detectable staining except for the founder 6 progeny, which showed an ectopic β-gal expression in the cerebellum. RPE65 is not expressed in brain (11) or cerebellum.

Analysis of the Rpe65 Promoter-driven Luciferase Activity in Vivo—To better understand the transcriptional regulation of the Rpe65 gene, we searched for a cellular model capable of activation of the mouse Rpe65 promoter. Although only traces of RPE65 mRNA are detected by PCR in nonconfluent cultures of the human RPE cell D407 (data not shown), it has been demonstrated that these cells are able to activate a human RPE65 promoter (22). Thus, to test its activity and specificity in vivo, the promoter fragment TR4 (−655 to +52) was cloned

3 T. M. Redmond, unpublished data.
Our results show that luciferase activity generated by pTR3 luc was very low and was similar in all of the cell lines tested. In HeLa, HepG2, and HS27, pTR4 luc activity was also similar to that produced by pTR3 luc (2.6, 2.2, and 2.6 times higher, respectively, than luciferase activity generated by pGL3-Basic alone). In contrast, in D407 cells, pTR4 luc generated activity 10–15 times higher than that generated by control plasmid alone (Fig. 4).

**Mutational Analysis of the Rpe65 Promoter**—To identify functionally important elements in the Rpe65 promoter, mutated derivatives of the TR4 promoter fragment (−655 to +52) cloned into pGL3-Basic (pTR4 luc) were constructed and transfected into D407 cells (oligonucleotides used for directed mutagenesis are shown in Table II). The mutation (TGGAAAATATAAAA) introduced into the NFI site (31) (located at position −178 to −165) had only a minor positive effect on promoter activity (Fig. 5A). In contrast, mutations of the core sequence of the two potential AP-4 binding sites, 1AP-4 (TCAGCTGAGG → TCCATGAGG) and 2AP-4 (TCAGCTCAGG → TCAATTAATT), located at positions −84 to −79 and −257 to −252, reduced promoter activity by 67% and 60%, respectively (Fig. 5A). In addition, mutations of the octamer sequence (ATGAAAAG → CCACACAA), located at position −498 to −491, reduced promoter activity by 67%.

Because none of these individual mutations completely abolished promoter activity, we next constructed combination mutants. The double combination of mutations in the 1AP-4 and 2AP-4 or octamer site had a greater effect than either mutation alone (Fig. 5B), dramatically reducing promoter activity by 78%, although the double mutant retained measurable promoter activity (3.5-fold the level of vector alone). Interestingly, a vector containing mutated 1AP-4, 2AP-4, and octamer sites further lowered luciferase activity (85% reduction compared with the wild type vector; Fig. 5C). This suggested that the E-box and octamer sequences are critical Rpe65 promoter elements. Finally, combinations that included the mutated NFI site together with an octamer mutation increased the luciferase activity obtained, compared with the values when this site was normal (Fig. 5, B and C).

**Specific Interactions of D407 and Bovine-RPE Nuclear Proteins with Elements in the Rpe65 Promoter**—To determine whether transcription factors binding to the potential NFI, octamer, and AP-4 sites in the Rpe65 promoter were present in vivo and in vitro, we performed EMSA using bovine-RPE nuclear proteins from freshly dissected bovine RPE tissue and D407 cells. A pattern consisting of three specific complexes was observed when either bovine RPE or D407 nuclear extracts were incubated with a labeled probe containing the NFI site. Each of these three complexes showed a similar mobility between the two nuclear extracts, and these were inhibited by addition of a 370-fold molar excess of cold competitor (Figs. 6B and 7B).

Incubation of bovine RPE or D407 nuclear extract with the downstream (1AP-4) (Figs. 6A and 7A) or the upstream (2AP-4) (Figs. 6C and 7C) AP-4 site resulted in the formation of a specific complex with each AP-4 probe. However, transcription factors bound to these sites with different affinities. In fact, the single complex formed with the 1AP-4 probe was observed only after 10 min of cross-linking (only traces were seen without cross-linking). A complex composed of a doublet was obtained with both nuclear extracts when 2AP-4 was used as a probe. In all cases, complexes were competed in the presence of an excess.
of cold competitor but not in the presence of a similar excess of mutated or nonspecific competitor.

Incubation of D407 nuclear extract with the Oct-1 probe resulted in the formation of two complexes. The lower molecular weight complex was competed away by a 250-fold molar excess of cold wild type competitor but not by cold mutant or irrelevant competitor, whereas the higher molecular weight complex was not inhibited by cold mutant competitor or nonspecific competitor.

The lack of available AP-4 antiserum precluded confirmation of AP-4 as a component of the complexes identified by EMSA with the two AP-4 probes. However, we performed UV crosslinking assays using the 1AP-4 probe and a D407 nuclear extract in order to determine the molecular weight of the complexes observed by EMSA. A higher band of 120 kDa and a lower band of 50 kDa were observed with both AP-4 probes (Fig. 8B). Both of the bands were competed away with a 2000-fold molar excess of cold probe but not with the same excess of an irrelevant competitor. These sizes correspond to those observed before for AP-4 by Mermod et al. (32).

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**DISCUSSION**

Rpe65 is, to date, the only known RPE-specific component of the RPE-specific all-trans- to 11-cis-retinoid isomerization mechanism known as the visual cycle. To understand the mechanism of this tissue specificity, we have characterized the Rpe65 promoter in vivo and in vitro. In this paper, we show that the −655 to +52 region of the mouse Rpe65 promoter confers tissue-specific expression in vivo and in vitro, and we define elements within this sequence that are crucial to this expression.

Our first goal was to identify a region of the 5′ flanking
region of the Rpe65 gene that reliably conferred in vivo RPE-specific expression. Our results from transgenic mice showed that the upstream region of the Rpe65 gene (–655 to +52) confers RPE-specific expression in adult animals, with no expression in nonocular tissues. Because transgenic animal production is a long-term procedure and unsuited to testing the effects of multiple mutations, we used an RPE cell line to perform in vitro experiments. However, expression levels of the Rpe65 gene are low or nonexistent in RPE cell lines (34) when compared with the in vivo expression in cells obtained from freshly dissected bovine eye. Variation in mRNA expression levels between in vivo and in vitro may result from changes due to immortalization and subsequent multiple passage of cell lines. Alternatively, repression of the endogenous Rpe65 gene in cell lines may be due to its context within chromatin. Because a transfected luciferase reporter gene is likely not to be in the same repressive chromatin context as the endogenous gene, its expression may be detected in a transient transfection assay if the transcriptional
elements necessary for this activation are present. A very low level of RPE65 mRNA was detectable by reverse transcription-PCR in 80% confluent cultures of the human RPE cell line D407 (not shown). Nicotelli et al. (22) reported that D407 is the RPE cell line that showed the highest transcriptional activity when transfected with a reporter human RPE65 promoter-luciferase vector. Accordingly, we used this cell line for transfection experiments to determine the minimal promoter sequence and to study the transcriptional elements necessary to induce luciferase expression. Our results showed that, as in vivo, the TR4 fragment induces the highest levels of luciferase expression, indicating that most, if not all, of the positive elements regulating the Rpe65 promoter activity in the RPE are indeed located in the −655 to +52 bp promoter sequence. Consequently, transcription factors binding to the AP-4, NFI, and octamer elements appeared to be obvious candidates for the Rpe65 specific gene expression in the RPE.

Specific and similar binding was detected by EMSA with nuclear extracts obtained either from freshly dissected bovine RPE cells or from D407, indicating that the same nuclear proteins may be involved in the Rpe65 gene expression both in vivo and in vitro. We found that mutations performed both in the AP-4 sites and in the octamer sequence completely disrupted the binding of nuclear proteins to these sites. In addition, these mutations introduced into the pTR4lac vector reduced the transcriptional activity of these potential elements. Although mutations of the upstream and downstream AP-4 sites separately reduced Rpe65 promoter activity by 67 and 60%, respectively, it was diminished by 78% by mutation of both together. Concurrent mutation of the octamer sequence further decreased transcriptional activity. This suggests a synergistic positive action of the transcription factors binding to these three sites in the transcriptional regulation of the Rpe65 gene.

Synergism of HLH and octamer binding proteins with themselves, between HLH and octamer binding proteins, and even of each with other proteins is consistent with previous studies. HLH proteins can act in concert with other HLH proteins to activate transcription. For instance, late transcription of SV40 activated by AP-4 is augmented by the addition of transcription factor AP-1 (32). Also, although MyoD can bind single sites, it must bind to multiple sites or adjacent to other transcription factors to activate muscle-specific genes (35, 36). Recently, tissue specific expression of the tyrosine hydroxylase gene has been shown to involve synergy between an HLH motif and an adjacent AP-1 site (37). In addition, the rat insulin gene is expressed and AP-4 is expressed relatively widely. Thus, one might account for the weak supershift observed in the presence of α-Oct-1 monoclonal antibody. Finally, it is conceivable that RPE65 expression in non-RPE cells is repressed by a negative-acting factor. One example of such a factor is neuronal restrictive silencer factor/RE1 (represor element 1)-silencing transcription factor (NRSF/REST), required for repression of multiple neuronal target genes in nonneuronal cells (50, 51).

The role of NFI in the Rpe65 transcriptional regulation is not clear. Our results indicated that NFI specifically binds to the Rpe65 promoter and also that luciferase reporter activity increases when the NFI site is mutated in combination with the octamer mutation. This might suggest a weakly negative effect of NFI in Rpe65 gene expression.

In summary, we have established that the proximal promoter region of the mouse Rpe65 gene can direct RPE-specific expression of a β-gal reporter construct in transgenic mice. We have also found that HLH and octamer-binding factors can synergistically regulate the Rpe65 gene and that mutations in these elements abolish transcriptional activity and prevent binding of the corresponding proteins. An NFI element is also involved but might act in a negative context. Although the regulation of Rpe65 gene expression provides a paradigm of tissue specific gene regulation, further investigation will be necessary to understand these mechanisms fully. In particular, identification and characterization of transcription factors binding to the transcriptional elements described is required. Such experiments are under way.

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