Regulatory Sequences in the 3′ Untranslated Region of the Human cGMP-Phosphodiesterase β-Subunit Gene

Mark R. Verardo,[1,2] *Andrea Viczian,*[1,3,4] *Natik Piri,*[1] *Novrouz B. Akhmedov,*[1] *Barry E. Knox,*[3,4] *and Debora B. Farber*[1,5]

Purpose. Rod cGMP-phosphodiesterase, a key enzyme in visual transduction, is important for retinal integrity and function. Mutations in the gene encoding the phosphodiesterase β-subunit (PDEβ) cause retinal degeneration in animals and humans. Here the authors tested the hypothesis that elements in the 3′ untranslated region (3′ UTR) of the *PDEβ* gene are involved in the regulation of *PDEβ* expression.

Methods. Involvement of the 3′ UTR of *PDEβ* mRNA in the regulation of *PDEβ* expression was assessed by Y-79 retinoblastoma cells or the heads of *Xenopus laevis* tadpoles with constructs containing the SV40 or *PDEβ* promoter, the luciferase cDNA, and either the SV40 or the *PDEβ* 3′ UTR (or fragments of its sequence).

Results. Compared with the SV40 3′ UTR (used as control), the entire *PDEβ* 3′ UTR decreased reporter gene expression in Y-79 retinoblastoma cells as well as in SY5Y neuroblastoma and 293 human embryonic kidney cell lines. However, the authors observed that two 100-nucleotide fragments from the *PDEβ* 3′ UTR increased while its noncanonical poly-adenylation signal abolished reporter gene expression in Y-79 retinoblastoma cells and in ex vivo experiments using *Xenopus* tadpole heads. In particular, an 11-nucleotide element (EURE) in one of the 100-nucleotide fragments was responsible for the upregulation of luciferase expression.

Conclusions. These studies indicate that the 3′ UTR of the *PDEβ* mRNA is involved in the complex regulation of this gene's expression in the retina. Moreover, the results show that the *PDEβ* poly-A signal has a dominant inhibitory effect over two other regions in the 3′ UTR that stimulate gene expression. (*Invest Ophthalmol Vis Sci.* 2009;50:2591–2598) DOI:10.1167/iovs.08-2010

The cGMP-phosphodiesterase (PDE) enzyme is involved in the conversion of light energy to an electrical signal in retinal photoreceptor cells, and is composed of at least four subunits, two with catalytic function (α and β) and two with inhibitory activity (γ).

Mutations in the coding region of the *PDEβ* subunit gene are known to cause retinal degeneration in mice and dogs[1,2] and, most important, result in autosomal recessive retinitis pigmentosa[3–7] and congenital stationary night blindness[8] in humans. A normal *PDEβ* subunit is, therefore, necessary to maintain the structure and function of photoreceptor cells.

Elements in the 5′ flanking region of the human *PDEβ* gene are necessary for efficient, cell-specific expression of *PDEβ* or reporter genes in cell cultures[9–13] and in *Xenopus*[9] retinas. Transcription factors binding to these elements and activating or repressing transcription are also important for retinal integrity and function. When mutated, these proteins may cause retinal degeneration (e.g., NRL,[14] CRX,[15,16] and Sp4[17]). In the past few years, considerable effort has been placed on characterizing these factors. In contrast, no information is available regarding the existence of regulatory elements in the 3′ UTR of *PDEβ*.

The 3′ UTR of several genes has been implicated in post-transcriptional regulation.[18,19] Specifically, it has been demonstrated that the 3′ UTR plays a role in message stability,[20] translation,[21] and intracellular transport[22] and that it is involved in polyadenylation.[23] Furthermore, the 3′ UTR has been acknowledged as a vital component of genes in the search for human mutations causing disease.[24]

We have previously shown that mouse *PDEβ* gene expression is also regulated at the translational level[25] and that the 3′ UTR of its mRNA increases the efficiency of protein synthesis.[26] In this study, we further analyze the function of this 3′ UTR and show that it contains specific sequences causing the upregulation or downregulation of expression of a reporter gene and that these events can be measured in transfections using different cell lines or the head of *Xenopus laevis* embryos.[27] This ex vivo transfection system has been used to characterize regulatory sequences in the 5′ flanking region of the *PDEβ*[9] and other retina-specific genes.[27,28] In addition, we report the identification of a novel 11-nucleotide element, EURE (eleven-nucleotide untranslated region element), that may regulate *PDEβ* gene expression.

Methods

Preparation of Luciferase Constructs

Several *PDEβ* 3′ UTR constructs were cloned into the pGL3-promoter vector (Promega, Madison, WI) between the luciferase reporter gene and the SV40 late poly-A signal. Transcription of the luciferase gene in this vector is controlled by the SV40 promoter.

- Construct p689 had the full-length *PDEβ* 3′ UTR (653 nt + 36 nt from the poly-A signal to the end of the poly-A tail). This was amplified from human retinal total RNA using the 3′ RACE kit, which provides the 3′ primer AUAP (Invitrogen, Carlsbad, CA), and the 5′ primer "a" (Table 1).
- Construct p228 contained the terminal 228 nt of *PDEβ* 3′ UTR (starting at the first putative poly-A signal) amplified by PCR with primer "b" (Table 1) and AUAP (3′ RACE Kit).

From the [1]Jules Stein Eye Institute, [2]Neuroscience IDP, and the [5]Molecular Biology Institute, University of California, Los Angeles, California; and the Departments of [3]Ophthalmology and [4]Biochemistry and Molecular Biology, SUNY Upstate Medical University, Syracuse, New York.

Supported by National Eye Institute Grant EY02651 (DBF) and Training Grants T32 EY07026 (MRV), EY11256, and EY12975 (BEK). Submitted in partial fulfillment of the requirements for a PhD at the University of California Los Angeles.

Submitted for publication March 11, 2008; revised November 5, 2008; accepted April 8, 2009.

Disclosure: **M.R. Verardo**, None; **A. Viczian**, None; **N. Piri**, None; **N.B. Akhmedov**, None; **B.E. Knox**, None; **D.B. Farber**, None

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Debora B. Farber, Jules Stein Eye Institute, 100 Stein Plaza, Room B237, University of California, Los Angeles, CA, 90095-7008; farber@jsei.ucla.edu.

TABLE 1. Primers Used for Generating Full-Length *PDEβ* 3′-UTR or Its Fragments and for Amplification of 3′ UTRs of Other Genes

Primer	Sequence
a	5′-GCACTGGTCCCGTGGGGACCCTAT
b	5′-AATAAACTGTAGCCTACATTAC
c	5′-GCGCTCGAGCTCAATCTTCACCCACTAGGA
d	3′-GCGGATCCTCACAGTTGGCTTCAGTTTA
Human cone arrestin	5′-GCGCTCGAGGGAGCTGAGCACCTCGCTCTG
	3′-GCGGGATCCCACATCTGAACAAACTGATTTATTAG
Human cone PDEα′	5′-CCGCTCGAGTATTATCTAACTGGTCTAACTGGTCTAAACTTC
	3′-CCGGATCCCAGGATTGCATGATTTTTT
Human SRB7	5′-CCGCTCGAGCCAGACTCATAGCATCAGTGG
	3′-CCGGATCCCATATGTTTCCTTATATTATGTTC
1	5′-ATCTAGACTCAATCTTCACCCACTAGG
2	3′-CGGATCCCAGAATGATCTTCAGTC
3	5′-GCTCTAGAGAAGATCATTCTGGATAT
4	3′-TAGGATCCTTGCAGTGAGCTGAGATC
5	5′-GATCTAGAATCTCAGCTCACTGCAACC
6	3′-CAGGATCCAAATTAGCCATGTGTGGTG
7	5′-ATCTAGACCACCACCACACATGGCTAA
8	3′-ATGGATCCCACTTCAGGAAGCTGAGGC
9	5′-CATCTAGAGCCTCAGCTTCCTGAAGTG
10	3′-CAGGATCCGGATGAGTAATGTAGGCTAC
11	5′-CTCTAGAGTAGCCTACATTACTCATCC
12	3′-TGGATCCCCCATCTGTCTACCTGTGTAC
13	5′-CTCTAGAGAACATTTGCAGCCACAC
14	3′-TGGATCCCTGAATTCCTGAGCATGT
15	3′-CGTCGACCTGTTTATTTTATTCTG
16	5′-GCTCTAGAGTTTTTATAAACGCTTCGAGCAGACATGATAA
17	3′-GCGTCGACTTTGTAGAGGTTTTACTTGCT

• Construct p431 contained nt 31 to 461 of *PDEβ* 3′ UTR (Fig. 1) and was obtained by RT-PCR of total RNA from Y-79 cells using primers "c" and "d" (Table 1).

• Seven approximately 100-nt *PDEβ* 3′ UTR fragments (F1–F7; Fig. 1) were generated by PCR with sequence-specific primers 1 and 2, 3 and 4, 5 and 6, 7 and 8, 9 and 10, 11 and 12, and 13 and 14, respectively (Table 1). These fragments were used to generate constructs pF1- to pF7-SV40 (see Fig. 6). In addition, construct pF7-PDEβ had F7 and the *PDEβ* poly-A signal. Primers 13 and 15 were used to obtain this fragment (see Fig. 5).

• A construct with the SV40 promoter in the pGL3 vector replaced by the *PDEβ* promoter (−93/+53) was used as backbone for the insertion of F1 or F5 or the 653-nt *PDEβ* 3′ UTR up to the poly-A signal (see Fig. 8).

• To generate construct pEURE containing the 11-nt element EURE identified in F5, the pGL3 promoter vector and primers 16 and 17 (Table 1) were used. Primer 16 has a sequence specific to the vector plus the EURE sequence, GTTTTTATAAA, and primer 17 is only from the pGL3 vector sequence. This resulted in a product with the 11 nt of *PDEβ* located immediately 5′ to the SV40 3′ UTR that was cloned back into the pGL3 promoter vector (see Fig. 9).

• For comparative studies, constructs with the full-length 3′ UTRs of cone PDEα′ (265 nt), cone arrestin (96 nt), and the SRB7 component of mammalian RNA polymerase II holoenzyme (306 nt) were prepared in the pGL3 promoter vector replacing the SV40 3′ UTR and polyA signal.

Cell Cultures and Transient Transfection

Y-79 human retinoblastoma (Y-79), 293 human embryonic kidney (HEK), and SY5Y human neuroblastoma cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Y-79 cells were propagated in suspension in RPMI 1640 medium (with L-glutamine and no sodium phosphate) supplemented with 15% fetal bovine serum (FBS). 293 HEK cells were propagated in Dulbecco's modified Eagle medium/F-12 supplemented with 10% FBS and penicillin/streptomycin mix (Invitrogen). SY5Y cells were maintained in a 1:1 mixture of Eagle minimum essential medium with nonessential amino acids and F-12 Nutrient Mixture (Ham) with 10% FBS. Y-79 cells were plated as described previously[12] at a density of 10^6 cells/60-mm plate. Similarly, 293 HEK and SY5Y cells were plated at a density of 10^6 cells/plate.

Transfections of Y-79 cells were carried out with 20 μg pGL3 construct/plate and the calcium phosphate precipitation method.[12] For 293 HEK and SY5Y cell transfections and for Y-79 cell transfections with constructs having EURE, 8.5 μg appropriate pGL3 construct/plate and lipofectamine were used (Invitrogen protocol). In all experiments, the pSV-β-galactosidase control vector (Promega) containing the bacterial *lacZ* gene driven by the SV40 early promoter was cotransfected with the construct being tested as an internal control for variations in transfection efficiency. A 15 μg/60-mm plate of pSV-β-galactosidase plasmid was used for calcium phosphate transfections and 6.3 μg/60-mm plate was used for lipofectamine transfections. Each construct was transfected in triplicate plates. Luciferase and β-galactosidase assays were performed, and relative luciferase activity was calculated as previously described.[10]

Ex Vivo Transfections in *Xenopus* Embryos

All experiments using *Xenopus* adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. *Xenopus* in vitro fertilizations and transfections were carried out as described by Batni et al.[27] Briefly, freshly laid *Xenopus* eggs were fertilized with crushed testicular tissue in vitro. Embryos grew until they reached stages 24 to 28, and groups of 10 to 12 dissected heads were transfected with 10 μg DNA and 30 μL DOTAP (Roche, Basel, Switzerland), each plasmid transfected in triplicate. After incubation for 72 to 80 hours postfertilization, heads were assayed for luciferase activity (Luciferase Assay System; Promega). The activity measured in these transfections has been shown to be accurate; hence, a normalization control[27] was not needed. Results are expressed as relative luciferase activity/head, ob-

tained by dividing total luciferase activity by the number of heads used for each plasmid tested.

Results

Cloning and Sequence Analysis of the Human *PDEβ* 3′ UTR

To obtain the full-length *PDEβ* 3′ UTR, 3′ RACE was performed on the retinal RNA of a human donor eye (Fig. 1). The resultant 689-nt product was longer than the previously reported *PDEβ* 3′ UTR[29] by 44 nt and contained the putative poly-A signal at position 654 and the poly-A tail. Its sequence (accession no. FJ417399; Fig. 1) was compared with that of the reported *PDEβ* cDNA[29] and with the *PDEβ* genomic sequence.[30] A few nucleotide differences were observed with the published sequences, but none fell within regions we found to be conserved in different species (Fig. 1, double underlines). The *alu* element (underlined) previously described[30] is not present in mouse, dog, or bovine *PDEβ* genes, suggesting its evolutionarily recent insertion into the human gene. In addition to the poly-A signal starting at position 654, another potential poly-A signal previously suggested[29] (dashed underlined) starts at position 461. However, no transcripts shorter than the sequence shown in Figure 1 have been found in human retinal mRNA samples. Further work is needed to determine whether both or only one poly-A signal is responsible for PDEβ polyadenylation.

Effects of the *PDEβ* 3′ UTR on Luciferase Expression

PDEβ is present in Y-79 retinoblastoma cells, indicating that these cells have the regulatory machinery necessary for *PDEβ* expression.[31] Therefore, Y-79 cells were chosen to analyze the effect of the 3′ UTR of *PDEβ* on gene expression. Transfection of construct p689 (containing the luciferase reporter gene driven by the SV40 promoter and the full-length 3′ UTR of *PDEβ*) into Y-79 cells resulted in luciferase activity that was approximately one-eighth of that produced by the pGL3 vector (Fig. 2). Moreover, transfection of the p228 construct containing the SV40 promoter, the luciferase reporter, and the terminal 228 nt of *PDEβ* 3′ UTR resulted in almost complete abolishment of luciferase expression (Fig. 2).

To determine whether this inhibitory effect is a feature of the *PDEβ* 3′ UTR, constructs with the SV40 promoter and the complete 3′ UTRs from three different genes—cone *arrestin* and cone *PDEα′* (both photoreceptor-specific[32,33]) and the *SRB7* hallmark component of the mammalian RNA polymerase II holoenzyme (a ubiquitously expressed mRNA[34])—were produced and transfected into Y-79 cells for comparison with p689 and pGL3. Luciferase activity of cells transfected with the *PDEβ* full-length 3′ UTR construct was approximately one-fifth, one-eighth, and one-twenty-fourth that of cells with the transfected *SRB7*, cone *arrestin*, and *PDEα′* 3′ UTR constructs, respectively (Fig. 3).

We also tested whether the *PDEβ* 3′ UTR downregulation of luciferase expression was specific to Y-79 cells by transfecting the p689 construct into 293 HEK and SY5Y neuroblastoma cells. In both cell lines, the *PDEβ* 3′ UTR-containing construct

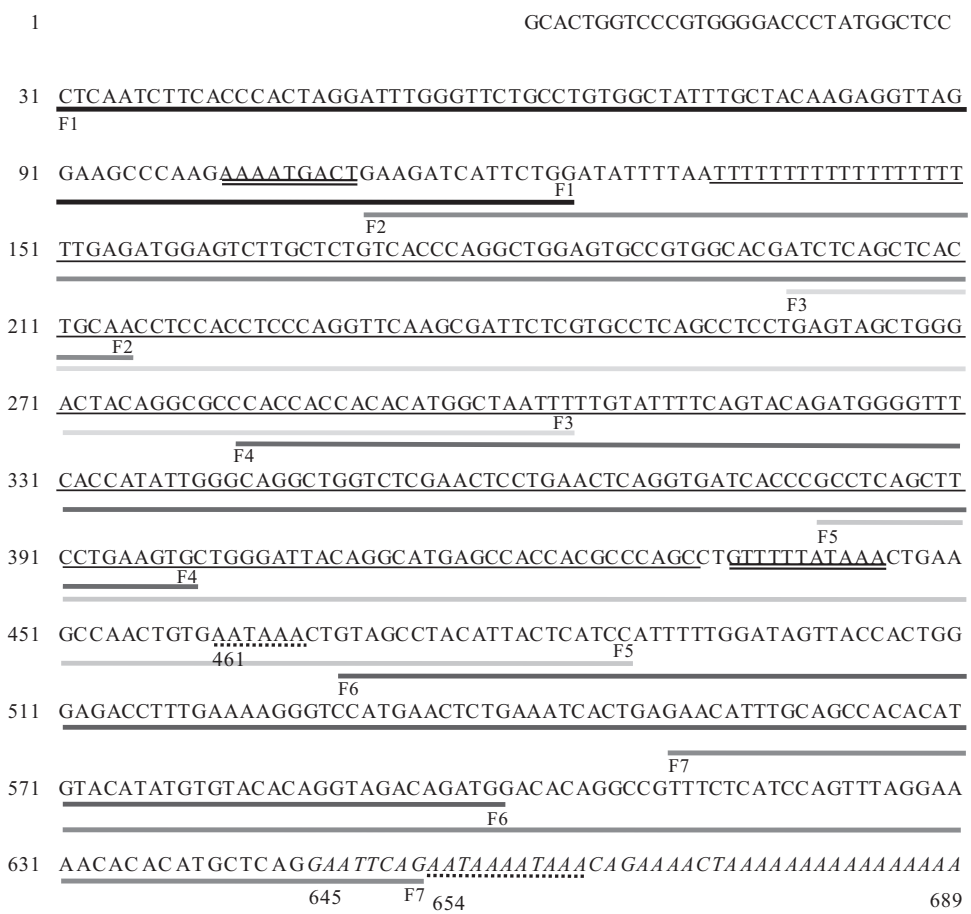

Figure 1. Sequence of the *PDEβ* 3′ UTR determined after 3′ RACE. The sequence shows the terminal 44 nt obtained after 3′ RACE, which complete the previously described *PDEβ* 3′ UTR. The previously reported alu-element is *underlined*. Two regions of high homology between mouse, dog, bovine, and human are *double underlined*. A potential poly-A signal is *dashed underlined*, as is the poly-A signal closest to the poly-A tail. The beginning and ending nucleotides of fragments F1 through F7, used in luciferase activity studies, are labeled, and lines of different shades of *gray* demarcate their sequences.

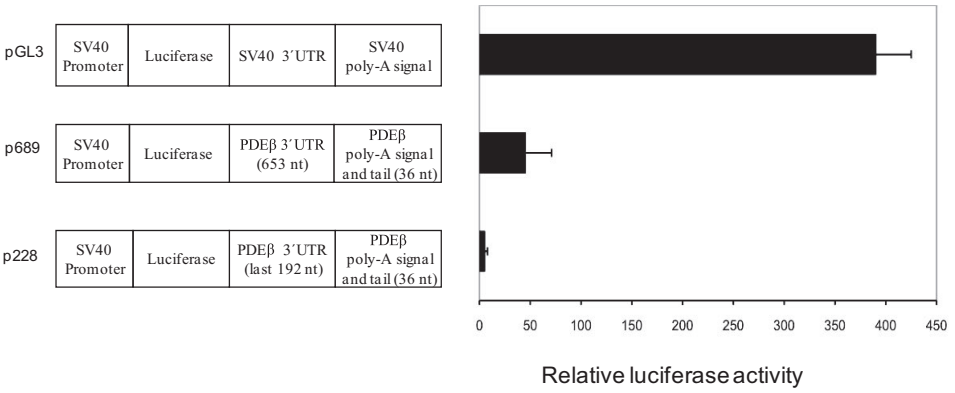

FIGURE 2. Both constructs with the full-length (689 nt) or terminal (228 nt) sequence of the *PDEβ* 3′ UTR significantly reduce the expression of the luciferase reporter gene produced by the pGL3 Promoter vector. Luciferase activity was measured in lysates of Y-79 cells transfected with pGL3 (containing the SV40 3′ UTR), p689 (containing the *PDEβ* full-length 3′ UTR: 653 nt plus 36 nt poly-A signal and poly-A tail), or p228 (containing the last 228 nt of the *PDEβ* 3′ UTR; 192 nt plus 36 nt poly-A signal and poly-A tail) and normalized to the corresponding β-galactosidase activity for each sample. Each transfection was performed in triplicate and repeated three to five times. Results, expressed as relative luciferase activity, represent the mean normalized luciferase activity measured for each sample ± SD. These results are statistically highly significant. Two-tailed *P* values are: pGL3 versus p689, $P < 0.0005$; pGL3 versus p228, $P < 0.0001$.

decreased gene expression by approximately 75% when compared with that produced by pGL3 (Fig. 4).

PDEβ Poly-A Signal

We studied the effect of the noncanonical *PDEβ* poly-A signal at position 654 on gene expression because both the p689 and the p228 constructs containing the full-length (653-nt and poly-A signal and poly-A tail) and terminal 228 nt (192-nt and poly-A signal and poly-A tail), respectively, have this sequence and decrease luciferase expression (Fig. 2). For these experiments, we compared luciferase activity from two constructs: pF7-SV40, prepared by replacing the SV40 3′ UTR with F7 (the last 110 nt of the *PDEβ* 3′ UTR before the poly-A signal) but keeping the SV40 poly-A signal, and pF7-PDEβ, in which the SV40 poly-A signal was substituted with the *PDEβ* poly-A signal. In both constructs, the luciferase reporter gene was driven by the SV40 promoter (Fig. 5). The relative luciferase activity produced in Y-79 cells by construct pF7-PDEβ was approximately one-sixth that produced by pF7-SV40. This activity was not significantly different from that generated by the p689 construct, which has the entire *PDEβ* 3′ UTR and its poly-A signal, whereas the luciferase activity produced by the pGL3 Promoter vector was similar to that obtained with pF7-SV40 (Fig. 5).

Fragments of the *PDEβ* 3′ UTR

Although the p689 construct dramatically reduced luciferase expression compared with that obtained with the pGL3 Promoter vector, the p431 construct upregulated the expression of luciferase by almost threefold (Fig. 6).

To narrow down the region(s) of the *PDEβ* 3′ UTR segment responsible for the observed effect on luciferase expression, we generated seven approximately 100-nt fragments of the 3′ UTR and subcloned each fragment into the pGL3 Promoter vector (Fig. 6). When transfected into Y-79 cells, fragment 1 (pF1) and fragment 5 (pF5, Fig. 1) increased luciferase expression to the level produced by p431 (Fig. 6). Fragments in pF2, pF3, pF4, pF6, and pF7 had no significant effect compared with pGL3.

To determine whether the upregulation caused by F1 and F5 is also observed in other cell lines, 293 HEK and SY5Y neuroblastoma cells were transfected with the pF1 and pF5 constructs (Fig. 7). In 293 HEK cells, both constructs increased expression, as they did in Y-79 cells. However, in SY5Y cells, pF5 increased expression over control levels whereas pF1 did not.

Potential Combined Effect of the *PDEβ* Promoter and 3′ UTR

The basal promoter of the *PDEβ* gene (−93/+53) has previously been characterized and shown to produce high levels of rod-specific PDEβ expression in Y-79 cells and in the developing retinas of *Xenopus* embryo heads maintained ex vivo.[9] To determine whether there is a combined effect of the *PDEβ* promoter and the 3′ UTR on luciferase expression, constructs depicted in Figure 8 were generated with this promoter cloned upstream of the luciferase reporter gene and the SV40 3′ UTR (construct pβ-3′SV40-SV40), fragments of the *PDEβ* 3′ UTR (F1, construct pβ-F1-SV40; F5, construct pβ-F5-SV40), or the full-length *PDEβ* 3′ UTR (construct pβ-3′β-PDEβ). When transiently transfected into dissected *Xenopus* embryo heads, construct pβ-3′β-PDEβ produced much lower luciferase activity than the other constructs and approximately one-tenth the activity generated by the control pβ-3′SV40-SV40. This activity was similar to that obtained by transfecting Y-79 cells with the

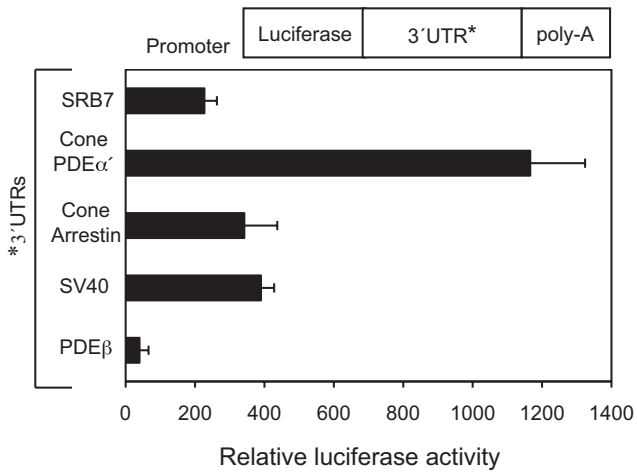

FIGURE 3. Comparison of the relative luciferase activity produced by constructs containing the *PDEβ* 3′ UTR or the 3′ UTRs of other genes. Transfections with pGL3 constructs that had the SV40 3′ UTR substituted by the 3′ UTR of the *SRB7* component of mammalian RNA polymerase II, cone *arrestin*, or *PDEα′* were carried out in triplicate in Y79 cells and were repeated three times. Results represent the mean relative luciferase activity measured for each sample ± SD. The full-length *PDEβ* 3′ UTR shows the downregulation of luciferase expression when compared to the 3′ UTR of any of the other genes studied.

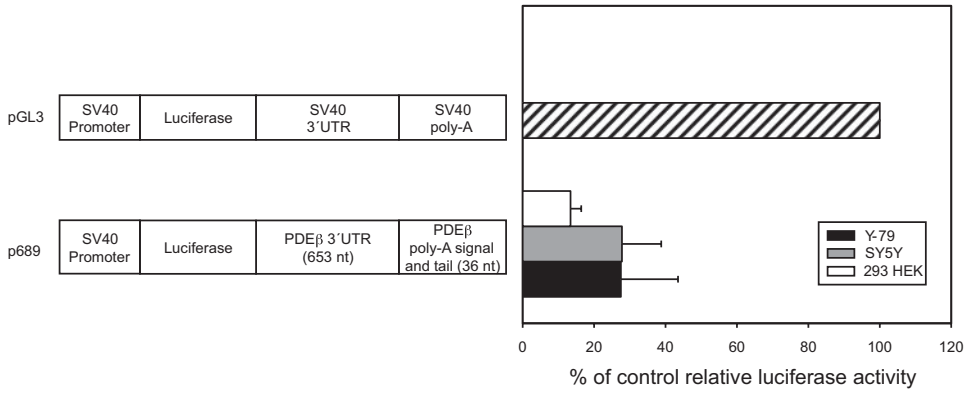

FIGURE 4. Comparison of the effect of *PDEβ* 3′ UTR on reporter gene expression in different cell lines. The pGL3 Promoter vector used as control was transfected into Y-79, SY5Y (neuroblastoma), and 293 HEK (human embryonic kidney) cell lines, and the luciferase activity obtained was considered 100% for each cell line (*striped bar*). Construct p689 was transfected in the same cell lines as pGL3 in triplicate, and the experiment was repeated three times. Relative luciferase activity generated by p689 in Y-79 (*black bar*), SY5Y (*gray bar*), and 293 HEK cells (*white bar*) is expressed as a percentage of the control activity and demonstrates that the *PDEβ* 3′ UTR reduces gene expression in these three cell lines. Error bars represent SD.

SV40 promoter-containing p689 (Fig. 2). In contrast, pβ-F1-SV40 and pβ-F5-SV40 showed an approximately threefold increase in expression of luciferase compared with pβ-3′SV40-SV40, similar to the results obtained by transfecting Y-79 cells with pF1-SV40 and pF5-SV40, which have the SV40 promoter (Fig. 6). Therefore, the data from the *Xenopus* transfections suggest that there is no combined effect of the *PDEβ* promoter and 3′ UTR that would significantly affect *PDEβ* gene expression.

An 11-nt Element in F5 Is Responsible for the Increase in Reporter Gene Expression

Sequence analysis demonstrated that an 11-nt sequence (EURE) in F5 is highly conserved across mouse, dog, cow, and human *PDEβ* 3′ UTR sequences (Fig. 9A). We have found that EURE is present in the 3′ UTR of several genes, including ADP-ribosylation-like factor 6 interacting protein 5, ubiquitin conjugating enzyme E2D1, and ethanolamine kinase transcript variant 1. Insertion of EURE between the luciferase cDNA and the SV40 3′ UTR of pGL3 led to approximately twofold higher luciferase activity in Y-79 cells than that obtained with the pGL3-Promoter vector (Fig. 9B). This suggests that the increase in luciferase expression after transfection of *Xenopus* heads with the F5 construct (Fig. 8) was caused by the presence of the EURE sequence in that fragment of the *PDEβ* 3′ UTR.

DISCUSSION

It is currently accepted that the 3′ UTR of mRNA is important in posttranscriptional events[18–21] and in translational control.[22–23] However, though many transcription enhancers and repressors have already been identified, few sequence elements have been described in the 3′ UTR controlling gene expression. With an increasing interest in 3′ UTRs,[24] tools to assay the function of such regions are becoming important.

We worked out a systematic approach that led to the discovery of at least two enhancers and one repressor in the 689-nt 3′ UTR of the human *PDEβ* mRNA. To determine the location of these sequences, we created seven approximately 100-nt segments of the *PDEβ* 3′ UTR that were introduced into reporter gene vectors and then tested each of them in Y79 cells for its effect on luciferase activity. Given that the length of the 3′ UTR has been reported to have an effect on gene expression,[35] we were careful to keep a similar number of nucleotides in our fragment constructs. We showed that two of these constructs containing F1 and F5 increased luciferase reporter

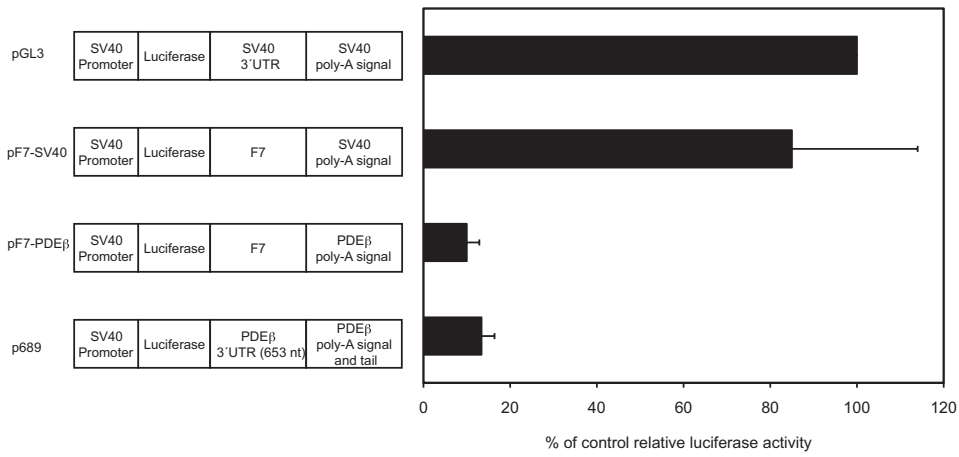

FIGURE 5. Effect of the *PDEβ 3′* UTR poly-A signal on gene expression. Relative luciferase activity generated by the control pGL3 Promoter construct was compared with that produced by constructs p689, pF7-SV40, and pF7-PDEβ (the latter two had F7 and the SV40 or PDEβ poly-A signal, respectively). Transfections in Y-79 cells were carried out in triplicate and repeated three to five times. Results, expressed as the mean percentage of control relative luciferase activity ± SD, show that the *PDEβ* 3′ UTR poly-A signal reduces by approximately 85% to 90% the relative luciferase activity produced by constructs that have the SV40 poly-A signal.

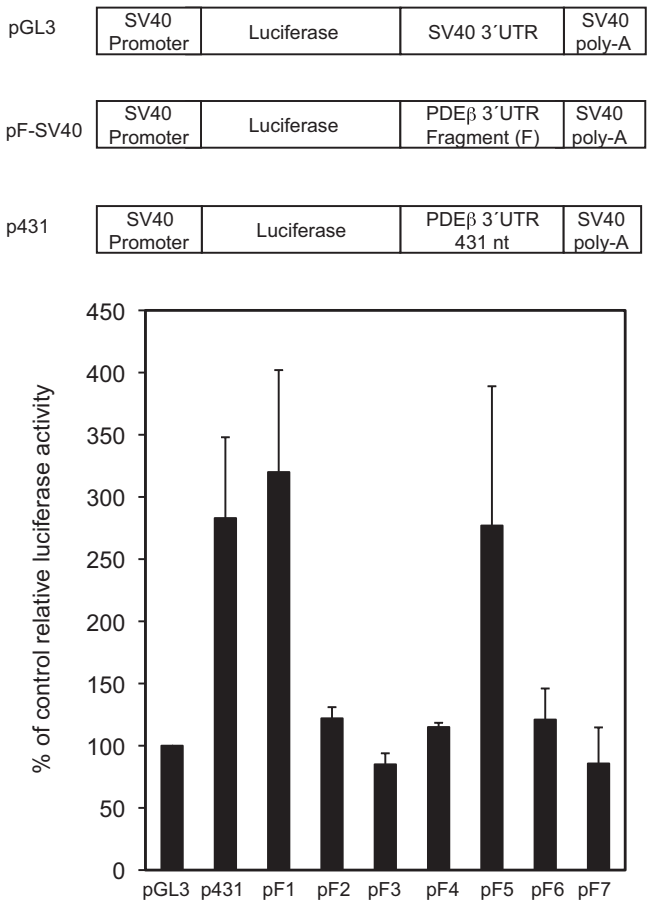

FIGURE 6. Effect of different segments of *PDEβ* 3′ UTR on reporter gene expression. Fragments of *PDEβ* 3′ UTR were cloned between the luciferase cDNA and the SV40 poly-A signal, and each construct was transfected in triplicate into Y-79 cells. This experiment was repeated three to five times. Relative luciferase activity is shown as a percentage of control ± SD (the pGL3 Promoter vector with the SV40 3′ UTR is used as 100%). Three constructs (pF1, pF5, and p431) increased reporter gene expression.

gene activity but that none of the other five constructs did. Therefore, specific sequences in F1 and F5 must be responsible for the increased gene expression.

When the sequences from human, cow, dog, and mouse *PDEβ* 3′ UTRs were compared, an 11-nt stretch was found to be conserved in all them. This 11-nt sequence, EURE, is present in F5. Transfection of Y-79 cells with pEURE, this EURE-containing pGL3 vector doubled the reporter gene activity of pGL3, similar to the upregulation of luciferase expression observed from the pF5 construct (Fig. 6). Thus, these 11 nucleotides define a novel *PDEβ* 3′ UTR enhancer.

Transfections of DNA constructs into *Xenopus* embryo heads have been used to characterize *Xenopus* and human gene promoters (*Xenopus rhodopsin*[27] and human *PDEβ*[9]). We corroborated the results of our transfections into Y-79 cells using ex vivo transfections into tadpole heads. We found that constructs containing the full-length *PDEβ* 3′ UTR produced a similar decrease in luciferase activity in either *Xenopus* embryo heads or Y-79 cells and that constructs with fragments F1 and EURE-containing F5 of the *PDEβ* 3′ UTR increased reporter gene expression in both transfection systems. This implied that similar *PDEβ* translational mechanisms are present in Y-79 retinoblastoma cells and *Xenopus* retina.

However, constructs containing F1 or F5 produced different results when transfected into various human cell types, suggesting the presence of two separate modules in the *PDEβ* 3′ UTR that control the increase in gene expression by distinct mechanisms, one of them responding to F1 and the other to F5. Although the module responding to F1 is absent or inhibited in neuroblastoma cells, the F1 and F5 modules are present in Y-79 and 293 HEK cells. Since it has been reported that 3′ UTRs regulate gene expression by binding *trans*-acting factors,[20] it is possible that elements in F1 and F5, such as EURE, are involved in these interactions. Moreover, EURE may be rod PDEβ-specific in the retina because we did not find it in any characterized retinal genes in a recent BLAST search.

In transfections of the construct containing the entire 3′ UTR of PDEβ mRNA into Y-79, 293 HEK and neuroblastoma

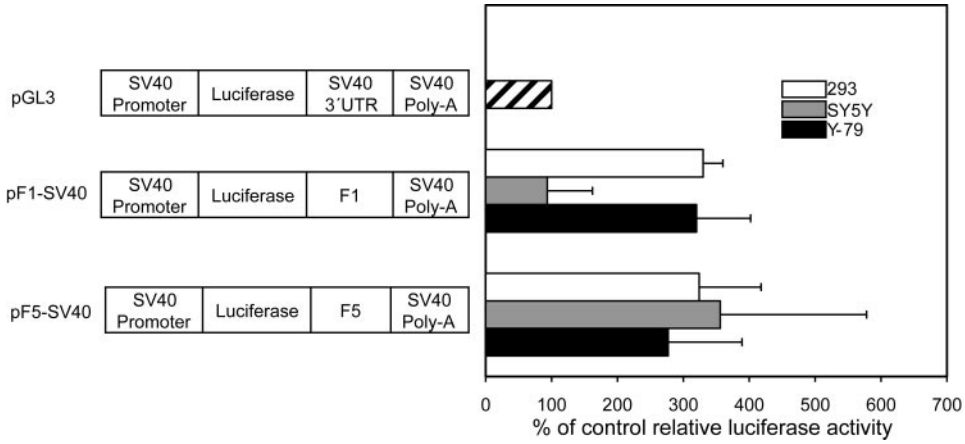

FIGURE 7. Comparison of F1 and F5 on reporter gene expression in different cell lines. The pGL3 Promoter vector used as control was transfected in Y-79, SY5Y, and 293 HEK cells, and the luciferase activity obtained was considered 100% for each cell line (*striped bar*). Constructs pF1 and pF5 were transfected in triplicate in the same cell lines, and transfections were repeated three times. The relative luciferase activity generated by these in Y-79 (*black bars*), SY5Y (*gray bars*), and 293 HEK (*white bars*) cells by pF1 and pF5 is expressed as a percentage of control activity and shows that F1 increased gene expression only in Y79 and 293 HEK cells, whereas F5 did increase expression in the three cells lines studied. Error bars represent SD.

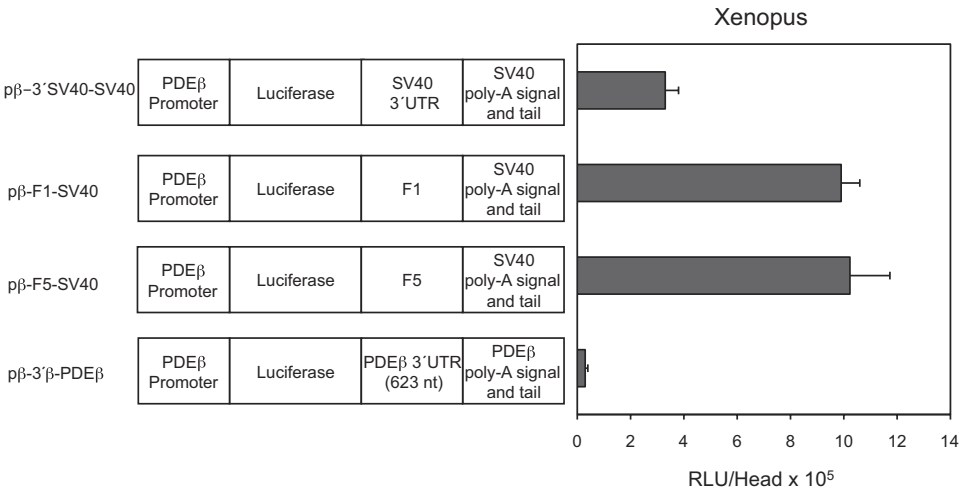

FIGURE 8. The *PDEβ* promoter does not modify the effect of the *PDEβ* 3′ UTR or its fragments on reporter gene expression. The SV40 promoter in pGL3 and the pF1-SV40 and pF5-SV40 constructs of Figure 7 were replaced by the *PDEβ* promoter, resulting in constructs pβ-3′UTR-SV40, pβ-F1-SV40, and pβ-F5-SV40. After transient transfection into *Xenopus* embryo heads (see Methods), the luciferase activity generated by pβ-F1-SV40 and pβ-F5-SV40 almost tripled that of the control pβ-3′UTR-SV40. Replacement of the SV40 3′ UTR and poly-A signal of pβ-3′SV40-SV40 with the *PDEβ* full-length 3′ UTR (construct pβ-3′β-PDEβ) decreased by 90% the relative luciferase activity of pβ-3′UTR-SV40. Results are expressed as relative luciferase (RLU) activity/head $\times 10^5$ and are statistically highly significant. Two-tailed *P* values are: pβ-3′SV40-SV40 versus pβ-3′β-PDEβ, $P = 0.0005$; pβ-3′SV40-SV40 versus pβ-F1-SV40, $P = 0.0002$; pβ-3′SV40-SV40 versus pβ-F5-SV40, $P = 0.0016$.

cells, as well as in *Xenopus* heads, a substantial decrease in expression of the luciferase reporter gene was observed. However, none of the individual 100-nt fragments of *PDEβ* 3′ UTR produced this effect, and neither did the 3′ UTRs of other genes (the photoreceptor-specific cone *PDEα′* and cone *arrestin* and the ubiquitously expressed *SRB7* component of mammalian RNA polymerase II). Further experiments showed that the noncanonical poly-A signal of the *PDEβ* 3′ UTR was responsible for this inhibition of expression. Constructs differing exclusively in the poly-A signal produced less luciferase activity when they had the *PDEβ* than the SV40 poly-A signal (Fig. 5). The mechanism of this downregulation may involve the binding to the poly-A signal of ubiquitous proteins found in every cell type, which may inhibit translation. Another possibility is that this sequence is important for mRNA processing. Mutations in the poly-A signal have been reported to disrupt RNA processing.[36] The noncanonical *PDEβ* poly-A signal could have the same effect.

If the *cis*-elements of the 3′ UTR of *PDEβ* are important regulators of gene expression, it is conceivable that mutations in these sequences could lead to degeneration of the photoreceptor cells. In addition, mutations in the genes encoding *trans*-acting factors binding to these elements may also cause retinal degeneration, similar to what is observed when mutated transcription factors such as NRL, CRX, and Sp4 bind to photoreceptor-specific promoters.[14–17]

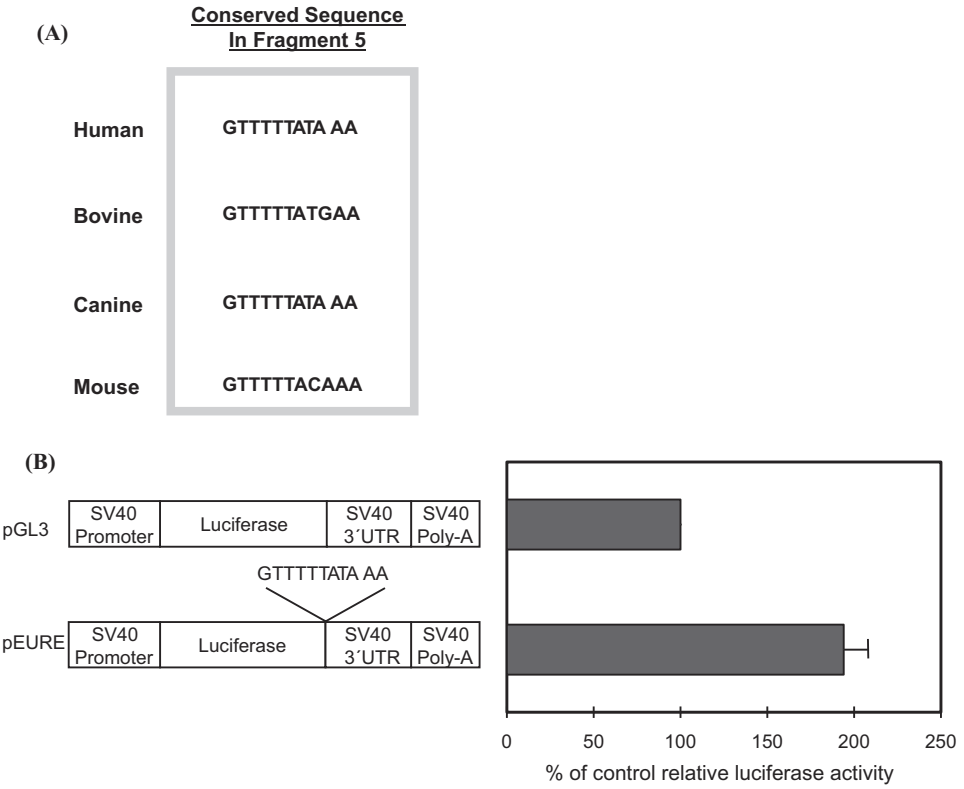

FIGURE 9. Insertion of the 11-nt EURE element found in F5 of *PDEβ* 3′ UTR into the pGL3 Promoter vector doubles its reporter gene expression in Y-79 cells. (**A**) Homologous sequence found in fragment 5 of the *PDEβ* 3′ UTR of several species. (**B**) Transfections of pGL3 and pEURE in Y79 cells were carried out in triplicate, and this experiment was repeated several times. Relative luciferase activity generated by the control pGL3 construct is considered 100%. Results are shown as a percentage of control relative luciferase activity ± SD.

In summary, our results illustrate that the 3′ UTR of the *PDEβ* mRNA is involved in the complex regulation of this gene's expression in the retina. Moreover, they show that the *PDEβ* poly-A signal has a dominant inhibitory effect over two other regions in the 3′ UTR that stimulate gene expression. However, we think the involvement of these regions in the regulation of *PDEβ* expression depends on the levels of *trans*-acting proteins that potentially bind those sequences. The level of these factors could vary, such as between developing and fully differentiated photoreceptors, leading to upregulation or downregulation of *PDEβ* expression.

Acknowledgments

The authors thank Leah Kang, Lili Kudo, and Nilofar Namia for technical assistance.

References

1. Bowes C, Li T, Danciger M, Baxter LC, Applebury ML, Farber DB. Retinal degeneration in the *rd* mouse is caused by a defect in the β subunit of rod cGMP-phosphodiesterase. *Nature.* 1990;347:677–680.
2. Suber ML, Pittler S, Qin N, et al. Irish setter dogs affected with rod-cone dysplasia contain a nonsense mutation in the rod cyclic GMP phosphodiesterase β subunit gene. *Proc Natl Acad Sci U S A.* 1993;90:3968–3972.
3. Bayes M, Giordano M, Balcells S, et al. Homozygous tandem duplication within the gene encoding the beta-subunit of rod phosphodiesterase as a cause for autosomal recessive retinitis pigmentosa. *Hum Mutat.* 1995;5:228–234.
4. Danciger M, Blaney J, Gao YQ, et al. Mutations in the PDE6B gene in autosomal recessive retinitis pigmentosa. *Genomics.* 1995;30:1–7.
5. Danciger M, Heilbron V, Gao YQ, Zhao DY, Jacobson SG, Farber DB. A homozygous PDE6B mutation in a family with autosomal recessive retinitis pigmentosa. *Mol Vision.* 1996;2:10.
6. McLaughlin ME, Sandberg MA, Berson EL, Dryja TP. Recessive mutations in the gene encoding the β-subunit of rod phosphodiesterase in patients with retinitis pigmentosa. *Nat Genet.* 1993;4:130–134.
7. McLaughlin ME, Ehrhart TL, Berson EL, Dryja TP. Mutation spectrum of the gene encoding the beta-subunit of rod phosphodiesterase among patients with autosomal recessive retinitis pigmentosa. *Proc Natl Acad Sci U S A.* 1995;92:3249–3253.
8. Gal A, Orth U, Baehr W, Schqwinger E, Rosenberg T. Heterozygous missense mutation in the rod cGMP phosphodiesterase β-subunit in autosomal dominant congenital stationary night blindness. *Nat Genet.* 1994;7:64–68.
9. Lerner LE, Gribanova YE, Ji M, Knox BE, Farber DB. Nrl and Sp nuclear proteins mediate transcription of rod-specific cGMP-phosphodiesterase beta-subunit gene: involvement of multiple response elements. *J Biol Chem.* 2001;276:34999–35007.
10. Lerner LE, Farber DB. Transcriptional regulation of the cGMP phosphodiesterase beta-subunit gene. *Methods Enzymol.* 2000;315:617–635.
11. Lerner LE, Gribanova YE, Whitaker L, Knox BE, Farber DB. The rod cGMP-phosphodiesterase beta-subunit promoter is a specific target for Sp4 and is not activated by other Sp proteins or CRX. *J Biol Chem.* 2002;277:25877–25883.
12. Di Polo A, Lerner L, Farber D. Transcriptional activation of the human rod cGMP-phosphodiesterase β-subunit is mediated by an upstream AP-1 element. *Nucleic Acids Res.* 1997;25:3863–3867.
13. Di Polo A, Bowes-Rickman C, Farber DB. Isolation and initial characterization of the 5′ flanking region of the human and murine cGMP phosphodiesterase β-subunit genes. *Invest Ophthalmol Vis Sci.* 1996;37:551–560.
14. Bessant DA, Payne AM, Mitton KP, et al. A mutation in NRL is associated with autosomal dominant retinitis pigmentosa. *Nat Genet.* 1999;21:355–356.
15. Freund C, Gregory-Evans C, Furukawa T, et al. Cone-rod dystrophy due to mutations in a novel photoreceptor-specific homeobox gene (CRX) essential for maintenance of the photoreceptor. *Cell.* 1997;91:543–553.
16. Freund C, Wang Q, Chen S, et al. De novo mutations in the CRX homeobox gene associated with Leber congenital amaurosis. *Nat Genet.* 1998;18:311–312.
17. Gao YQ, Danciger M, Ozgul RK, Gribanova Y, Jacobson S, Farber DB. Association of the Asn306Ser variant of the SP4 transcription factor and an intronic variant in the beta-subunit of transducin with digenic disease. *Mol Vis.* 2007;13:287–292.
18. Pesole G, Mignone F, Gissi C, Grillo G, Licciulli F, Liuni S. Structural and functional features of eukaryotic mRNA untranslated regions. *Gene.* 2001;276:73–81.
19. Grzybowska EA, Wilczynska A, Siedlecki JA. Regulatory functions of 3′ UTRs. *Biochem Biophys Res Commun.* 2001;288:291–295.
20. Boado RJ, Pardridge WM. Ten nucleotide cis element in the 3′-untranslated region of the GLUT1 glucose transporter mRNA increases gene expression via mRNA stabilization. *Brain Res Mol Brain Res.* 1998;59:109–113.
21. Harford JB, Morris DR, eds. *mRNA Metabolism and Post-Transcriptional Gene Regulation.* New York: Wiley-Liss; 1997.
22. Liu S, Redmond T. Role of the 3′-untranslated region of RPE65 mRNA in the translational regulation of the RPE65 gene: identification of a specific translation inhibitory element. *Arch Biochem Biophys.* 1998;357:37–44.
23. Keene JD. Why is Hu where? Shuttling of early-response-gene messenger RNA subsets. *Proc Natl Acad Sci U S A.* 1999;96:5–7.
24. Conne B, Stutz A, Vassalli JD. The 3′ untranslated region of messenger RNA: a molecular 'hotspot' for pathology? *Nat Med.* 2000;6:637–641.
25. Piri N, Yamashita CK, Shih J, Akhmedov NB, Farber DB. Differential expression of rod photoreceptor cGMP-phosphodiesterase alpha and beta subunits: mRNA and protein levels. *J Biol Chem.* 2003;278:36999–37005.
26. Piri N, Mendoza E, Shih J, Yamashita CK, Akhmedov NB, Farber DB. Translational regulation of the rod photoreceptor cGMP-phosphodiesterase: the role of the 5′- and 3′-untranslated regions. *Exp Eye Res.* 2006;83:844–848.
27. Batni S, Scalzetti L, Moody SA, Knox BE. Characterization of the *Xenopus* rhodopsin gene. *J Biol Chem.* 1996;271:3179–3186.
28. Mani SS, Besharse JC, Knox BE. Immediate upstream sequence of arrestin directs rod-specific expression in *Xenopus*. *J Biol Chem.* 1999;274:15590–15597.
29. Collins C, Hutchinson G, Kowbel D, Riess O, Weber B, Hayden MR. The human β-subunit of rod photoreceptor cGMP phosphodiesterase: complete retinal cDNA sequence and evidence for expression in brain. *Genomics.* 1992;13:698–704.
30. Weber B, Riess O, Hutchinson G, et al. Genomic organization and complete sequence of the human gene encoding the β-subunit of the cGMP phosphodiesterase and its localization to 4p16.3. *Nucleic Acids Res.* 1992;19:6263–6268.
31. Di Polo A, Farber DB. Rod photoreceptor-specific gene expression in human retinoblastoma cells. *Proc Natl Acad Sci U S A.* 1995;92:4016–4020.
32. Hurwitz RL, Bunt-Milam AH, Beavo JA. Immunologic characterization of the photoreceptor outer segment cyclic GMP phosphodiesterase. *J Biol Chem.* 1984;259:8612–8618.
33. Craft CM, Whitmore DH, Wiechmann AF. Cone arrestin identified by targeting expression of a functional family. *J Biol Chem.* 1994;269:4613–4619.
34. Chao DM, Gadbois EL, Murray PJ, et al. A mammalian SRB protein associated with an RNA polymerase II holoenzyme. *Nature.* 1996;380:82–85.
35. Tanguay RL, Gallie DR. Translational efficiency is regulated by the length of the 3′ untranslated region. *Mol Cell Biol.* 1996;16:146–156.
36. Sheets MD, Ogg SC, Wickens MP. Point mutations in AAUAAA and the poly (A) addition site: effects on the accuracy and efficiency of cleavage and polyadenylation in vitro. *Nucleic Acids Res.* 1990;18:5799–5805.