Differential Expression of Rod Photoreceptor cGMP-Phosphodiesteraseα and β Subunits

mRNA AND PROTEIN LEVELS

Natik Pirì, Clyde K. Yamashita, Jennifer Shih, Novrouz B. Akhmedov, and Debra B. Farber

Received for publication, April 9, 2003, and in revised form, July 15, 2003
Published, JBC Papers in Press, July 18, 2003, DOI 10.1074/jbc.M303710200

The catalytic core of photoreceptor-specific cGMP-phosphodiesterase (PDE) consists of two subunits, PDEα and PDEβ, that are homologous and have similar domain organization but are encoded by different genes. We have examined the PDEα and PDEβ mRNA steady-state and protein levels as well as the biosynthesis rate of these proteins in developing and fully differentiated retinas. We have also determined the translational efficiency of PDE subunits and the role of their mRNA structures in regulating protein synthesis. In mature retinas, PDEα and PDEβ are represented by \(1.5 \times 10^8\) and \(7.5 \times 10^8\) copies/μg retinal mRNA, respectively. The levels of these transcripts in developing photoreceptors (P10) are approximately 75% of those at P30. Quantification of protein concentration indicated that PDEα and PDEβ are equally expressed in developing and fully differentiated photoreceptors. Furthermore, the PDEα/PDEβ ratios obtained throughout a 2-h pulse-chase period revealed a similar turnover rate for both subunits. The observed discordance between the mRNA and protein levels of PDEα and PDEβ suggested post-transcriptional regulation of their expression. We found that PDEα mRNA is translated more efficiently than either of the two PDEβ transcripts expressed in retina. Therefore, the lower level of PDEα mRNA is compensated by its more efficient translation to achieve equimolar expression with PDEβ. We also analyzed the effect of PDEα and PDEβ mRNA 5’- and 3’-untranslated regions as well as that of their coding regions on protein synthesis. We determined that the PDE-coding regions play a critical role in the differential translation of these subunits.

Signal transduction in the vertebrate rod and cone photoreceptors is mediated by a cascade of protein interactions/activations that lead to hydrolysis of cGMP by a photoreceptor-specific cGMP-phosphodiesterase (PDE) followed by closure of the cGMP-gated channels on the plasma membrane. The catalytic core of cone PDE is composed of two identical α’ subunits, whereas that of the rod enzyme is a heterodimer (PDEα, 88 kDa and PDEβ, 84 kDa). Although each of these three proteins is encoded by a different gene, they share significant homology in both amino acid and nucleotide sequences. Cone and rod PDEs have similar domain organization and contain two cGMP-binding site motifs, a catalytic site and a CAAAX box signal for post-translational modification. Why the rod holoenzyme consists of two different subunits is still unknown. Based on classical biochemical studies carried out with the bovine rod cGMP-PDE, it has been accepted that PDEα and PDEβ are present in a 1:1 ratio and that they are assembled in a heterodimeric catalytic core. This coordinated expression of PDEα and PDEβ in rod photoreceptors must be regulated at the transcriptional and/or post-transcriptional level so that a final equimolar ratio of these proteins is achieved. However, there is some indirect evidence suggesting the possibility that the rod PDE catalytic core is not only present as a heterodimer but also as αα and ββ homodimers, similar to the rod α’/α’ PDE. We have previously demonstrated that the individually expressed catalytic subunits have PDE enzymatic activity, supporting the idea of independent catalytic motifs on PDEα and PDEβ. Thus, PDEα and PDEβ may be present in photoreceptor cells at any ratio depending on their expression "strength."

The proteins of the phototransduction cascade, PDE in particular, have been extensively studied for many years. However, very little is known regarding the regulation of PDEα and PDEβ expression at either the transcriptional or translational level. Although the 5’-flanking regions of PDEα and PDEβ genes have been cloned and sequenced, a detailed analysis that would allow us to speculate about the possible mechanisms of coordinated expression of these genes has not been carried out. A sequence analysis of the 5’-flanking regions of the PDEα and PDEβ revealed two common potential regulatory elements: a Ret1-like site, the binding site for the retina-specific Ret1 transcription factor (8), and a GC box, the binding site for the SP family of transcription factors. Work coming out from our laboratory has implicated Sp4 in the transcriptional regulation of PDEβ (9, 10), but the effect of this factor on PDEα has not yet been established.

This study was initiated with the intention of: 1) determining the PDEα and PDEβ mRNA steady-state levels as well as transcriptase; CRS, competitive reference standard; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; UTR, untranslated region; ROS, rod outer segments; IOD, integrated areas of the optical density peaks; NLS, nuclear localization sequence.

This paper is available on line at http://www.jbc.org
Expression of C-GMP-phosphodiesterase Subunits

RNA Secondary Structure Prediction and Analysis—5'-UTRs of PDEα and PDEβ mRNAs were analyzed for the presence of stem-loop structures using the RNA Secondary Structure Prediction Program of Genebee Services (www.genebee.msu.su/services/rna2_reduced.html) that determines the most stable structures and their free energy level. PSORT II (psort.nibb.ac.jp), SubLoc v1.0 (www.bioinfo.tsinghua.edu.cn/SubLoc), and the Predict NLS (ubic.bioc.umb.edu/predict-NLS) were used to determine subcellular localization of the PDEα and PDEβ subunits.

Preparation of Mouse Rod Outer Segments (ROS) and Immunoblot Analysis—An analysis of the stoichiometric amounts of PDEα and PDEβ subunits was performed on retinas from 10- and 30-day-old animals and on sealed ROS isolated from 48 adult (greater than 30 days of age) C57BL/6J/Rt mice. The ROS were centrifuged in a Beckman SW28.1 rotor to the method of Papermaster (14). Proteins (50 μg of total protein/lane from retinal homogenates and 8 μg of total protein/lane from ROS) were separated by Tris-Tricine-buffered inverted gradient gel electrophoresis (13) and transferred to nitrocellulose membrane (15). Membranes were blocked in either 3% bovine serum albumin, 0.1% Tween 20 in Tris-buffered saline (500 mM NaCl, 20 mM Tris, pH 7.6) or 0.3% Tween 20 in Tris-buffered saline for colloidal gold staining. Immunoblots were incubated with a polyclonal peptide antiserum (αP1) previously characterized (7), and the signal was amplified with a goat anti-rabbit biotinylated secondary antibody and AvidinD/biotin-coupled horseradish peroxidase (Vector Laboratories, Burlingame, CA). Blots were visualized with the ECL kit (Amersham Biosciences) on Hyperfilm-MP pre-flashed according to the Sensizite™ protocol (Amersham Biosciences) to increase the range of film exposure linearity. Films were developed and scanned densitometrically on a Pharmacia/LKB XL densitometer (Amersham Biosciences).

Labeling and Immunoprecipitation—Mice were sacrificed at 10 postnatal days (according to the guidelines of the UCLA Committee on Animal Research and the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research) and enucleated, and the eyes were hemisected. The resulting eyecups were incubated in HEPES-buffered Medium 199 with Hanks' salts without dl-methionine (fivitrogen) for 30 min at 37 °C in a 5% CO2 incubator to methionine-deplete the tissue. The eyecups were then incubated with L-[35S]methionine (1000 Ci/mmol and 100 μCi/ml media, Amersham Biosciences) for 3 h and subsequently washed and incubated in fresh Medium 199 supplemented with cold l-methionine at 500 μCi final concentration. At the appropriate times, eyecups were removed and the retinas were dissected and immediately frozen at −80 °C on dry ice to prevent further incorporation of label and protein degradation. Retinas were homogenized with hypotonic buffer, and aliquots were removed to determine trihaloracetic acid-precipitable counts and protein concentration by the method of Peterson (16) using bovine serum albumin as standard.

Immunoprecipitation was carried out as described previously (17) using a polyclonal rod cGMP-PDE-specific antiserum, MOE (18). Proteins were separated by a Tris-Tricine-buffered inverted gradient sodium dodecyl sulfate-polyacrylamide gel as described previously (13) for 22 h at 35 mA constant current. Proteins were then blotted to nitrocellulose membrane, stained with Aurodye total protein stain (Bio-Rad), and exposed to autoradiography on pre-flashed Hyperfilm-MP.

RESULTS

Quantification of PDEα and PDEβ mRNAs by Northern Blot Hybridization—This method for absolute quantification of specific mRNAs is based on the generation of a standard curve using known amounts of an external standard (synthetic sense strand RNA with identical sequence to the target mRNA) and interpolation of the concentration of the target mRNA from the curve. The external standards for PDEα and PDEβ mRNA quantification were synthesized in vitro from constructs containing full-length PDEα and PDEβ cDNAs. mRNA samples isolated from 10- and 30-day-old mouse retinas were hybridized with the random-labeled PDEα and PDEβ cDNA fragments. Two transcripts with molecular masses of 3.0 and 2.8 kb corresponding to the PDEα and PDEβ mRNAs, respectively, were hybridized. Using the external standard curve, we determined that in the 30-day-old mouse retina the level of PDEα transcript was 1.5 ± 0.05 ng and that of PDEβ transcript was 0.34 ± 0.02 ng/1 μg of retinal mRNA (Fig. 1). Based on the molecular masses of PDEα and PDEβ transcripts (1.03 × 106

Experimental Procedures

RNA Isolation and in Vitro Transcription—Total RNA was extracted from 10- and 30-day-old normal mouse retinas using RNazol B (Tel-Test, Friendswood, TX). mRNA was isolated using the mRNA purification kit (Amersham Biosciences). Concentrations of the RNA samples were determined by measuring their optical density.

For in vitro transcription, the cDNAs were subcloned into the pGEMZ vector (Promega, Madison, WI) between BamHI and SalI. In vitro transcription was performed using 1 μg of linearized DNA and the T7 mMessage mMachine kit (Ambion, Austin, TX) according to the manufacturer’s protocol. The length and the concentration of the synthesized transcripts were determined by running an aliquot on a denaturing agarose gel as recommended by Ambion. Quantification of the synthesized transcripts was performed using the two-dimensional spot densitometry program of the Alpha Imager 2000 (Alpha Innotech, San Leandro, CA).

RT-PCR Analysis—First strand cDNA was synthesized using murine leukemia virus reverse transcriptase (PerkinElmer Life Sciences), 0.75 mM of antisense primer, 1.5 mM dNTPs, 10 units of RNasin (Promega), 5 mM MgCl2, 10 mM Tris-HCl, pH 8.3, and 50 mM KCl. The reaction mixture was incubated at 42 °C for 15 min. PCR was performed with AmpliTaq DNA polymerase (PerkinElmer Life Sciences), 0.75 mM antisense and sense primers, 2 mM MgCl2, 10 mM Tris-HCl, pH 8.3, and 50 mM KCl. The PCR profile and temperature were as follows: denaturation at 94 °C for 2 min, annealing at 57 °C for 1 min 20 s, and extension at 72 °C for 1 min 30 s with a final elongation step at 72 °C for 5 min.

Quantitative Northern Blot and Quantitative RT-PCR—mRNAs (2 μg) were separated by electrophoresis in a 1.2% agarose gel containing 2.2% formaldehyde, transferred to Hybond-N+ (Amersham Biosciences), cross-linked, and hybridized with random-labeled PDEα and PDEβ cDNA fragments. Autoradiographs were analyzed using the two-dimensional spot densitometry program of the Alpha Imager 2000 to determine the intensity of the hybridization signals.

Quantification of the PDEβ transcripts differing by the length of their 5'-UTRs was based on the PCR method described by Riedy et al. (11). A competitive reference standard RNA (RNA-CRS) was obtained as follows: cDNA fragments with a 75-nucleotide deletion were synthesized by PCR using primers MB7 (5'-CGCGGATCCTGAGGACATCCTGTACC-3') and MB45 (5'-TGCGGTGGAGAGAAGATCGGATCG-3') and MB25 for both the long and short transcripts to obtain data for mRNA quantification curves. Conditions for RT-PCR were the same as described above. Aliquots from each reaction were electrophoresed in a 2% agarose gel and stained with 0.5 mg/ml ethidium bromide, and the bands were quantified using the GeneRuler system of the Alpha Imager 2000. Since the RNA-CRS cDNA is smaller in size than the wild type cDNA, the decrease in ethidium bromide incorporation was taken into account by using a correction equation (12).

In Vitro Translation—Equal amounts of in vitro synthesized RNAs were used for all of the in vitro translation studies. In vitro translation reactions were performed in the presence of [35S]methionine with the retic lysate IVT kit (Ambion) according to the manufacturer’s protocol. Translation products were separated by SDS-polyacrylamide gel electrophoresis. The cDNAs were cotransfected using the Tris-Tricine buffer system as previously described (13). The gels were then fixed, stained with Coomassie Blue G-250, dried, and exposed to x-ray film. Proteins were quantified by densitometry of the autoradiograph or direct scintillation counting of the excised bands.

Protein levels in the mouse retina at different stages of photoreceptor development; 2) examining the turnover of translated PDEα and PDEβ by pulse-chase labeling and immunoprecipitation to determine whether the final stoichiometric amounts of the catalytic subunits are the result of differences in either efficiency of translation or post-translational protein stability; and 3) determining the PDEα and PDEβ mRNA translational efficiency and the role of their 5'-UTRs, coding regions, and 3'-UTRs in protein synthesis.
Expression of cGMP-phosphodiesterase Subunits

Fig. 1. Quantitative Northern blot analysis of mouse rod PDEα (a) and PDEβ (b) mRNAs. Randomly labeled PDEα (1–300 nucleotides) and PDEβ (1–298 nucleotides) cDNA fragments were used as probes. Lanes 1 and 2 contain 2 μg of retinal mRNA isolated from 10- and 30-day-old mouse retinas, respectively. Lanes 3–6 contain serial dilutions of the in vitro synthesized PDEα and PDEβ RNAs. Lane 3, 2 ng; lane 4, 4 ng; lane 5, 8 ng; and lane 6, 16 ng.

and 0.87 × 10⁶, respectively) we estimated that 1 μg of retinal mRNA contains −1.5 × 10⁸ (±0.2 × 10⁸) and 7.5 × 10⁶ (±0.5 × 10⁶) PDEα and PDEβ transcripts, respectively. Similar quantification carried out with mRNA samples of 10-day-old mouse retinas. The levels reported are the average of those determined in three different experiments.

Quantitative RT-PCR Analysis of mRNAs—It had been reported previously (19) that mouse PDEβ has two different transcription start sites represented in the retina by two transcripts, one with a 59-nucleotide 5'-UTR and the other with a 34-nucleotide 5'-UTR. Because the 5'-UTR is a major element implicated in mRNA translation, the two PDEβ transcripts could have different translation initiation strengths. Therefore, any changes in the molar ratio of these transcripts during the development of photoreceptor cells could be responsible for modulation of PDEβ protein concentration.

Quantitative RT-PCR was used to determine the level of the PDEβ transcript with long 5'-UTR and the total concentration of PDEβ mRNA (the sum of the long and short transcripts) in the 10- and 30-day-old mouse retinas. This technique involves the use of an RNA-CRS that is identical in sequence to the wild type mRNA with the exception of an internal nucleotide deletion. Primers MB4 and MB25 were used to amplify the long transcript, and primers MB5 and MB25 were used to amplify at the same time both the long and the short transcripts. PCR products were separated by gel electrophoresis, stained with ethidium bromide, and analyzed densitometrically (Fig. 2, Ia, Ib, Ia, and Iib). The concentration of the RNA-CRS (x axis) was plotted versus the ratio of the RNA-CRS to the wild type mRNA band densities (Fig. 2, Ic and Iic, y axis). The concentration of PDEβ mRNA corresponds to the point on the x axis where the ratio RNA-CRS:PDEβ mRNA is 1. The results of these experiments indicate that both long and short PDEβ transcripts are represented at equimolar concentrations in the photoreceptor cells of the 10- and 30-day-old animals.

Western Blot Analysis—Given the 5-fold difference in transcript abundance observed by quantitative Northern blots, we had to establish the protein subunit stoichiometry in the mouse retina. We accomplished this by utilizing αT1, an antibody that recognizes the same epitope on both PDEα and PDEβ. The relative amounts of PDEα and PDEβ were obtained by determining the corresponding integrated areas of the optical density peaks (IOD) and then the ratio of PDEα IOD to PDEβ IOD (Fig. 3). Ratios of ∼1:1 were obtained for all three of the samples analyzed, confirming equimolar expression of PDEα and PDEβ subunits in mouse rod photoreceptors, similar to what is seen for the bovine enzyme.

Biosynthesis and Turnover—Using 1,4-[35S]methionine, we performed pulse-chase labeling of newly synthesized PDE subunits followed by immunoprecipitation with a specific polyclonal rod cGMP-PDE antiserum to confirm that the differences observed between protein and corresponding mRNA levels were the result of differential protein translation and/or turnover. The autoradiograph of the gel containing the immuno-precipitated proteins (Fig. 4) was scanned densitometrically, and the IODs were obtained. The rate of 1,4-[35S]methionine incorporation was higher for PDEα than PDEβ as indicated by values greater than 1.0 throughout the course of the experiment for the PDEα/PDEβ-calculated IOD ratios. However, the range of observed IOD ratios from 1.20 to 1.41 suggests that the turnover rate for PDEβ is not significantly greater than for PDEα, particularly when the difference in the molar methionine content of PDEα and PDEβ, 34 residues and 29 residues, respectively, is taken into account.

Translational Efficiency of the Full-length PDEα and PDEβ mRNAs—To determine the translational efficiency of the PDEα and PDEβ mRNAs, three constructs were prepared by subcloning the PDEα and PDEβ (B5s and B5l) cDNAs into the pGEM3Z vector (Fig. 5, Ia). The PDEβ subunit is represented by two constructs since human photoreceptor cells express two PDEβ transcripts that differ in the length of their 5'-UTRs. Capped mRNAs were in vitro transcribed from the A, B5s, and B5l templates, transcripts were quantified by both spectrophotometry and spot densitometry, and equal amounts of each mRNA were used for in vitro translation in the presence of 1,4-[35S]methionine. As a first step in the analysis of the translational efficiency of PDEα and PDEβ, we determined the approximate linear range of protein synthesis and the relative stability of the corresponding mRNAs in the reticulocyte lysate. In pilot studies, the in vitro translation reactions with A, B5s, and B5l mRNAs were incubated for 20, 40, or 60 min. The RNA from each reaction was then extracted and analyzed by slot-blot hybridization. No noticeable mRNA degradation was observed during incubation for up to 60 min. Synthesized proteins were separated by PAGE, and their amount was determined by measuring 1,4-[35S]methionine incorporation. The results obtained showed that at 60 min but not after 20 or 40 min of incubation, protein synthesis reached saturation. Based on these observations, all of the reactions for quantitative analysis were performed for 30 min.

mRNA synthesized from constructs A, B5s, and B5l yielded products with the expected molecular masses of 88 and 84 kDa, corresponding to PDEα and PDEβ, respectively, but exhibited differential translational efficiency (Fig. 5, Ib). The synthesized PDEα was ∼4.5-fold more abundant than PDEβ synthesized from the short transcript (B5s) and 5.0-fold more abundant than PDEβ produced from the long transcript (B5l) (Fig. 5, Ib and Ic). A low level of protein synthesis from B5l was anticipated since this mRNA contains in the 5'-UTR an upstream AUG known to have a negative effect on translation. Thus, constructs A, B5s, and B5l enabled us to determine the differences in PDEα and PDEβ protein synthesis efficiency resulting from the contribution of the full-length mRNAs. The input of the coding regions and 5'- and 3'-UTRs of each of these mRNAs in the differential translation of PDEα and PDEβ was assessed in the experiments described below.

The Role of the 5'-UTR in PDEα and PDEβ mRNA Translation—The 5'-UTR is known to be an essential factor in determining the rate of translation initiation (20, 21) and consequently the overall protein synthesis efficiency. We
hypothesized that if the 5'UTRs of PDEα or PDEβ were solely responsible for the observed differences in efficiency of translation, the exchange of these regions between the subunits would lead to opposite results to those obtained with the original A, B5s, and B5l constructs. That is, the exchange of 5'UTRs would produce approximately 5 times more PDEα than PDEβ. However, the chimeric constructs shown in Fig. 5, IIa, gave results different from what was expected. The amount of protein synthesized using construct A5B (containing PDEα 5'-UTR, PDEβ-coding region, and 3'-UTR) was ~5.5-fold lower than that produced by the native PDEα transcript (construct A) and slightly less than that produced by B5s or B5l (Fig. 5, IIb and Ic). In contrast, the placement of the PDEβ short 5'-UTR in front of the PDEα-coding region and 3'-UTR (construct B5sA) resulted in greater protein synthesis than that produced by the original A (~2-fold), B5s (~8-fold), or B5l (~10-fold) constructs. Similarly, the placement of the...
PDEβ long 5'-UTR in front of the PDEα-coding region and 3'-UTR (construct B5lA) also increased protein synthesis above the levels produced by the native mRNAs, although this construct was ~1.8-fold less efficient than B5sA with the PDEβ short 5'-UTR. This reduction in efficiency of translation of construct B5lA when compared with B5sA is possibly due to the presence of the AUG upstream of the translation initiation site. These experiments clearly demonstrated the negative impact of the PDEα 5'-UTR on the rate of protein synthesis.

The Effect of a Non-consensus Translation Initiation Sequence and of the Upstream AUG in the 5'-UTR on PDEβ mRNA Translation—It has been shown that both the absence of a "strong" translation initiation sequence (22–24) and the presence of an upstream AUG (25–27) can result in lower protein synthesis efficiency. In addition to the upstream AUG in its long 5'-UTR, the PDEβ mRNA translation initiation sequence is not in absolute agreement with the Kozak consensus sequence (there is an A instead of G at the +4 position). To find out whether these sequences have a negative effect on PDEβ translation, three new constructs were created by site-directed mutagenesis (Fig. 5, IIIa). Two of the constructs, B5sM and B5lM, were obtained by replacing the A at the +4 position of PDEβ mRNA with G, creating in this way the consensus translation initiation sequence. As expected, the amount of protein synthesized from the mutated short transcript was ~2.6-fold higher than that obtained from B5s mRNA (Fig. 5, IIIb and IIIc and Ib and Ic). Thus, the absence of a Kozak sequence reduces translational efficiency of PDEβ. However, the A to G mutation at the +4 position in the transcript with the long 5'-UTR did not affect its translational rate. This may again be attributed to the presence of the upstream AUG in the long 5'-UTR. To test this possibility, we generated the third construct, B5lM2, by mutating the upstream AUG of the long PDEβ transcript to AAG. Indeed, protein synthesis from this construct was 2.2 times more abundant than that from the wild type B5l transcript (Fig. 5, Ib and Ic and IIIb and IIIc).

The Role of the 3'-UTRs in PDEα and PDEβ Translation Efficiency—Along with the 5'-UTR, the 3'-UTR is emerging as another important mRNA sequence controlling its translation (28–30). To determine the contribution of the PDEα and PDEβ mRNA 3'-UTR on protein synthesis, three chimeras were constructed by exchanging the 3'-UTRs of constructs A and B5s or B5l (Fig. 5, IVa). AB3 was translated only 1.3 times more efficiently than construct A. In contrast, the placement of PDEα 3'-UTR in the B5sA3 construct increased protein production by close to 3-fold (Fig. 5, IVb and IVc). However, protein synthesis by B5lA3 construct was similar to that produced by B5l, again suggesting a repressing effect of the upstream AUG in the long PDEβ 5'-UTR. Swapping 3'-UTRs between PDE subunits did not reverse their translational efficiency, i.e., protein synthesis from PDEα mRNA was greater than that from PDEβ.
Expression of cGMP-phosphodiesterase Subunits

A comparison of the levels of protein synthesized from all of the constructs described in this study demonstrates that those with the PDEα-coding region, regardless of which flanking 5′- or 3′-UTR they may contain, produce more protein than any construct with the PDEβ-coding region.

DISCUSSION

As a first step toward understanding the mechanisms of PDEα and PDEβ gene expression, we determined the steady-state levels of PDEα and PDEβ mRNAs and examined the biosynthesis/turover of the translated PDEα and PDEβ in the developing and fully differentiated mouse retina. Our results indicate that the mRNA for PDEβ at both 10- and 30-post-natal days is ~5-fold more abundant than the mRNA for PDEα. Despite the fact that the PDEα:PDEβ mRNA ratio we observed by quantitative Northern analysis is larger than that observed by Phelan and Bok (31) in 10-day-old mouse retinas using quantitative RT-PCR (this variability could be due to the different techniques used to determine the mRNA levels), both studies as well as results from Y79 cells (32) indicate a higher number of transcripts for PDEβ than for PDEα. In contrast, the examination of protein levels showed equimolar expression and similar turnover rates of PDEα and PDEβ subunits. This discordance between PDEα and PDEβ steady-state mRNA and protein levels suggested that photoreceptor cells must have a mechanism for post-transcriptional regulation of PDE expression.

In this study, we were able to conclusively demonstrate that PDEα mRNA is translated ~5 times more efficiently than its PDEβ counterpart. Thus, the low level of PDEα mRNA found in retina could be counterbalanced by its efficient translation if PDEα and PDEβ mRNAs are translated in photoreceptor cells with a similar efficiency to that we observed in vitro. Our data also point to possible regulation of PDE expression in photoreceptor cells by the feedback mechanism, i.e. if one of the subunits is being translated in excess it could by itself or through interaction with other factors stimulate or inhibit transcription of the less or more abundant subunit mRNA, respectively. This regulatory mechanism would require translocation of transcription factors from the cytoplasm to the nucleus. Alternatively, signals could be delivered from the cytosol to the nucleus by protein kinases that would activate transcription factors by phosphorylation. It has been shown that proteins with a molecular size of ~50 kDa can freely enter the nucleus but that larger proteins must have a nuclear localization sequence (NLS) that is recognized by the members of the importin/karyopherin superfamily. The complex between importin/karyopherin and the NLS-containing protein can then be actively transported through the nuclear pore complex (33, 34). To check the possibility of PDE subunits (PDEα, 88 kDa and PDEβ, 84 kDa) directly regulating their own transcription, we analyzed these proteins using Predict NLS: Prediction and Analysis of NLSs, PSORT II: Prediction of Protein Sorting Signals and Localization Sites in Amino Acid Sequences, and SubLoc (Version 1.0): Subcellular Localization Prediction of Eukaryotic Proteins. This analysis predicted no nuclear localization of PDEα and PDEβ and revealed no potential NLS in their sequences, suggesting that the proposed feedback mechanism would require the involvement of other factors regulating their transcription.

After determining the translational efficiency of PDEα and PDEβ mRNAs, we evaluated the roles of their 5′- and 3′-UTRs known to be involved in controlling protein synthesis as well as that of their coding sequences on this process. Since neither of the 5′-UTRs of PDEα and PDEβ mRNAs contains stable secondary structures that can reduce the rate of protein synthesis, we first hypothesized that the presence of an upstream AUG and the absence of a strong initiation sequence in the 5′-UTR of PDEβ mRNA could account for its lower protein synthesis efficiency. Indeed, when the upstream AUG was mutated and the consensus translation initiation sequence in the PDEβ mRNA was restored, the protein production was increased. However, using several chimeric constructs, we demonstrated that in fact the 5′-UTR of PDEα leads to lower protein synthesis than the 5′-UTR of PDEβ mRNA. Therefore, the differential translation of the PDE subunits cannot be solely explained based upon the primary or secondary structures of their 5′-UTRs.

The results of our studies undoubtedly implicate the involvement of the coding regions in the differential translation of PDEα and PDEβ mRNAs. All of the eight constructs containing the PDEβ-coding region resulted in lower protein expression than the four constructs containing the PDEα-coding region, regardless of the flanking 5′- or 3′-UTR they had. Regulation of protein synthesis by the mRNA-coding region is not a widespread phenomenon. One example of this type of regulation is provided by the response of HSP70 to a heat shock, which involves an increase of the elongation rate (35). It has also been shown that some mRNAs contain in the coding region secondary structures that lead to “ribosomal pause” (36). Liebhaber et al. (37) have shown that extensive secondary structures in the coding region suppresses translation to a minimal or to a substantial degree depending on the distance of these structures from the initiation codon. Coding region duplexes in close proximity to the AUG dramatically suppress translation, whereas duplexes further downstream in the coding region have a milder effect on protein synthesis. We have analyzed PDEα and PDEβ mRNAs for the presence of stable secondary structures. The overall ΔG of PDEα mRNA is ~680.7 kcal/mol, and the most stable structure (ΔG = −17.8 kcal/mol) proximal to the initiation codon is created by the coding region sequences 5′-GAGGAGGT-3′ (position +15 to +22) and 5′-CTCCTCCA-3′ (position +107 to +110). Structures with lower ΔG (~20.4 and −20.3 kcal/mol) are localized ~900 and 1800 nucleotides from the AUG start codon, respectively. The total free energy of the PDEβ mRNA is ~593.4 kcal/mol. It has six structures with ΔGs ranging from ~19.0 to ~23.5 kcal/mol. One of these structures (ΔG = −19.0 kcal/mol) is in very close proximity to the AUG start codon and involves sequences 5′-GGACACGC-3′ (position −20 to −13 in the 5′-UTR) and 5′-CCGTGTCG-3′ (+164 to +147 in the coding region). It will be interesting to determine the involvement of these predicted structures in the regulation of PDE translation.

In summary, our results indicate that the low level of PDEα mRNA found in retina can be compensated by its more efficient translation to achieve equimolar expression with PDEβ. Moreover, our results indicate that the PDEα- and PDEβ-coding regions are responsible for the differential expression of these subunits.

Acknowledgments—We thank Dr. Bernard Fung for the gift of the polyclonal antiserum MOE against PDE.

REFERENCES
1. Beavo, J. A. (1995) Physiol. Rev. 75, 725–748
2. Baehr, W., Devlin, M. J., and Applebury, M. L. (1979) J. Biol. Chem. 254, 11669–11677
3. Deterre, B., Bigay, J., Forquet, F., Robert, M., and Chabre, M. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 2424–2428
4. Stroop, S., and Beavo, J. A. (1992) in Advances in Second Messenger and Phosphoprotein Research (Strada, S. J., and Hidaka, H., eds) Vol. 25, pp. 55–71, Raven Press, Ltd., New York
5. Fang, B. K., Young, J. H., Yamane, H. K., and Griswold-Prenner, I. (1990) Biochemistry 29, 2637–2644
6. Artemyev, N. O., Surendran, R., Lee, J. C., and Hamm, H. E. (1996) J. Biol. Chem. 271, 25382–25388
7. Piriev, N. I., Yamashita, C., Samuel, G., and Farber, D. B. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 9340–9344
8. Morimoto, M. A., Yu, X., and Barnstable C. J. (1991) J. Biol. Chem. 266,
Expression of cGMP-phosphodiesterase Subunits

9667–9672
9. Lerner, L. E., Grihantova, Y. E., Ji, M., Knox, B. E., and Farber, D. B. (2001) J. Biol. Chem. 276, 34999–35007
10. Lerner, L. E., Grihantova, Y. E., Whitaker, L., Knox, B. E., and Farber, D. B. (2002) J. Biol. Chem. 277, 25877–25883
11. Riedy, M. C., Timm, E. A., Jr., and Stewart, C. C. (1995) J. Clin. Microbiol. 30, 70–76
12. Menzo, S., Bannarelli, P., Giacca, M., Manzin, A., Varaldo, P. E., and Clementi, M. (1992) BioTechniques 18, 70–76
13. Tsang, S. H., Gouras, P., Yamashita, C. K., Kjeldbye, H., Fisher, J., Farber, D. B., and Goff, S. P. (1996) Science 272, 1026–1029
14. Papermaster, D. S. (1982) Methods Enzymol. 81, 48–52
15. Towbin, H., Staehlin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
16. Peterson, G. L. (1977) Anal. Biochem. 83, 346–356
17. Farber, D. B., Park, S., and Yamashita, C. (1988) Exp. Eye Res. 4, 363–374
18. Fung, B. K. K., and Grewel-Frenner, I. (1989) Biochemistry 28, 3133–3137
19. Di Polo, A., Rickman, C. B., and Farber, D. B. (1996) Invest. Ophthalmol. Visual Sci. 37, 551–560
20. Day, D. A., and Tuite, M. F. (1998) J. Endocrinol. 157, 361–371
21. van der Velden, A. W., and Thomas, A. A. (1999) Int. J. Biochem. Cell Biol. 31, 87–106
22. Kozak, M. (1987) J. Mol. Biol. 196, 947–950
23. Kozak, M. (1986) Cell 44, 283–292
24. Kozak, M. (1997) EMBO J. 16, 2482–2492
25. Kozak, M. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7134
26. Gehalle, A. P., and Morris, D. R. (1994) Trends Biochem. Sci. 19, 159–164
27. Morris, D. R., and Gehalle, A. P. (2000) Mol. Cell Biol. 20, 8635–8642
28. Jackson, R. J., and Standart, N. (1990) Cell 62, 15–24
29. Conze, B., Stutz, A., and Vassalli, J. D. (2000) Nat. Med. 6, 637–641
30. Stuart, J. J., Egy, L. A., Wong, G. H., and Kaspar, B. L. (2000) Biochem. Biophys. Res. Commun. 274, 641–648
31. Phelan, J. K., and Bok, B. (2000) Exp. Eye Res. 71, 119–128
32. Di Polo, A., and Farber, D. B. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 4016–4020
33. Cyert, M. S. (2001) J. Biol. Chem. 276, 20805–20808
34. Jans, D. A., Xiao, C.-Y., and Lam, M. H. C. (2000) BioEssays 22, 532–544
35. Theodorakis, N. G., Banerji, S. S., and Morimoto, R. I. (1988) J. Biol. Chem. 263, 14579–14585
36. Jansen, M., de Moor, C. H., Sussenbach, J. S., and van den Brande, J. L (1995) Pediatr. Res. 37, 681–686
37. Liebhaber, S. A., Cash, F., and Eshleman, S. S. (1992) J. Mol. Biol. 226, 609–621