THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 19, Issue of May 7, pp. 19800–19807, 2004
Printed in U.S.A.

Functional Analysis of the Rod Photoreceptor cGMP Phosphodiesterase α-Subunit Gene Promoter

Nrl AND Crx ARE REQUIRED FOR FULL TRANSCRIPTIONAL ACTIVITY*

Received for publication, February 20, 2004
Published, JBC Papers in Press, March 4, 2004, DOI 10.1074/jbc.M401864200

Steven J. Pittler,[a,b] Youwen Zhang,[a] Shiming Chen,[c,d] Alan J. Mears,[e,f] Donald J. Zack,[g,h] Zhiyong Ren,[a] Prabodh K. Swain,[e,i] Suxia Yao,[a] Anand Swaroop,[e,j] and J. Brandon White[a,k]

From the [a]Department of Physiological Optics, Vision Science Research Center, School of Optometry, University of Alabama at Birmingham, Birmingham, Alabama 35294, [c]Department of Ophthalmology and Visual Sciences, Washington University School of Medicine, St. Louis, Missouri 63110, [e]Departments of Ophthalmology and Human Genetics, Kellogg Eye Center, University of Michigan, Ann Arbor, Michigan 48105, and [g]Departments of Ophthalmology, Molecular Biology and Genetics, and Neuroscience, The Johns Hopkins Medical School, Baltimore, Maryland 21287

To understand the factors controlling expression of the cGMP phosphodiesterase type 6 (PDE6) genes, we have characterized the promoter of the human PDE6A gene that encodes the catalytic α-subunit. *In vivo* DNase I hypersensitivity assays revealed two sites immediately upstream of the PDE6A core promoter region. Transient transfection assay in Y79 cells of constructs containing varying lengths of the promoter region showed a decrease in promoter activity with increasing length. The most active segment contained a 177-bp upstream sequence including apparent Crx and Nrl transcription factor binding sites. Both Crx and Nrl transactivated the PDE6A promoter in HEK293 cells and showed a >100-fold increase when coexpressed. Coexpression of a dominant negative inhibitor of Nrl abolished Nrl transactivation but had no effect on Crx. DNase I footprinting assays identified three potential Crx binding sites within a 55-bp segment beginning 29 bp upstream of the transcription start point. Mutation of two of these sites reduced reporter gene activity by as much as 69%. Gel shifts showed that all three Crx sites required a TAAT sequence for efficient binding. Consistent with a requirement for Crx and Nrl in *Pde6a* promoter activity, *Pde6a* mRNA is reduced by 87% in the retina of $Crx^{-/-}$ mice and is undetectable in $Nrl^{-/-}$ mice at postnatal day 10. These results establish that both Nrl and Crx are required for full transcriptional activity of the PDE6A gene.

The type 6 cGMP phosphodiesterase (PDE6)[1] in rod photoreceptors is a heterotrimeric enzyme that is essential for phototransduction, functioning to lower cytoplasmic cGMP levels in response to light activation of the receptor rhodopsin (1–3). The α- and β-subunits comprise the catalytic core of the enzyme, and the γ-subunit partially inhibits activity to maintain basal levels in the dark. We have focused our studies on the PDE6A gene, which encodes the α-subunit of PDE6. Defects in the coding region of this gene account for a subset of autosomal recessive retinitis pigmentosa (4). Because all three genes that encode PDE6 subunits are required for subunit assembly (4–6), the regulation of gene expression for each subunit could be essential to maximize enzyme production. Dissection of promoter architecture may also be important to develop safe gene therapy vectors with controlled targeted expression in specific cell types. Our initial characterization of the PDE6 α-subunit gene promoter indicated that it shows very weak activity compared with other photoreceptor gene promoters including the PDE6B gene promoter (7). We suggest that the relatively weak expression of this promoter could be useful where overexpression with the well-characterized, most commonly used rhodopsin promoter could induce, rather than reverse, retinal degeneration (8, 9).

We reported previously that the PDE6A gene promoter is contained within an ~4-kb region upstream of the transcription start point, contains several potential cis-elements, and can bind tissue-specific factors in a retinal nuclear extract (10). A DNase I footprinting region was identified within the first 100 upstream nucleotides that was confirmed by the demonstration of a retina-specific mobility shift. The promoter region was delimited to within a 177-bp upstream region that is active both *in vitro* (11) and *in vivo* in transgenic mice (12). Contained within this region are several potential cis-elements that may bind retina-specific transcription factors including CRX sites (Crx binding element (CBE)) and a non-consensus NRL-like site (Nrl response element (NRE)). CRX binds to the consensus sequence (C or T)TAATC(C or A) (13, 14) and is critical for photoreceptor maturation (15, 16) and expression of many photoreceptor genes in adult retina (14–18). NRL is required for normal maturation of rod photoreceptors, as indicated by an Nrl knockout mouse that is devoid of functional rods (19) and patients with cone-rod dystrophy (20). Nrl binds to the consensus sequence GATGCNTCAGCC and can transactivate rhodop-

* This work was supported in part by National Institutes of Health Grants EY09924 (to S. J. P.), EY11115 (to A. S.), EY12543 (to S. C.), and EY09769 (to D. J. Z.); The Foundation Fighting Blindness; Research to Prevent Blindness; and the University of Alabama Health Services Foundation (to S. J. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[b] To whom correspondence should be addressed: University of Alabama at Birmingham, 924 18th St. S., Birmingham, AL 35294-4390. Tel.: 205-934-6744; E-mail: pittler@uab.edu.

[d] Recipient of a Research to Prevent Blindness career development award.

[f] Present address: University of Ottawa Eye Institute, Ottawa Health Research Institute, Ottawa, Canada.

[h] The Guerrieri Professor of Genetic Engineering and Molecular Ophthalmology and a Research to Prevent Blindness Senior Scientific Investigator.

[i] Present address: National Brain Research Center, Gurgaon, India.

[j] The Harold F. Falls Collegiate Professor.

[k] Present address: The Salk Institute, Regulatory Biology, La Jolla, CA 92037.

[1] The abbreviations used are: PDE6, cGMP phosphodiesterase type 6; CBE, Crx binding element; NRE, Nrl response element; PN, postnatal day; GST, glutathione *S*-transferase; pAT, poly[d(A-T)].

sin promoter activity synergistically with Crx (14) by physical interaction with Crx (21). In this paper, we show that the PDE6A promoter is contained within a 100-bp segment of the upstream region and requires Nrl and Crx for full transcriptional activity. Our data and that from studies of the PDE6B gene promoter (7), which shows greater activity but contains essentially the same binding sites, indicate that promoter strength is at least in part due to the specific sequence and the combinatorial array of cis-elements within a given promoter.

EXPERIMENTAL PROCEDURES

Cell Lines and Cell Culture—Media and antibiotics for tissue culture were purchased from Invitrogen. Fetal bovine serum was purchased from Hyclone. HEK293 and Y79 cells were purchased from American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum and 100 units/ml penicillin and streptomycin or in RPMI 1640 supplemented with 10% fetal bovine serum.

DNase I Hypersensitivity Assay—Y79 or HEK293 cell nuclei were isolated at 4 °C using a nuclei isolation kit (Sigma) following the manufacturers' protocol. The nuclei were pelleted at 2000 rpm in an IEC clinical centrifuge and resuspended in 1× phosphate-buffered saline. Approximately 5×10^6 nuclei were digested with DNase I at varying concentrations ranging from 0 to 100 μg/ml. The reaction was incubated for 10 min on ice, and reactions were quenched by the addition of EDTA to a final concentration of 25 mM. Genomic DNA was extracted using the DNeasy Tissue Kit (Qiagen). For Southern blot analysis, ~15 μg of DNA was co-digested with AflII and XhoI at 37 °C for 4 h, with one addition of each enzyme after 2 h, and the digestion products were electrophoresed in a 0.7% agarose gel. DNA was transferred to a nylon membrane and hybridized with an [α-^{32}P]dCTP-labeled 3′-end probe. The results were visualized by autoradiography after a 5-day exposure.

Generation of Promoter Constructs—The following reporter constructs were used: (*a*) pGL3-Control encoding the firefly luciferase gene under control of the SV40 strong promoter and SV40 enhancer; and (*b*) pGL3-Basic containing no promoter and no enhancer. A 4.1-kb XbaI/XhoI restriction fragment of the upstream region of the PDE6A gene cloned into pBS II KS (10) was amplified with primers U-Sal (5′-TTTGTCGACGCTCTGTGCAGACATCTCTTCT-3′) and U-Xba (5′-AAATCTAGAACCTCCTCTGCTGTCACCT-3′), generating a 322-bp segment upstream of the ATG initiation codon. This fragment was cloned into the pBS II KS SmaI site (pBS-322). Eight promoter constructs were made driving luciferase reporter gene expression in the pGL3-Basic plasmid vector. These constructs were called pGL3-260, pGL3-260r (reverse orientation of pGL3-260), pGL3-300, pGL3-592, pGL3-1060, pGL3-1542, pGL3-3800, and pGL3-PL (promoterless). These eight constructs are representative of different lengths of the upstream region of the PDE6A gene. The pGL3-260 and pGL3-260r constructs were prepared by amplification with Pfu polymerase (Stratagene) using U-Sal and U-Xba primers with the pBS-322 vector as template. The PCR product was then cut with StuI, and the 260-bp fragment obtained was cloned into the SmaI site of pGL3-Basic. Forward and reverse orientation was obtained and verified by PCR and restriction digests. The pGL3-300 construct was generated by restriction digest of the pBS-322 plasmid with SalI and NcoI and inserted into the XhoI/NcoI site of pGL3-Basic vector. The pGL3-1060 construct was generated by amplifying a ~1279-bp fragment from pBS-4.1 using primers designed to amplify that portion of the upstream region (HAU6-R, 5′-GTGCTGGGATTACAGGCGTGAG-3′; HARE-3, 5′-CTGTAAATTCTCCTGAAAGTCCCGCAGGAG-3′). The product was partially digested with NcoI to obtain a 1060-bp fragment, which was cloned into the SmaI and NcoI sites of pGL3-Basic. The pGL3-3800 was prepared by digesting pBS-4.1 with XbaI and NcoI and cloning the ~3.3-kb fragment into the NheI and NcoI sites of pGL3-Basic vector. A ~500-bp NcoI to NcoI fragment was then cloned into this intermediate construct to generate the pGL3-3800 construct. The pGL3-593 construct was made by digesting pGL3-3800 with SacI and ApaI and purifying the vector with the insert using a Qiagen gel extraction kit following the manufacturer's protocol. The ends of the vector were filled in using T4 DNA polymerase and blunt-end-ligated together. The pGL3-1542 plasmid was generated by digesting the pGL3-3800 construct with NheI and MluI, followed by purifying the vector with insert using a Qiagen gel extraction kit. The ends of the vector were filled in using T4 DNA polymerase and blunt-end-ligated together. Plasmid preparations were performed using Qiafilter Endofree Maxi or Mega kits (Qiagen) following the manufacturer's recommended protocol. All plasmids were sequenced to verify integrity.

Plasmids containing mutated potential Crx binding sites (TAA to CCG) were generated using mutagenic primers with mutA1-R primer (5′-AGAGGGAGCAAGCTTGGGAGA<u>CCG</u>AGTCATTAA-3′) or mutB-1-R primer (5′-AGAGGGAGCAAGCTTGGGAGATTAAGTCA<u>CCG</u>ATATTCTCA-3′) using pBS-322 as template and a reverse primer, GL2 (5′-ACCTTCTGCGGTTTTTGTATTTC-3′). *Underlined sequences* in the primers indicate mutated sites. Promoter constructs were created from the mutated PCR product from the pBS-322 vector by restriction digests with SalI and NcoI and inserted into the XhoI/NcoI site of pGL3-Basic vector. These promoter constructs are designated pGL3-M13 (m13) and pGL3-M16 (m16), respectively. The plasmids were sequenced to verify mutations. Crx, Nrl, and DD10 expression constructs have been described previously (14, 22).

Adenofection of Y79 Cells—Y79 cells were transfected using the adenofection method described previously for this cell line (11). Cells were harvested 48 h after infection and lysed in 100 μl of 1× reporter lysis buffer (Promega), and light emission was measured immediately in a Turner Designs 20/20 luminometer (Promega). Values were normalized to viral β-galactosidase expression. All experiments were performed in triplicate a minimum of three times ($n \geq 9$).

Transfection of HEK293 Cells with Calcium Phosphate—HEK293 cells were transfected with calcium phosphate using a standard protocol. Briefly, ~6×10^5 HEK293 cells were plated in a 60-mm Petri dish in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum and 1% penicillin/streptomycin and grown for 24–36 h at 37 °C to achieve 50–75% confluence. The medium was changed once, 3 h before transfection. Up to 16 μg of DNA in 300 μl of 250 μM $CaCl_2$ was combined dropwise with 300 μl of 2× Hank's balanced salt buffer and incubated for 30 min. The DNA consisted of 1 μg of β-galactosidase internal control, 4–5 μg of test plasmid, and 2–5 μg of Crx or Nrl expression construct. The DNA mixture was then added dropwise to the cells and incubated for 48 h, with one media change after 12 h of incubation.

Luciferase and β-Galactosidase Assays—Cells were harvested and lysed in 350 μl of Galacto-Star reporter lysis buffer (Tropix, Inc.), and cell debris was pelleted at 12,000 × *g* for 2 min at 4 °C. For luciferase assays, 20 μl of cytosolic extract was diluted to 300 μl, and light emission was measured immediately in the luminometer using a luciferase activity kit (Promega) following the suggested protocol. β-Galactosidase activity was assayed with the Galacto-Star activity kit (Applied Biosystems) using 20 μl of the same cytosolic extract diluted to 300 μl. The luminometer was set to 30% (for HEK293 cells) or 40% sensitivity (for Y79 cells), with a delay time of 2 s and an integration time of 10 s. All transfections were done in triplicate a minimum of three times.

DNase I Footprinting of Crx—The plasmid pGL3-300 was used as a template for PCR separately with either sense or antisense radiolabeled primers to generate DNase I footprinting probes. The reaction was performed in binding buffer containing 12.5 mM Hepes, pH 7.6, 100 mM KCl, 5 mM $ZnSO_4$, 0.5 mM dithiothreitol, 2% polyvinyl alcohol, and 10% glycerol. Binding reactions were performed on ice for 20 min in a total volume of 50 μl including labeled probe and 1× binding buffer with or without 100 ng of purified bovine CrxHD-GST fusion protein (14). For DNase I digestion, $MgCl_2$ and $CaCl_2$ were added to final concentrations of 5 and 2.5 mM, respectively, followed by 0.005, 0.01, or 0.02 unit of DNase I for 1 min at room temperature. The reactions were terminated with 90 μl of 1× stop buffer (20 mM EDTA, pH 8.0, 1% SDS, 0.2 M NaCl, and 250 μg/ml glycogen). Ten μl of 2.5 mg/ml proteinase K was added and incubated at room temperature for 5 min followed by phenol chloroform extraction, precipitation, and resuspension in 1× DNA sequencing gel loading buffer. DNA sequencing reactions were run alongside in a 6% denaturing acrylamide gel.

Electrophoretic Mobility Shift Assay—Gel shift assays were performed essentially as described previously (10), with minor modification. KCl optimum was determined to be 75 mM, and 5% glycerol was included in the reactions. Incubations with and without label were done for 15 min. The binding reaction was run on a nondenaturing 6% acrylamide gel in 0.5× TBE for 2 h at 380 V. CrxHD-GST protein was purified on a glutathione affinity column as previously described (14)

Real-time Quantitative PCR—$Crx^{-/-}$ mice were obtained from Dr. Connie Cepko (Department of Genetics, Harvard Medical School). The $Crx^{-/-}$ mouse was outcrossed to a congenic C57Bl/6 × SJL hybrid strain that is +/+ at the *rd1* locus (6). The mice were bred to homozygosity for the Crx knockout allele and maintained for >6 generations. Total RNA was isolated from the eyes of 10-day-old $Crx^{-/-}$ and $Nrl^{-/-}$ mice as well as background matched controls using RNAqueous RNA isolation kit (Ambion) or TRIzol (Invitrogen). Reverse transcription reactions (23)

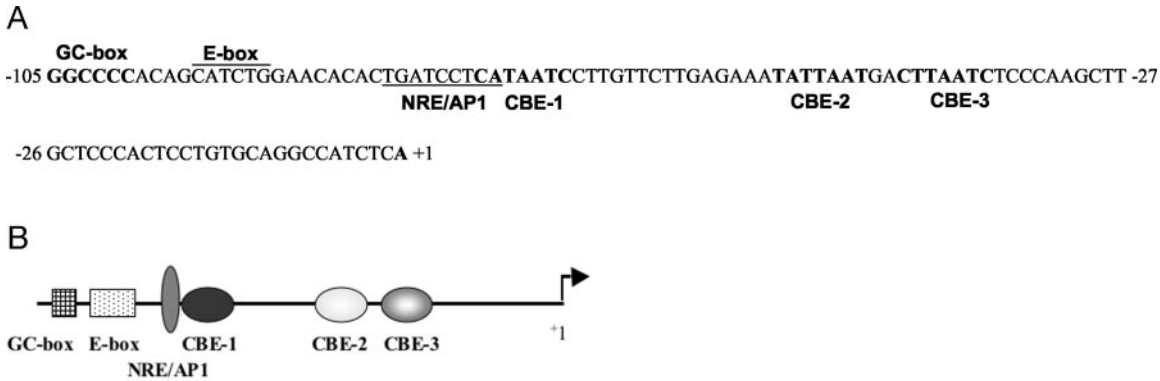

FIG. 1. **DNA sequence and schematic representation of the human PDE6A core promoter region.** *A*, PDE6A promoter region sequence. The nucleotide sequence of the upstream region from the transcription start point (+1) to −105 is shown with annotation of cis-elements that may be of functional significance. All of the putative cis-elements are highly conserved in mouse and rat. A primary focus of this study is on three potential Crx binding sites, CBE-1, CBE-2, and CBE-3. NRE/AP1 is a site similar to Nrl and AP1 binding sites that were shown to be functionally important in rhodopsin and PDE6B promoters (22, 28, 31). The E-box site was reported to be important for promoter activity in other photoreceptor genes (32). *B*, schematic diagram of PDE6A core promoter region. CBE sites are *shaded* to represent relative binding affinities for Crx determined by gel shift assays in this study.

were performed with 2 μg of total RNA using Moloney murine leukemia virus (Ambion) or Superscript II (Invitrogen) reverse transcriptase following the manufacturer's guidelines. The reaction was diluted to 100 μl with water. One μl of the diluted cDNA was used in each PCR reaction using either the Dynamo SYBR green qPCR kit (MJ Research) or Platinum Taq polymerase (Invitrogen) with SYBR green I (Molecular Beacons). Primer pairs were used that amplify portions of *Pde6a*-F (5′-CCATGCTGGATGGGATCACTAAC) and PDE6A-R (5′-CAGGGTGACCTCCTCCTTCCTG), *Pde6b*-F (5′-GTATACAAGGAATTTTCTCG) and PDE6B-R (5′-CAAATTGCTATAGGCAGAGTCCG), *rhodopsin*-F (*Rho*) (5′-ATCCCTGAGGGCATGCAATGTTCATG) and *Rho*-R (5′-CTGCTTGTTCAACATGATGTAGATGAC), and *Gapdh*-F (5′-TGATGACATCAAGAAGGTGGTGAAG) and *Gapdh*-R (5′-TCCTTGGAGGCCATGTAGGCCAT). The reactions were run in the real-time PCR Opticon II system (MJ Research) or iCycler (Bio-Rad) and analyzed using the comparative C_t method ($\Delta\Delta C_t$) with *Gapdh* as the normalizer (dorakmt.tripod.com/genetics/realtime.html).

RESULTS

The PDE6A Promoter Region Is in an Open Chromatin State in Y79 Cells—Promoter regions of genes that are transcriptionally active are often demarcated by a DNase I-accessible open chromatin region (24, 25). Shown in Fig. 1 is the portion of the PDE6A core promoter region analyzed in this study. This region contains three potential Crx binding elements (CBE1–3), one site resembling an Nrl/AP1 binding element (NRE/AP1), and one additional segment that is conserved in the upstream regions of both human and mouse (10). To assess the chromatin status of the region around the PDE6A promoter region *in vivo*, a DNase I hypersensitivity assay was performed with Y79 cells, which transcribe PDE6A mRNA (26). Treatment of intact Y79 nuclei with DNase I revealed two fragments in close proximity (Fig. 2*B*, *lanes 6* and *7*) within a 6.4-kb AflII/XhoI genomic fragment spanning the promoter region (Fig. 2*A*). No cleavage products were observed using HEK293 nuclei, which do not express PDE6A (data not shown). The estimated size of these fragments places the hypersensitive sites immediately upstream of the predicted core promoter region. This result is consistent with an active promoter within the region previously identified *in vitro* (10).

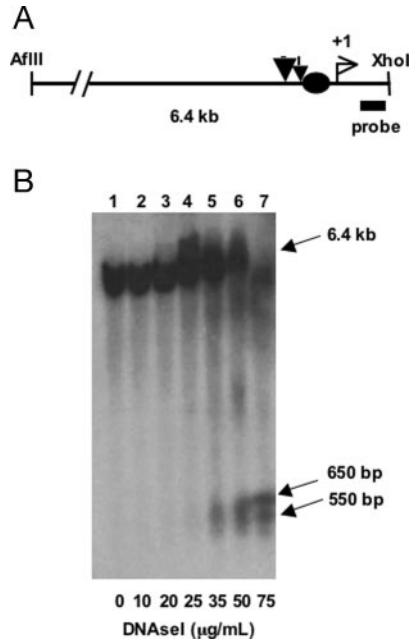

FIG. 2. **DNase I hypersensitivity mapping of the PDE6A core promoter region.** Nuclei were isolated from Y79 Rb cells and treated with increasing amounts of DNase I followed by Southern blot analysis. *A*, shown is a diagram of the results. DNase I hypersensitive sites (*vertical arrows*) were found ~550 and 650 bp upstream of the transcription start point at +1. The 650-bp band appears first (*A*, *thicker vertical arrow*; *B*, *lane 5*). *B*, after DNase I digestion, the DNA was extracted and digested with restriction enzymes AflII and XhoI that yield a 6.4-kb fragment spanning the PDE6A promoter region identified *in vitro*. Southern blot analysis was performed with a 272-bp PCR-amplified probe designated in *A*. Product in *lane 1* was not predigested with DNase I, resulting in an intact 6.4-kb fragment. *Lanes 2–7* contain increasing amounts of DNase I as noted. Two bands of 650 and 550 bp become increasingly visible in *lanes 5–7*.

A 177-bp Segment of the Upstream Region Yields the Highest Reporter Gene Activity in Vitro—To delimit the core promoter region, luciferase reporter gene constructs containing varying lengths of the upstream region were assayed by transient transfection in Y79 Rb cells. Due to the relatively low promoter activity, a modified adenofection method was devised to increase the efficiency of gene transfer severalfold over previously reported methods (11). As shown in Fig. 3, the luciferase activity was highest with the shortest fragments tested, pGL3-300 and pGL3-260. Both of these constructs contain 177 bp of sequence extending upstream from the transcription start point and either the entire 5′-untranslated region (pGL3-300) or a downstream truncation of 40 bp (pGL3-260), respectively. Reversing the orientation of the pGL3-260 construct (pGL3-260r) reduced activity equivalent to the promoterless control (pGL3-pl). The remaining four constructs tested (pGL3-593, pGL3-1060, pGL3-1542, and pGL3-3800) all contain upstream repetitive elements (10) that appear to attenuate promoter activity.

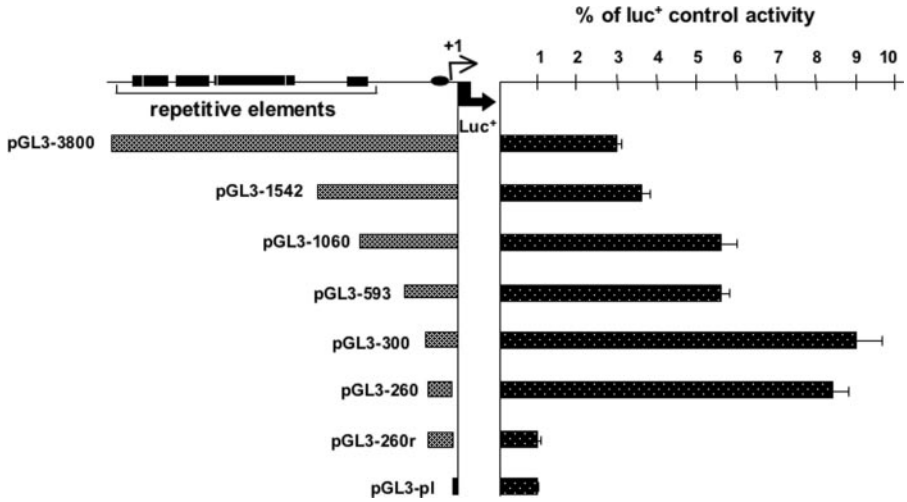

FIG. 3. **Relative activity of PDE6A upstream promoter fragments in Y79 cells.** Transient adenofection analysis (11) was performed in Y79 cells using six constructs (pGL3-260 to pGL-3800) that contain varying lengths of the upstream region. Plasmid pGL3-260r contains the same 260-bp fragment in pGL3-260 in the reverse orientation, and pGL3-pl was used as a promoterless control. Luciferase activity was normalized to control (β-galactosidase), and the results are shown as a percentage of a pGL3-SV40-luc$^+$ control plasmid. Values represent the average of three independent adenofections done in triplicate. *Error* is shown as S.D.

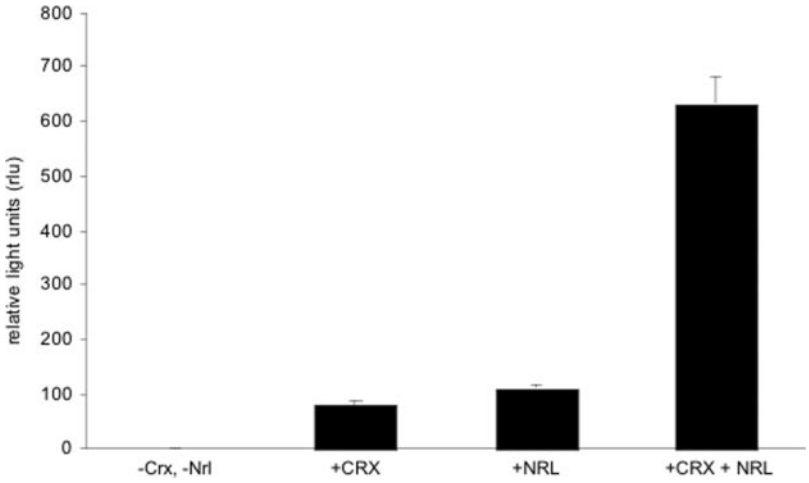

FIG. 4. **Crx and Nrl transactivation of the PDE6A promoter.** Transient transfection was performed in HEK293 cells using plasmid pGL3-300 with Crx and/or Nrl expression plasmids. Crx (+*CRX*) or Nrl (+*NRL*) alone stimulated promoter activity as much as 15–20-fold. Together, Crx and Nrl (+*CRX,* +*NRL*) synergistically activated promoter activity up to 150-fold. Values were normalized to β-galactosidase activity. *Error* is shown as S.E.

Crx and Nrl Transactivate the PDE6A Promoter—To identify cis-elements necessary for transcriptional activity, we first tested transactivation activity of the photoreceptor-specific transcription factors, Crx and Nrl. Transient transactivation assays were performed in HEK293 cells with and without Crx and Nrl expression constructs. As shown in Fig. 4, cotransfection of the pGL3-300 PDE6A promoter construct in the absence of Crx or Nrl expression is below detectable limits. In contrast, the inclusion of the Crx or NRL expression construct increased luciferase activity at least 15–20-fold above background levels. Inclusion in the assay of both Crx and Nrl expression constructs further stimulated activity (>100-fold over background), similar to the synergistic activation of rhodopsin (21).

Crx Binds within a 55-bp Segment Containing Three Potential Binding Sites—To identify the precise sequence within the PDE6A promoter where Crx binds, DNase I footprinting was done with a purified recombinant Crx fragment containing the DNA binding domain (14). An extended binding region was observed on both strands of the DNA in the region from −29 to −84 (Fig. 5, compare *lanes 2* and *3* with *lanes 4* and *5*). This region consists of three potential Crx binding sites, all containing a TAAT core consensus Crx binding sequence (see Fig. 1) (13, 14).

Crx Binds More Tightly to CBE-1—The relative binding strength of each potential Crx binding site was determined by electrophoretic mobility shift assay. Eleven double-stranded segments of varying lengths were generated containing various combinations of mutated and intact CBE sites (Fig. 6*A*). The shorter oligomers (Fig. 6*B*, *lanes 4–11*) showed comparable labeling with the longer oligomers (*lanes 1–3*) when electrophoresed for shorter periods of time (data not shown). For each oligomer, the AAT of the core TAAT Crx binding site was mutated to CCG. Mutation of CBE-1 alone (Fig. 6*B*, *lane 11*) abolishes binding, in contrast to the significant binding observed with an intact CBE-1 (*lane 8*) and a longer segment containing all three CBE sites (*lane 1*). Intact isolated CBE-2 or CBE-3 showed no binding to CBE-2 and only weak binding to CBE-3 (*lanes 9* and *10*), however, a longer oligomer with CBE-2 and CBE-3 showed strong binding (*lane 6*), suggesting possible cooperativity of binding or involvement of secondary structure. CBE-3 shows weak binding when included with a mutated CBE-2 site (*lane 7*). CBE-2, however shows no binding with a mutated CBE-3 site (*lane 4*), but moderate binding is observed when mutated CBE-1 and CBE-3 sites are included in the oligomer (*lane 3*). As predicted, mutation of all CBE sites within an oligomer abolishes binding (*lanes 2, 5*, and *11*), consistent with the requirement for the TAAT core for Crx binding. Each isolated CBE was then tested for binding in the presence of increasing amounts of poly[d(A-T)] (pAT). As shown in Fig. 6*C*, CBE-1 binding is unaffected even with 3× levels of pAT. Mutation of the CBE-1 TAAT binding core sequence shows nonspecific binding in the absence of pAT (*CBE-1m*) that is abolished in the presence of pAT. CBE-2 shows very weak binding even in the absence of pAT and is competed completely with 2× pAT. CBE-3 shows intermediate binding strength being competed with 3× pAT (*CBE-3*, *lane 4*). Taken together, the electrophoretic mobility shift assay results show that Crx has the greatest affinity for binding to the CBE-1 site in isola-

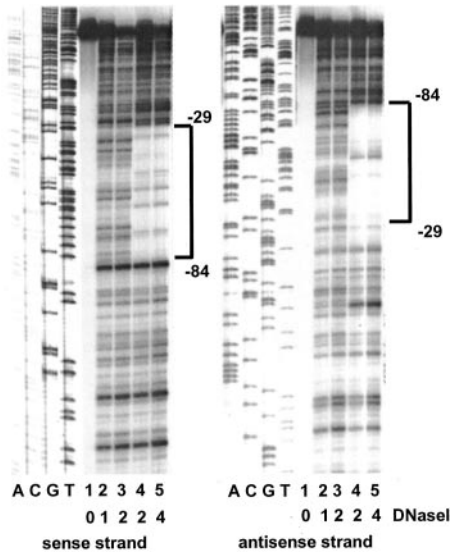

FIG. 5. **DNase I footprinting of Crx.** A bovine Crx homeobox domain region-GST fusion protein was used for DNase I footprinting of the PDE6A promoter fragment contained in pGL3-300. The footprinted region (*bracketed*) extends over 70 bp (−29 to −84) containing three possible Crx binding sites. DNA sequencing runs are shown (*lanes A*, *C*, *G*, and *T*) alongside the footprinting reactions. *Lane 1* for both sense and antisense probes represents the absence of DNase I, *lanes 2–5* show the effects of DNase I in the absence (*lanes 2* and *3*) and presence of Crx (*lanes 4* and *5*). DNase I concentration varied from 5 microunits (1×) to 20 microunits (4×), as indicated *below* the footprint.

tion but that the presence of intact CBE-2 and CBE-3 shows binding comparable with CBE-1. These results indicate that DNA bending and cooperativity of binding may be factors in the observed Crx binding.

Crx and Nrl Are Required for Full Promoter Activity in Transfected Cells—The contribution of Crx and Nrl to promoter activity was assessed by site-directed mutagenesis of the TAAT core sequence recognized by Crx and with an Nrl dominant negative expression construct shown to abolish Nrl activity (22). The Crx core binding sequence TAAT was mutated to CCGT at sites CBE-2 and CBE-3 to generate constructs m13 and m16, respectively. Transient transfection analysis with the normal or mutated sequences in the absence of Crx (Fig. 7*A*, *300-CRX, m13-CRX*, and *m16-CRX*) showed very low background activity. Mutation of the CBE-2 site (*m13+CRX*) showed a 50% reduction in activity, and mutation of CBE-3 (*m16+Crx*) reduced activity by 69%. Inclusion of the CBE-2 mutant with Crx and Nrl expression constructs (*m13+CRX+NRL*) caused a 61% reduction in reporter gene expression, and the CBE-3 mutant with Nrl and Crx (*m16+CRX+NRL*) caused a 79% reduction compared with the activity seen with the pGL3-300 construct, Crx, and Nrl (*300+CRX+NRL*). The CBE-3 mutant construct cotransfected with Nrl and Crx reduced reporter gene activity close to the level seen with Nrl alone (*300+NRL*), suggesting that the synergism between Crx and Nrl was abolished. Nrl transactivation of the promoter mutants in the absence of Crx was not significantly different than that observed with the intact promoter (data not shown).

The expression construct DD10 generates a naturally occurring truncated Nrl protein that inhibits Nrl activity (22). Use of this construct with the pGL3-300 construct and an Nrl expression construct abolished Nrl mediated transactivation (Fig. 7*B*, *+Nrl +DD10*). Inclusion of all three expression constructs (*+Crx +Nrl +DD10*) reduced promoter activity to a level similar to that seen with Crx alone (*+Crx*), thereby removing the synergistic activation of the promoter.

Crx and Nrl Are Required for Full Transcriptional Activity in in Vivo Knockout Mouse Studies—To assess the requirement of Nrl and Crx for PDE6A transcription, we determined the relative levels of transcript in Crx (15) and Nrl knockout mouse retinas. Total RNA isolated from whole eyes of postnatal day 10 (PN10) $Crx^{-/-}$ and $Nrl^{-/-}$ mice and from adult $Nrl^{-/-}$ mice was converted to cDNA, amplified with primer pairs that amplify short segments (300–500 bp) of the coding region of *Pde6a*, and visualized in real time. *Pde6b* and *Rho* were used as positive controls, and *Gapdh* was used for normalization. In the $Crx^{-/-}$ mouse, *Pde6b* and *Rho* transcript levels were reported to be at 75% and 8%, respectively, of the levels observed in $Crx^{+/+}$ littermates (15). As shown in Fig. 8, the mRNA levels of *Pde6a*, *Pde6b*, and rhodopsin in the Crx knockout mice were 13%, 55%, and 11%, respectively, of that observed in the $Crx^{+/+}$ littermates. Thus, Crx is necessary to achieve wild type transcription levels. In contrast, of the three genes analyzed, only rhodopsin was barely detectable in the PN10 retina of Nrl knockout mice, suggesting that Nrl may be essential for transcription of the *Pde6a* and *Pde6b* genes. Surprisingly, *Pde6a* (7% of wild type level) and, to a lesser extent, Pde6b (2% of wild type level) mRNA were detected in the adult retina of Nrl knockout mice. Determining the mechanism for this observation will require further study. Together, these reverse transcription-PCR results indicate that both Crx and Nrl are required *in vivo* for full transcriptional activity of the *Pde6a* gene.

DISCUSSION

Whereas several photoreceptor gene promoters have been analyzed *in vitro* and outside of the native promoter context in transgenic mice, in none of these studies have the data been confirmed *in vivo* in the native promoter. Using the DNase I hypersensitivity assay in Y79 cell nuclei, we found a strong correlation with the location of the minimal promoter delimited by the *in vitro* analysis. In addition to the primary DNase I hypersensitive sites, we also consistently observed a slower migrating, very weak signal that maps further upstream, indicating another possible open chromatin regulatory region. Because Rb cells represent a mixed population of cells, this region may contain a binding site active in cells not entered into a photoreceptor commitment pathway to act as a negative regulator of transcription.

An unusual feature of the PDE6A promoter relative to other photoreceptor promoters studied to date is the presence of numerous repetitive elements in close proximity, beginning ~900 bp from the transcription start point. The presence of these elements seems to attenuate gene expression and at least partly explains why promoter activity decreases with increasing length of the promoter. It also suggests that no other positive cis-elements are likely to be found within at least 3800 bp of the upstream region.

Our Crx reverse transcription-PCR data, coupled with previously published data (15), average to a 38% reduction of *Pde6b* mRNA, and we found an 87% reduction in *Pde6a* mRNA, indicating a critical role for Crx in achieving full promoter activity of both the *Pde6a* and *Pde6b* promoters. This is in contrast to other published results suggesting that Crx does not have a significant involvement in the activation of the *Pde6b* promoter (27, 28). This result is based on data from transient transfection assays showing that Nrl and Sp4 did transactivate the promoter but that Crx or combinations of Crx with Nrl or Sp4 did not, and on the lack of effect upon introduction of the promoter with a mutated CRE in both Y79 and *Xenopus* embryo transient transfections. This could reflect a difference between the native environment of the promoter within chromatin as in the Crx knockout mice compared with the artificial environment created with transient or stable

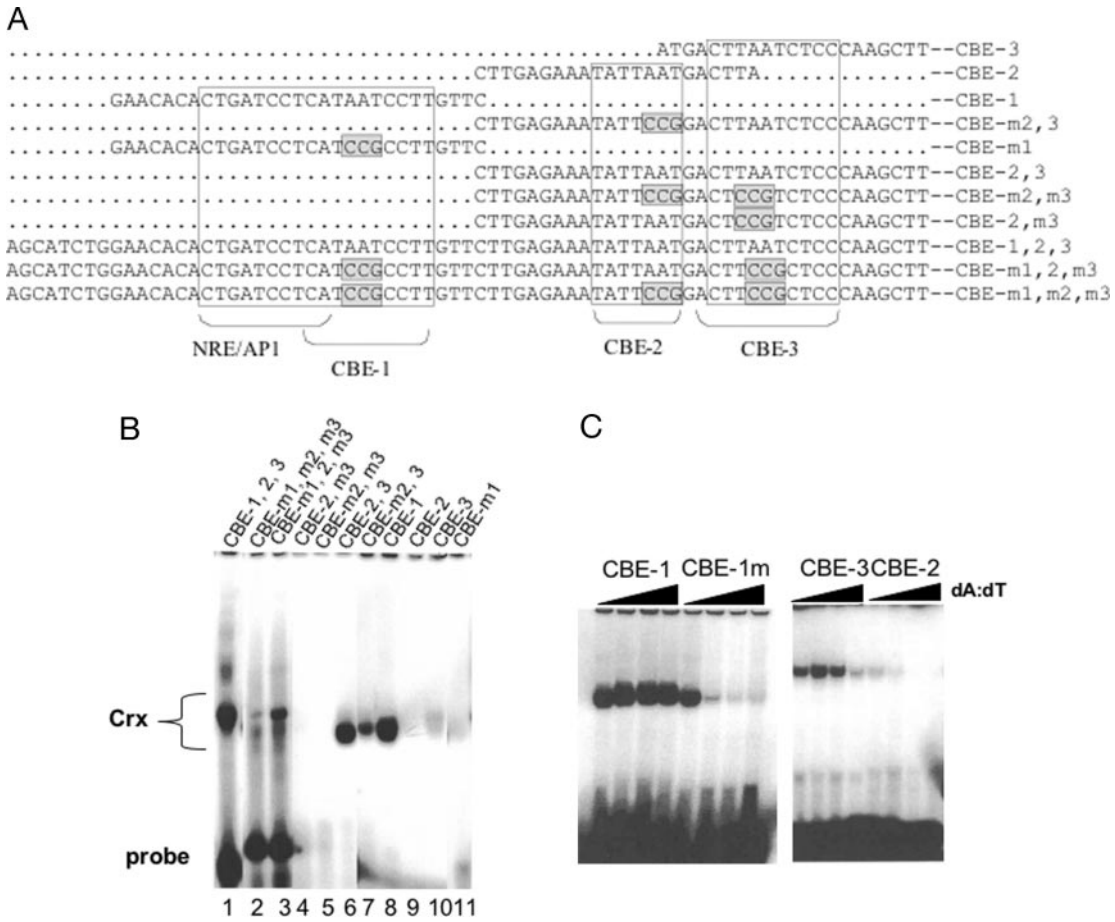

FIG. 6. **Gel shift assays of Crx binding to CBE sites in the PDE6A promoter.** *A*, oligomers used for gel shift assays. Eleven double-stranded oligomers were synthesized according to the sequence of the human PDE6A promoter region of varying lengths and containing different combinations of intact and mutated CBE sites. The positions of the CBE sites and the predicted *Nrl* binding site are shown *below* the sequences. Mutated residues are *boxed* and *shaded*. *B*, Crx binding to intact and mutated CBE. Binding reactions were performed in the presence of 2× pAT and electrophoresed through nondenaturing polyacrylamide gels. All of the shorter unshifted probes that ran off the gel (*lanes 4–11*) were observed in a shorter run (data not shown). *C*, CBE-1 in isolation shows the highest relative binding affinity. Binding assays were performed in the presence of increasing amounts of pAT (0, 1×, 2×, and 3×) for each of the isolated CBE sequences and a mutated CBE-1. CBE-1 binding was unaffected at even the highest level of competitor (*CBE-1*, *lane 4*).

transfections. Alternatively, the discrepancy in results may be resolved by a recent preliminary study (33) showing that inclusion of Crx with Nrl and Sp4 showed a greater stimulation of *Pde6b* promoter activity than did Nrl and Sp4 together without Crx. This concurs with a requirement for Crx for full activity of the *Pde6a* and *Pde6b* promoters.

In the absence of Nrl, there is no detectable *Pde6a* mRNA at PN10; however, a weak signal is detected in the adult mouse, based on microarray analysis and real-time reverse transcription-PCR. These data are consistent with Affymetrix microarray analysis showing that *Pde6a* turns on after PN4 and before PN8 (www.scripps.edu/cb/friedlander/gene_expression/search.html, Affy# 100696_at). The cellular origin of the transcripts, however, at any given time point has not been determined. Despite the presence of rudimentary discs contained within shortened outer segments, the $Nrl^{-/-}$ mouse displays very low to no expression of several rod photoreceptor-specific markers including *Pde6b* (19). It seems most likely that the *Pde6a* signal detected in Nrl-null mice is from inner retinal cells, consistent with our previous report showing *in situ* hybridization labeling in the inner nuclear layer of adult mouse retina (12).

Comparison of the anatomy of the *Pde6a* and *Pde6b* promoters reveals a striking similarity. Both core promoters are contained within 105 bp upstream of the transcription start point, both initiate transcription at one major site and one minor site, and both consist of a similar array of cis-elements including a GC box, an E-box, and Crx and Nrl binding sites, yet there is a 5–8-fold lower activity of the *Pde6a* promoter (7, 11). The primary differences are the spatial arrangement of the cis-elements, the sequence of the NRE/AP1 site, and the absence of repetitive elements upstream of the *Pde6b* promoter. We have found that the difference in promoter activities can be accounted for by the repetitive elements and the NRE/AP1 sequence. Conversion of the *Pde6a* AP1 sequence to that of *Pde6b* and removal of the repetitive elements raise the activity of *Pde6a* to the level of *Pde6b*.[2] We also found that the position of the cis binding sequence is critical for promoter activity. Moving the *Pde6b* NRE/AP1 site further upstream did not elevate *Pde6a* activity. These results and the results reported here indicate that there is a direct correlation between the spatial array of cis-elements and promoter strength. Thus, it may be possible to develop a series of promoters with defined strengths simply by rearranging the same cis-elements, a hypothesis that is worth testing for the development of safe, predictable vehicles for gene therapy.

Our results show that of three CBE binding sites in the *Pde6a* promoter, a site that is overlapping with the NRE/AP1-like site (CBE-1) binds Crx very tightly in isolation; however, DNase I footprinting, transient transfection assays, and gel

[2] J. B. White and S. J. Pittler, unpublished observations.

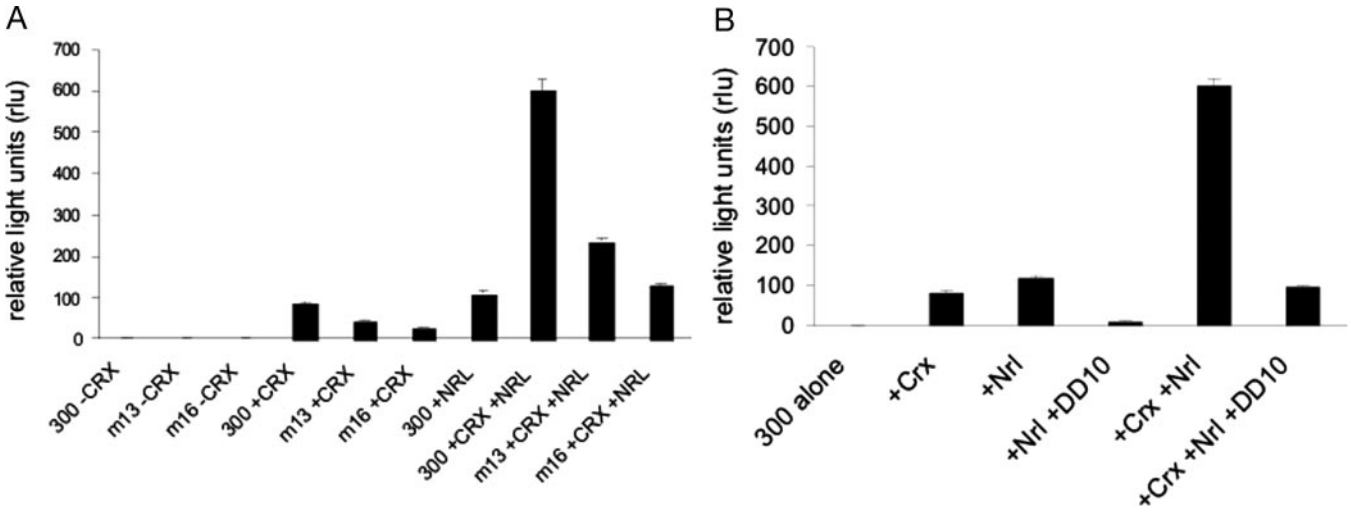

FIG. 7. **Transient transfection analysis of the effects of altering Crx and Nrl binding and interaction.** *A*, mutation analysis of Crx binding sites. Using the 300-bp PDE6A promoter fragment, relative luciferase activity of the native promoter was compared with plasmids with a mutated CBE-2 (m13) or CBE-3 (m16) site. Transient transfection of each plasmid was performed in HEK293 cells with the addition of Crx and/or Nrl expression plasmids. Crx stimulated activity was reduced 53% and 80% in the m13 and m16 mutants, respectively. Promoter activation by Crx and Nrl was also significantly reduced in the mutants, but Nrl activation appears unaffected. *B*, effects of mutant Nrl expression on PDE6A promoter transactivation. DD10 is a dominant negative acting, naturally occurring deletion mutant of Nrl that may be important in regulating Nrl activity *in vivo* (22). Transient transfection assays were performed in HEK293 cells using the pGL3-300 promoter construct (*300*) with or without plasmids expressing Nrl and DD10. The stimulatory effect observed with Nrl is abolished in the presence of DD10. DD10 also abolished the Crx/Nrl synergistic activation of the promoter but did not affect Crx transactivation. All transfections were done in triplicate and repeated at least twice. *Error* is shown as S.D.

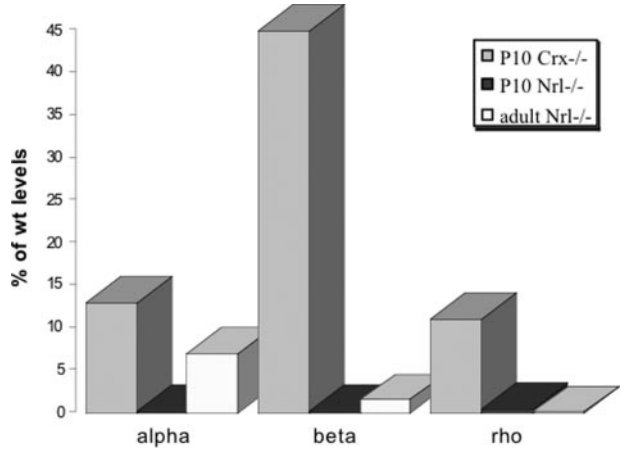

FIG. 8. **Quantitative analysis of *Pde6a* transcript levels in Crx and Nrl knockout mice.** Real-time quantitative PCR analysis was used to determine the relative transcript levels of rhodopsin (*rho*), *Pde6a* (*alpha*), and *Pde6b* (*beta*) in retinas of PN10 Crx and Nrl mice and adult Nrl-null mice (15, 19). Values were normalized to levels of *Gapdh* and are shown as a percentage of the wild type values arbitrarily set to 100%.

shift assays indicate that the CBE-2 and CBE-3 sites together can also show strong binding. The close proximity of Nrl and Crx could promote protein-protein interaction that has been demonstrated with these factors (21). The gel shift assay result in which CBE-1 and CBE-2 show weak binding in isolation (Fig. 6*A*, *lanes 9* and *10*) but relatively strong binding at each site when the sites are combined (*lanes 3, 6*, and *7*) is seemingly contradictory to the other binding data with CBE-1. The data are consistent, however, with a requirement for defined DNA bending and possible cooperativity of Crx binding. Also consistent with this idea is our previous finding using human retina nuclear extracts in gel shift assays, in which a retina-specific shift was only observed when the entire promoter region was included in the probe (10). The rhodopsin proximal promoter region is composed of a very similar array of cis-elements with an NRE flanked on both sides by Crx binding sites (29), and this promoter also shows weak activity in the absence of an upstream enhancer (30). Overall, the data indicate that binding of Crx to all three CBE sites, Nrl binding to the NRE/AP1 site, and presumably the basal transcription machinery are required for full activation of the *Pde6a* promoter.

Acknowledgments—We thank Russell Taylor and Kathy Bonness for some of the preliminary work developing the transient transfection assays; Telisha Millender-Swain for technical assistance; the Cystic Fibrosis Gene Therapy Core Center (National Institutes of Health National Institute of Diabetes and Digestive and Kidney Diseases T30 DK54781) at University of Alabama at Birmingham for providing the LacZ adenovirus; and Dr. Connie Cepko for providing *Crx* knockout mice. Human retina tissue was obtained from the Alabama Eye Bank through the Age-related Histopathology Laboratory, a service funded by the International Retinal Research Foundation, the National Eye Institute (EY06109), and the Vision Science Research Center (P30EY03039) at the University of Alabama at Birmingham School of Medicine.

REFERENCES

1. Burns, M. E., and Baylor, D. A. (2001) *Annu. Rev. Neurosci.* **24,** 779–805
2. Koutalos, Y., Nakatani, K., and Yau, K. W. (1995) *J. Gen. Physiol.* **106,** 891–921
3. Nakatani, K., Chen, C., Yau, K. W., and Koutalos, Y. (2002) *Adv. Exp. Med. Biol.* **514,** 1–20
4. Huang, S. H., Pittler, S. J., Huang, X., Oliveira, L., Berson, E. L., and Dryja, T. P. (1995) *Nat. Genet.* **11,** 468–471
5. Tsang, S. H., Gouras, P., Yamashita, C. K., Kjeldbye, H., Fisher, J., Farber, D. B., and Goff, S. P. (1996) *Science* **272,** 1026–1029
6. Pittler, S. J., and Baehr, W. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 8322–8326
7. Piri, N., Yamashita, C. K., Shih, J., Akhmedov, N. B., and Farber, D. B. (2003) *J. Biol. Chem.* **278,** 36999–37005
8. Olsson, J. E., Gordon, J. W., Pawlyk, B. S., Roof, D., Hayes, A., Molday, R. S., Mukai, S., Cowley, G. S., Berson, E. L., and Dryja, T. P. (1992) *Neuron* **9,** 815–830
9. Tan, E., Wang, Q., Quiambao, A. B., Xu, X., Qtaishat, N. M., Peachey, N. S., Lem, J., Fliesler, S. J., Pepperberg, D. R., Naash, M. I., and Al Ubaidi, M. R. (2001) *Investig. Ophthalmol. Vis. Sci.* **42,** 589–600
10. Mohamed, M. K., Taylor, R. E., Feinstein, D. S., Huang, X., and Pittler, S. J. (1998) *J. Mol. Neurosci.* **10,** 235–250
11. White, J. B., Taylor, R. E., and Pittler, S. J. (2001) *J. Neurosci. Methods* **106,** 1–7
12. Taylor, R. E., Shows, K. H., Zhao, Y., and Pittler, S. J. (2001) *Biochem. Biophys. Res. Commun.* **282,** 543–547
13. Furukawa, T., Morrow, E. M., and Cepko, C. L. (1997) *Cell* **91,** 531–541
14. Chen, S., Wang, Q. L., Nie, Z., Sun, H., Lennon, G., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., and Zack, D. J. (1997) *Neuron* **19,** 1017–1030
15. Furukawa, T., Morrow, E. M., Li, T., Davis, F. C., and Cepko, C. L. (1999) *Nat. Genet.* **23,** 466–470

16. Furukawa, A., Koike, C., Lippincott, P., Cepko, C. L., and Furukawa, T. (2002) *J. Neurosci.* **22,** 1640–1647
17. Livesey, F. J., Furukawa, T., Steffen, M. A., Church, G. M., and Cepko, C. L. (2000) *Curr. Biol.* **10,** 301–310
18. Boatright, J. H., Borst, D. E., Peoples, J. W., Bruno, J., Edwards, C. L., Si, J. S., and Nickerson, J. M. (1997) *Mol. Vis.* **3,** 15–24
19. Mears, A. J., Kondo, M., Swain, P. K., Takada, Y., Bush, R. A., Saunders, T. L., Sieving, P. A., and Swaroop, A. (2001) *Nat. Genet.* **29,** 447–452
20. Bessant, D. A., Payne, A. M., Mitton, K. P., Wang, Q. L., Swain, P. K., Plant, C., Bird, A. C., Zack, D. J., Swaroop, A., and Bhattacharya, S. S. (1999) *Nat. Genet.* **21,** 355–356
21. Mitton, K. P., Swain, P. K., Chen, S., Xu, S., Zack, D. J., and Swaroop, A. (2000) *J. Biol. Chem.* **275,** 29794–29799
22. Rehemtulla, A., Warwar, R., Kumar, R., Ji, X., Zack, D. J., and Swaroop, A. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 191–195
23. Yu, J., Farjo, R., MacNee, S. P., Baehr, W., Stambolian, D. E., and Swaroop, A. *Genome Biology* http://genomebiology.com/2003/4/10/R65
24. Montecino, M., Pockwinse, S., Lian, J., Stein, G., and Stein, J. (1994) *Biochemistry* **33,** 348–353
25. Mirkovitch, J., and Darnell, J. E., Jr. (1991) *Genes Dev.* **5,** 83–93
26. Di Polo, A., and Farber, D. B. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 4016–4020
27. Lerner, L. E., Gribanova, Y. E., Whitaker, L., Knox, B. E., and Farber, D. B. (2002) *J. Biol. Chem.* **277,** 25877–25883
28. Lerner, L. E., Gribanova, Y. E., Ji, M., Knox, B. E., and Farber, D. B. (2001) *J. Biol. Chem.* **276,** 34999–35007
29. Chen, S., and Zack, D. J. (1996) *J. Biol. Chem.* **271,** 28549–28557
30. Nie, Z., Chen, S., Kumar, R., and Zack, D. J. (1996) *J. Biol. Chem.* **271,** 2667–2675
31. Di Polo, A., Lerner, L. E., and Farber, D. B. (1997) *Nucleic Acids Res.* **25,** 3863–3867
32. Ahmad, I. (1995) *Brain Res. Dev. Brain Res.* **90,** 184–189
33. Lerner, L. E., Gribanova, Y., and Farber, D. B. (2003) *Investig. Ophthalmol. Vis. Sci.* **44,** 3535