Functional analysis of the rod photoreceptor cGMP phosphodiesterase alpha subunit gene promoter: Nrl and Crx are required for full transcriptional activity*

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Running Title: Crx and Nrl are required for full PDE6A promoter activity
Rod photoreceptor cGMP phosphodiesterase is an essential enzyme in the phototransduction cascade. To understand the factors controlling expression of the PDE6 genes, we have characterized the promoter of the PDE6A gene which encodes the catalytic $\alpha$-subunit. In vivo DNaseI hypersensitivity assays revealed two sites in close proximity immediately upstream of the PDE6A core promoter region. Transient transfection assay in Y79 retinoblastoma cells of constructs containing varying lengths of the promoter region showed a proportional decrease in promoter activity with increasing length. The most active segment contained 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. DNaseI 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 undetectable in Nrl$^{-/}$ mice at postnatal day 10. Taken together, the results establish that both Nrl and Crx are required for full transcriptional activity of the PDE6A gene.
INTRODUCTION

The type 6 cGMP phosphodiesterase (PDE6) 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 RP (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 to other photoreceptor gene promoters including the PDE6B gene promoter (7). We suggest that the relatively weak expression of this promoter could be useful where over-expression with the well-characterized, most commonly used rhodopsin promoter could induce, rather than reverse retinal degeneration (8;9).

We previously reported 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 DNaseI 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 (CBE) and a non-consensus NRL-like site (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 rhodopsin 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) that 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
Life Technologies. Fetal bovine serum (FBS) was purchased from Hyclone. HEK293 and Y79
cells were purchased from American Type Culture Collection and maintained in DMEM
supplemented with 5% FBS, and 100 units/ml of penicillin and streptomycin, or RPMI 1640
supplemented with 10% FBS.

DNaseI 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 2K
rpm in an IEC clinical centrifuge and resuspended in 1x PBS. Approximately 5x10^6 nuclei were
digested with DNaseI at varying concentrations ranging from 0-100 µg/ml. The reaction was
incubated 10’ 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 & Xhol at 37°C for 4 hours with one addition of each enzyme after 2 hours, and the digestion products were electrophoresed in a 0.7% agarose gel. DNA was transferred to a Nylor membrane, and hybridized with an α[32P]-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: pGL3-Control encoding the firefly luciferase gene under control of the SV40 strong promoter and SV40 enhancer and 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 it into PBS II KS (10) was amplified with primers, U-Sal (5’-TTTGTCGACGCTCTGTGCAGACATCTCTTCT-3’) and U-Xba (5’-AAATCTAGAACCTCCTGTGCACCT-3’) generating a 322-bp segment upstream of the ATG initiation codon. This fragment was cloned into the pBS II KS Smal 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 amplifying 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 Smal 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 Sall and NcoI and was inserted in to 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’- GTGCTGGGATTACAGGCGTGA-
3’ and HARE-3; 5’- CTGTAAATTCTCTGAAAGTCCCGAGAG-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 was made by digesting pGL3-3800 with SacI and ApaI and purifying the vector with the insert using a Qiagen (Valencia, CA) 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 preps were performed using Qiafilter Endofree Maxi or Mega kits (Qiagen) following 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’-
AGAGGGAGCAAGCTTGGAGACCGAGTCATTAA-3’) or mutB1-R (5’-
AGAGGGAGCAAGCTTGGAGATTTACCGAGTCATTAA-3’) primer using pBS-322 as template and a reverse primer, GL2 (5’- ACCTTCTGCGTTTTGTATTTTC-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 was 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 previously described (14;22).
Adenofection of Y79 cells—Y79 cells were transfected using the adenofection method previously described for this cell line (11). Cells were harvested 48 h after infection and lysed in 100 µl of 1 X reporter lysis buffer (Promega) and light emission was measured immediately in a Turner Designs 20/20 luminometer (Promega). Values were normalized to viral β-gal expression. All experiments were performed in triplicate a minimum of three times (n≥9).

Transfection of HEK293 cells with calcium phosphate—HEK293 cells were transfected with calcium phosphate using a standard protocol. Briefly, ~6 x 10⁵ HEK293 cells were plated in a 60 mm Petri dish in DMEM supplemented with 5% FBS and 1% penicillin/streptomycin and grown for 24 to 36 hours at 37°C to achieve 50 to 75% confluency. The medium was changed once 3 hours prior to transfection. Up to 16 µg DNA in 300 µl 250 µM CaCl₂ was combined dropwise with 300 µl 2X HBS and incubated 30 min. The DNA consisted of 1 µg β-gal internal control, 4-5 mg test plasmid, and 2-5 µg of Crx or Nrl expression construct. The DNA mixture was then added dropwise to the cells, incubated for 48 hours with one media change after 12 hours incubation.

Luciferase and β-Galactosidase assays--Cells were harvested and lysed in 350 µl of Galacto-Star reporter lysis buffer (Tropix, Inc.), cell debris was pelleted at 12K xg 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 3 times.

DNaseI footprinting of Crx—The plasmid pGL3-300 was used as a template for PCR separately with either sense or antisense radiolabeled primers to generate DNaseI 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 DTT, 2% polyvinyl alcohol, and 10% glycerol. Binding reactions were performed on ice for 20 min. in a total volume of 50 ul including labeled probe, 1x binding buffer with or without 100 ng purified bovine CrxHD-GST fusion protein (14). For DNaseI 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 0.02U DNaseI for 1 min. at RT. The reactions were terminated with 90 uL of 1x stop buffer (20 mM EDTA, pH 8.0, 1% SDS, 0.2 M NaCl, and 250 ug/mL glycogen). Ten µL of 2.5 mg/mL proteinase K was added and incubated at RT for 5 min. followed by phenol chloroform extraction, precipitation, and resuspension in 1x DNA sequencing gel loading buffer. DNA sequencing reactions were run along side in a 6% denaturing acrylamide gel.

**Electrophoretic mobility shift assay**—Gel shift assays were performed essentially as described (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 non-denaturing 6% acrylamide gel in 0.5x TBE for two hours at 380 volts.

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, Dept. of Genetics, Harvard Medical School. The Crx$^{-/-}$ mouse was outcrossed to a congenic C57Bl/6 x SJL hybrid strain that is +/+ at the rd1 locus (6). The mice were bred to homozygosity for the Crx knockout allele and maintained for greater than six generations. Total RNA was isolated from eyes of 10-day old Crx$^{-/-}$ and Nrl$^{-/-}$ mice as well as background matched controls using RNAqueous RNA isolation kit (Ambion, Inc.) or TRIZOL (Invitrogen). Reverse transcription reactions (23) were performed with 2 µg total RNA using MMLV (Ambion) or Superscript II (Invitrogen) reverse transcriptase following 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, PDE6A-R, 5’-CAGGGTGACCTCCTCCTTCTTG ;
Pde6b-F, 5’-GTATACAAGGAATTTTCTCG, PDE6B-R, 5’-
CAAAATTGCTATAGGCAGAGTCCG ; rhodopsin-F (Rho), 5’-
ATCCCTGAGGGCATGCAATGTTCATG, Rho-R, 5’-
CTGCTTTGTCAACATGATGATGAC; Gapdh-F, 5’-
TGATGACATCAAGGAAGGTGTAAG, Gapdh-R, 5’-TCCTTGGAGGCCATGTAGGCCAT.
The reactions were run in the real time PCR Opticon II system (MJ Research) or iCycler (BioRad) and analyzed using the comparative Ct method (ΔΔCt) with Gapdh as the normalizer (http://dorakmt.tripod.com/genetics/realtime.html). Error values are expressed as standard error of the mean (n=8).

RESULTS

<<<Figure 1>>>  
*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 DNaseI accessible open chromatin region (24;25). Shown in Figure 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). <<<Figure 2>>> To assess the chromatin status of the region around the PDE6A promoter region *in vivo*, a DNaseI hypersensitivity assay
was performed with Y79 cells, that transcribe PDE6A mRNA (26). Treatment of intact Y79 nuclei with DNaseI revealed two fragments in close proximity (Fig. 2B, lanes 6, 7) within a 6.4 kb AflII/XhoI genomic fragment spanning the promoter region (Fig. 2A). No cleavage products were observed using HEK293 nuclei, which do not express PDE6A (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).

**Figure 3**

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 several fold over previously reported methods (11). As shown in Figure 3, the luciferase activity was highest with the shortest fragments tested, pGL-300 and, pGL-260. Both of these constructs contain 177 bp of sequence extending upstream from the transcription start point, and either the entire 5’-untranslated region (pGL-300) or a downstream truncation of 40 bp (pGL-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, -1060, -1542, -3800) all contain upstream repetitive elements (10) that appear to attenuate promoter activity.

*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 Figure 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, DNaseI 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 (Figure 5, cf. lanes 2, 3 to lanes 4, 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 EMSA. Eleven double stranded segments of varying lengths were generated containing various combinations of mutated and intact CBE sites (Fig. 6A). The shorter oligomers (Fig. 6B, lanes 4-11) showed comparable labeling to the longer oligomers (lanes 1-3) when electrophoresed for shorter periods of time (not shown). For each oligomer, the AAT of the core TAAT Crx binding site was mutated to CCG. Mutation of CBE-1 alone (Fig. 6B, 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, 10), however, a longer oligo 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 abolish binding (lanes 2, 5, 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[dA:dT] (pAT). As shown in Figure 6C, CBE-1 binding is unaffected even with 3x 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 2x pAT. CBE-3 shows intermediate binding strength being competed with 3x pAT (CBE-3, lane 4). Taken together the EMSA results show that Crx has the greatest affinity for binding to the CBE-1 site in isolation, but the presence of intact CBE-2 and CBE-3 shows binding comparable to CBE-1. These results indicate that DNA bending and cooperativity of binding may be a factor 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 (300-Crx, m13-Crx, m16-Crx, Fig. 7A) 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 to the activity seen with the 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 (not shown).

<<<Figure 7>>>

The expression construct DD10 generates a naturally occurring truncated Nrl protein that inhibits Nrl activity (22). Use of this construct with the 300 construct and an Nrl expression construct abolished Nrl mediated transactivation (+Nrl+DD10, Fig. 7B). 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.

<<<Figure 8>>>

*Crx and Nrl are required for full transcriptional activity 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 post natal 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, 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 mouse, suggesting that Nrl may be essential for transcription of the Pde6a and Pde6b genes. Surprisingly, Pde6a (7% of wt level) and to a lesser extent Pde6b (2% of wt level) mRNA were detected in the adult retina of Nrl knockout mice. Determining the mechanism for this observation will require further study. Together, these RT-PCR results indicate that both Crx and Nrl are required in vivo for full transcriptional activity of the Pde6a gene.

DISCUSSION

While several photoreceptor gene promoters have been analyzed in vitro and outside of the native promoter context in transgenic mice, in none of these studies has the data been confirmed in vivo in the native promoter. Using the DNaseI 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 DNaseI hypersensitive sites, we also consistently observed a slower migrating very weak signal that maps further upstream indicating another possible open chromatin regulatory region. Since 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 at least within 3800 bp of the upstream region.

Our Crx RT-PCR data coupled with that previously published (15) averages 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 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 to the artificial environment created with transient or stable transfections. Alternatively, the discrepancy in results may be resolved by a recent preliminary study 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 RT-PCR. This data is consistent with Affymetrix microarray analysis showing that *Pde6a* turns on after PN4 and prior to PN8 (http://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*
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 tsp, both initiate transcription at one major and one minor site, and both consist of a similar array of cis elements including a GC box, an E-box, 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 raises the activity of Pde6a to the level of Pde6b$^3$. 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 DNaseI footprinting, transient transfection assays and gel shift assays indicate that 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
assays result where CBE-1 and CBE-2 show weak binding in isolation (Fig. 6A, lanes 9, 10), but binding at each site is relatively strong when the sites are combined (lanes 3, 6, 7), is seemingly contradictory to the other binding data with CBE-1. The data is 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, where a retina specific shift was only observed when the entire promoter region was included in the probe (10). The rhodopsin proximal promoter region is a comprised 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.

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Footnotes

1 Abbreviations Used: PDE6, rod photoreceptor cGMP-phosphodiesterase type 6; CBE, Crx binding element; NRE, Nrl response element; PN, postnatal day; DNaseI, deoxyribonuclease I.

2 Lerner et al., 2003 Invest. Ophthalmol. Vis. Sci. Abstract #B238

3 White and Pittler, unpublished observations
Figure legends

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.

Fig. 2 **DNaseI hypersensitivity mapping of the PDE6A core promoter region.** Nuclei were isolated from Y79 Rb cells and treated with increasing amounts of DNaseI followed by Southern blot analysis. A, Shown above is a diagram of the results. DNaseI 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, Following DNaseI 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 panel A. Product in lane 1 was not predigested with DNaseI resulting in an intact 6.4 kb fragment. Lanes 2-7 contain increasing amounts of DNaseI as noted. Two bands of 650 and 550 bp become increasingly visible in lanes 5-7.

Fig. 3 **Relative activity of PDE6A upstream promoter fragments in Y79 cells.** Transient adenofection analysis (11) was performed in Y79 cells using 6 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-gal 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 in SD.

Fig. 4 Crx and Nrl transactivation of the PDE6A promoter. Transient transfection was performed in HEK293 cells using plasmid pGL-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-gal activity. Error is shown as SEM.

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

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 position of the CBE sites and the predicted Nrl binding site is 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 2x poly[dA:dT]
and electrophoresed through non-denaturing polyacrylamide gels. All of the shorter unshifted probes that ran off the gel (lanes 4-11) were observed in a shorter run (not shown). C, CBE-1 in isolation shows the highest relative binding affinity. Binding assays were performed in the presence of increasing amounts of poly[dA:dT] (0, 1x, 2x, and 3x) 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).

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 to 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 percent 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 reported in SD.

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 shown as a percent of the wild type values arbitrarily set to 100%. Error is shown as SEM (n=8).
Figure 1

A

-105 GGCCCCACAGCATCTGGAAACACACTGATCCTCAATACTCTTGTTCTTGAGAAATATTATGACTTAATCTCCCAAGCTT(-27)

| NRE/AP1 | CBE-1 | CBE-2 | CBE-3 |
|---------|-------|-------|-------|

-26-GCTCCCACTCCTGTGCAGCCATCTCA +1

B

GC-box | E-box | CBE-1 | CBE-2 | CBE-3
NRE/AP1

+1
Figure 2

A

AflII XhoI

6.4 kb probe

B

DNAseI (µg/mL)

0 10 20 25 35 50 75

6.4 kb

650 bp

550 bp
Figure 3

% of luc⁺ control activity

| Repetitive Elements | % of luc⁺ control activity |
|---------------------|---------------------------|
| pGL3-3800           |                           |
| pGL3-1542           |                           |
| pGL3-1060           |                           |
| pGL3-593            |                           |
| pGL3-300            |                           |
| pGL3-260            |                           |
| pGL3-260r           |                           |
| pGL3-pl             |                           |
Figure 5
Figure 7

A

B
Figure 8

The diagram shows the percentage of wt levels of alpha, beta, and rho proteins in different conditions:

- **P10 Crx-/-**
- **P10 Nrl-/-**
- **adult Nrl-/-**

The bars indicate the percentage of wt levels for each protein in the respective conditions.
Functional analysis of the rod photoreceptor cGMP phosphodiesterase α-subunit gene promoter: Nrl and Crx are required for full transcriptional activity

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