



The 5' flanking sequence of the human retGC1 gene acquires a photoreceptor cell restricted activity pattern over the course of retinal development

Jason E. Coleman,¹ Kebin Wu,² Hans Jürgen Fülle,^{2,3} Susan L. Semple-Rowland¹

¹Department of Neuroscience, University of Florida McKnight Brain Institute and College of Medicine, Gainesville, FL; ²Department of Cell and Neurobiology and ³Department of Ophthalmology, Mary D. Allen Laboratories for Vision Research, Doheny Eye Institute, University of Southern California Keck School of Medicine, Los Angeles, CA

Purpose: Specific mutations of the retinal guanylyl cyclase-1 (retGC1) gene have been linked to Leber congenital amaurosis type 1 (LCA1) and cone-rod dystrophies in humans, diseases that are amenable to treatments using molecular based therapies. As a step towards developing a therapeutic transgene for LCA1, we analyzed the cell specific and developmental activity profiles of fragments of the human retGC1 5' flanking region *in vivo*.

Methods: We generated self inactivating lentiviral vector constructs carrying three different fragments of the human retGC1 promoter fused to a nuclear localized β -galactosidase reporter gene (nlacZ). The transgenes were packaged into lentiviral vectors, which were then used to transduce retinal progenitor cells of the developing chick. We monitored the expression of nlacZ in the retina over the course of development and in the retina, brain and pineal gland just prior to hatching.

Results: A 1.8 kb fragment of the retGC1 5' flanking region upstream of Exon 2 was capable of targeting nlacZ expression to photoreceptor cells *in vivo* and its activity was augmented by the presence of intron 1. We also demonstrated that the cell specific activity of this fragment arises, at least in part, by silencing expression in non-photoreceptor cells during the final stages of retinal development.

Conclusions: We have identified a human retGC1 promoter fragment that exhibits photoreceptor cell specific activity *in vivo*. Our results suggest that an element located in the proximal promoter may play a role in silencing expression of this gene in non-photoreceptor cells, thereby by shaping the restricted expression pattern of GC1 in the retina.

Signaling cascades mediated by the intracellular second messenger cyclic guanosine 3',5'-monophosphate (cGMP) have been described in numerous cells types and tissues [1]. The role of cGMP is perhaps best understood in the outer segments of vertebrate retinal photoreceptor cells where cGMP serves as the key second messenger of visual signal transduction [2,3]. In photoreceptor cells, retinal guanylyl cyclases (GCs) catalyze the formation of cGMP in response to decreases in intracellular calcium levels that result from light triggered hydrolysis of cGMP and closure of cGMP gated ion channels. The regulation of retinal GC activity by calcium is mediated by calcium binding proteins called guanylate cyclase activating proteins [4,5].

At least two eye specific membrane bound GC isoforms, termed retGC1 and retGC2 in human, are predominantly, but not exclusively, localized to the photoreceptor outer segments [6-8]. The relative amounts of retGC1 in the rod and cone

photoreceptors of the rod-dominant rat retina and the cone rich retina of chicken appear similar [9], while levels of retGC1 appear to be higher in cone cells in cat, monkey and human retinas [6,9,10]. Immunocytochemical and biochemical studies show that retGC1 and retGC2 are co-expressed in rat rod photoreceptor cells and that these two proteins preferentially form homomers *in vivo* [11]. RetGC1, but not retGC2, is also expressed in the pineal gland and may play a role in pinealocyte function [8,12,13].

The gene encoding retGC1 has been mapped to human chromosome 17p13 and its genomic organization has been described [14]. Specific mutations of the human retGC1 gene have been causally linked to autosomal recessive Leber congenital amaurosis type 1 (LCA1) [15,16] and to autosomal dominant cone-rod dystrophy (CORD6) [15-19]. With recent advances in molecular technologies and the availability of both naturally occurring and transgenic animal models carrying null mutations in retGC1 [20,21], it is now feasible to conduct detailed cellular and molecular studies of retGC1 in normal and diseased retina. One important aspect of these studies is to determine how the expression of GC1 is regulated in normal retina. In this series of experiments, we cloned and analyzed the 5' flanking region of the human retGC1 gene and examined the cell specific expression characteristics of selected retGC1 promoter fragments in the developing chicken retina. Using a lentiviral vector system to infect retinal progenitor

Correspondence to: Susan L. Semple-Rowland, University of Florida McKnight Brain Institute, Department of Neuroscience, 100 Newell Drive, Building 59, Room L1-100, Gainesville, FL, 32610-0255; Phone: (352) 392-3598; FAX: (352) 392-8347; email: rowland@mbi.ufl.edu

Dr. Coleman is now at The Picower Center for Learning and Memory, Massachusetts Institute of Technology, Cambridge, MA.

cells, we found that segments located in both the proximal and distal regions of the human retGC1 promoter are required to achieve photoreceptor specific expression of a lentiviral transgene over the course of development.

METHODS

Isolation of human BAC clones: Bacterial Artificial Chromosome (BAC) genomic clones containing the human retGC1 gene were obtained from Research Genetics after screening human BAC plasmid DNA pools by PCR with specific oligonucleotides. Initial primer design was based on human retGC1 cDNA (GenBank accession number M92432) and genomic sequences.

Genome walking, DNA sequencing and analysis: Human retGC1 5' flanking region was amplified and subcloned using a TOPO® Walker Kit (Invitrogen, Carlsbad, CA). The nucleotide sequence of both strands was determined using an ABI Prism dRhodamine terminator cyclase sequencing kit and an ABI Prism 310 Genetic Analyzer with POP-6 separation polymer according to the manufacturer's instructions (Applied Biosystems, Foster City, CA).

Generation of DNA constructs: Selected retGC1 promoter fragments were amplified using PCR and recombinant *Pfu* DNA polymerase (Stratagene, La Jolla, CA) and sequence was verified as described above. The core sequences of the primers for the designated retGC1 promoter fragments and their positions are shown in Table 1. Short sequence extensions that included *NotI* and *PmeI* restriction sites were added to the 5' ends of the core sequence of the sense and antisense primers, respectively. The following cycle parameters were used: 94 °C for 1 min (once); 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min/kb (30 times); 72 °C for 10 min (once); 4 °C soak. The CMV promoter was excised from the pTYF.CMV.nlacZ vector using *NotI* and *PmeI* and the selected retGC1 promoter fragments flanked by *NotI* and *PmeI* were inserted to generate the promoter-nlacZ expression vectors depicted in Figure 1A. The pTYF vector backbone (self inactivating (SIN) lentiviral vector transducing vector) used in these studies has been described elsewhere [22]. Transfection grade plasmid DNA was prepared using Qiagen endotoxin-free MaxiPrep kits (Valencia, CA).

Production of lentiviral vector and titers: The production, concentration and titering of viruses used for experiments in this study were performed as described previously [22]. Particle titers were determined using a p24 ELISA (Becton Dickinson, Franklin Lakes, NJ) following the protocol provided by the manufacturer. The p24 concentrations for each viral stock used in the experiments were as follows: 250 ng/ml pTYF.GCE1.nlacZ, 1610 ng/ml pTYF.GCE7.nlacZ, 1830 ng/ml pTYF.GCE8.nlacZ.

Embryonic injections: Neural tube injections of Hamberger-Hamilton stage 10-12 embryos [23] were performed as described previously [24]. Briefly, SPF grade White Leghorn eggs (Charles River Laboratories, Wilmington, MA) were set on day 0 and incubated on their sides without rotation at 37.5 °C and 60% humidity. A small opening was made in the eggshell overlying the embryo and about 0.5 µl virus

was slowly injected into the ventricular space of the neural tube. The egg was then sealed with parafilm and incubation was continued until the embryo reached the desired age for analysis. In a typical experiment, six embryos were injected with viral vector. The survival rate averaged greater than 80% and 3-4 of the surviving embryos (at least 6 retinas) were used for analysis.

Tissue preparation, X-gal histochemistry and microscopy: The neural retinas were dissected from the eyes of injected embryos at selected ages and dispase (Sigma, St. Louis, MO) was used as necessary to aid in the removal of the pigmented epithelium. Retinal whole mounts were prepared by placing the retinas photoreceptor side down on a Millicell (Millipore, Billerica, MA) insert containing PBS and using fine tipped glass rods to flatten the tissue. To detect expression of nlacZ, retinas were fixed by gently immersing the Millicell membrane in 4% paraformaldehyde for 15 min at room temperature. The retinas and membrane were then rinsed three times in PBS and incubated in PBS (pH 7.9) containing 35 mM potassium ferrocyanide, 35 mM potassium ferricyanide, 2 mM magnesium chloride, 0.02% NP-40 and 40 mg/ml 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) at 37 °C for 16-18 h. Following this incubation, the retinas were rinsed three times in PBS, cryoprotected with 30% sucrose and mounted in OCT compound (Tissue-Tek, Tokyo, Japan) for cryosectioning. Serial sections (20 µm) were obtained through areas positive for X-gal staining, mounted on slides, and counterstained with 4,6-diamidino-2-phenylindole (DAPI; Molecular Probes, Eugene, OR). The sections were then coverslipped using an aqueous based mounting medium (Gel Mount, BioMedia, Foster City, CA). Thirty to eighty sections, taken from the retinas of at least three different animals injected with the human retGC1 promoter-nlacZ viral vectors were analyzed for each time point. In some cases the pineal glands and brains of E18 embryos that had been injected with virus were removed and fixed in 4% paraformaldehyde. Pineal glands were stained with X-gal in toto and processed for cryosectioning as described above. The brains were cut into 100 to 200 µm sections using a vibratome and placed in the wells of a 12 well

TABLE 1. PCR PRIMER DATA

Fragment	Primer sequence and position (5' to 3')	
	Sense	Antisense
GCE1	CACTTGTTACTTCTGGCTGA (-680 to -700)	GGTCATTGCCGGCTT (-9 to +6)
GCE7	TCTGCTCTCATCCAACATTT (-1746 to -1726)	GGTCATTGCCGGCTT (-9 to +6)
GCE8	TCTGCTCTCATCCAACATTT (-1746 to -1726)	CACAGGCTTCTCTTGCCA (-403 to -386)
nlacZ456	GCGCACGGCGTTAAAGTTGTTCTG	CGCGCTGACTGGAGGCTGAA

The primers listed were used for cloning the human GC1 promoter fragments (GenBank accession number M92432) and for genomic DNA analysis. The GCE primers were used to amplify selected fragments of the human GC1 5' flanking region. The positions of the GCE1 and GCE7 sense primers were selected to generate two fragments containing intron 1 that differ only in the size of region upstream of exon 1. The positions of the GCE7 and GCE8 antisense primers were selected to amplify two promoter fragments that contain identical regions upstream of exon 2, but that carry or lack intron 1, respectively. The nlacZ456 primer set was used to amplify a 456 bp region of the nlacZ reporter gene from mouse genomic DNA.

tissue culture plate. The brain sections were stained with X-gal for 16-18 h, rinsed in PBS and viewed under a Zeiss Stemi SV6 dissecting microscope. Brightfield and fluorescence microscopy were performed as described previously [24].

Genomic PCR: Following X-gal staining, E18 retinal cross-sections (20 μ m) were microdissected under a Zeiss Stemi SV6 microscope using a fine razor blade shard fractured from a blade cooled in liquid nitrogen [25]. The cells in the ONL were cut away from the cells in the INL and GCL. Approximately five tissue fragments 5-7 mm in length (ONL only, INL/GCL only or all retinal layers) were cut from sections and collected into a 0.5 ml tube. For pineal gland analyses, pineal cross sections were scraped from the slides and collected into 0.5 ml tubes. Genomic DNA was extracted from these tissues by digestion with 70 μ l of proteinase K solution (Arcturus Picopure DNA Extraction Kit, Carlsbad, CA). Genomic DNA was isolated from fixed and X-gal stained flat mounted retinas using a Qiagen DNeasy kit. Integrated lentiviral vector transgenes were detected using PCR primers flanking a 456 bp region of the nlacZ gene as shown in Table 1. DNA (5 μ l from proteinase K samples and 100-200 ng DNA from fixed whole retinas) was amplified using Amplitaq Gold (Applied Biosystems, Foster City, CA) and the following cycle parameters: 95 °C for 3 min once; 95 °C for 1 min, 60 °C for 1 min, 72 °C for 30 s 30 times; 72 °C for 10 min once; 4 °C soak. Amplified products were resolved on 2.0% agarose gels.

RESULTS

Primary analyses of upstream regulatory sequences: The organization of the 5' UTR in the human retGC1 gene is summarized in Figure 1A and matches that of bovine retGC1 sequences (GenBank accession number AH005629). The gene contains a non-coding exon (Exon 1) that is interrupted by an intron, thereby placing the translation initiation site (ATG = +1 to +3) in Exon 2. Our retGC1 sequence was identical to the corresponding region of human chromosome 17p13 (GenBank accession number NT_010718) and the only other human retGC1 promoter sequence in GenBank (accession number AY037782). Comparisons of all three human sequences revealed that the AY037782 entry contains 500 bp of duplicated sequence located between nucleotides 1615 and 2113 that was not present in either our sequence or the genomic contig entry.

The human and bovine retGC1 5' UTRs share 48% identity and some conserved cis-elements. Two putative, head to tail cone-rod homeobox protein (CRX) binding elements (CBEs; consensus CTAATNAGCTY [26], shown in orange) were identified in the human retGC1 promoter, one between nucleotides -459 to -469 (CBE1) and one further upstream between nucleotides -1539 to -1549 (CBE2; Figure 1). The sequence and location of the downstream head to tail CBE is 100% conserved in the bovine retGC1 promoter, as is a putative Ret-1 binding site [27] between nucleotides -463 and -469, indicated by the green highlight in Figure 1B. Two additional inverted CRX/OTX-like binding sites were identified at positions -452 to -446 and -480 to -474 indicated by the brown highlights on Figure 1B. A putative transcription start

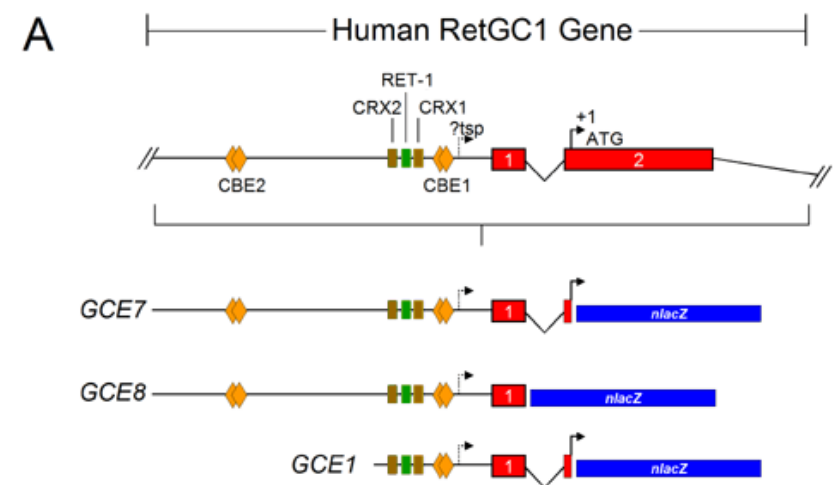
point (tsp) of the human retGC1 gene was identified (Eukaryotic Neural Network Promoter Prediction program) within Exon 1 at nucleotide -347 (score=1.00) just downstream of the site that corresponds to the empirically determined bovine tsp [28] (shown as the turquoise "A" at position -425 in Figure 1B). The tsp at -425 is associated with a putative transcription initiator (Inr) site (Figure 1B, shown in lowercase at position -421 to -427) [28]. Five 12 bp AT rich repeated elements (5'-TAT ATA ATT GCT-3') of unknown function were also identified further upstream (shown in purple in Figure 1B).

Expression of the human RetGC1 gene promoter in vivo:

We examined the cell specific and developmental activity of the human retGC1 promoter *in vivo*, taking advantage of the unique properties of a (SIN) lentiviral vector system [22,24]. The LTR regions of the SIN vector have been modified so that the viral promoter elements are lost following integration of the proviral SIN transgene into the host cell genome. This feature permits the analyses of the activities of promoters placed within the vector transgene without interference from viral promoter elements [24,29,30]. In addition, intron containing promoters remain intact in the packaged vector genomes and are retained in the integrated transgenes [30]. Activity data obtained from previous transient transfection assays of human retGC1 promoter constructs in WERI-Rb1 cells [31] were used as a guide to select the following promoter fragments for further study *in vivo*: (1) the proximal promoter including Intron 1 (GCE1), (2) the distal and proximal promoter including Intron 1 (GCE7), and (3) the distal and proximal promoter without Intron 1 (GCE8). The selected promoter fragments were cloned upstream of a nuclear localized β -galactosidase reporter gene (nlacZ) and were packaged into lentiviral vectors. The packaged virus was injected into the neural tubes of Hamberger-Hamilton stage 10-12 chicken embryos, and the activities of the various promoters were qualitatively monitored in retinas of embryos at selected stages of development using routine X-gal histochemistry and light microscopy.

Tissue specificity and relative strength of expression: In a previous study, we showed that the lentiviral vector system used in the current study is capable of achieving retinal transduction efficiencies of up to 80% following neural tube injection [22]. Although variable, the transduction efficiencies of all the promoter-nlacZ vectors were high enough to analyze the cell specificity of expression in clusters of nlacZ positive cells, an issue that was discussed in a previous study where we employed a similar experimental paradigm [24].

Additionally, we examined brain and pineal gland at E18 because lentiviral vectors injected into the neural tube also transduce these tissues [22]. None of the retGC1 promoters were active in brain at this age (data not shown). The GCE7 promoter was the only promoter that exhibited activity outside of the retina. Analyses of embryos injected with GCE7-nlacZ vector revealed that a small number of cells were positively stained in serial sections of the pineal gland. These cells were positioned near the lumens within the gland suggesting that the transgene was being expressed in pinealocytes (Figure 2A). In contrast, neither GCE1 nor GCE8 was active in



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NotI
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GCGGCCGCTC TGCTCCTCAT CCAACATTTC CCCAGCTTT AGAATCCACT
GATGATTCTT ACCTGATCCA ATCTTTGCCA CCAAGTCTGA AAATGATTCA -1655
TTTAAAAGTT TTTAAGTTTT ATTTTTCACA TATAGATCTT TGACCTGGAA
TAGATTTTGT GTATGGTGTG AGATAGGGAT TGAATTCTAT TTTCCCCCA -1555

CBE2
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GATGGCTAAT CAGCTCCATT TGTGAAGTT TATTCTTTC AAGTTGGTAA
GAAATGTCCA CGTCTCACA CATTTGCTGT GTTGTTTTCA GACTGTATA -1455
TTCTATTTC TGGTCCATT TGTTTATCCT TGTGCCAAA CTATGAAGTC
TTTATTGCCA TAGCTTTATA ATATATATTT ATATAAATAT ATAATTGCTG -1355
TCACTACATA ATGTGGATT ATATAAATAT ATAAATATATA ATTGCTATAG
CTTTATAGTA TATATTATATA TAATTGCTGT TTTATATATC ATATAATTAT -1255
ATAAAATATAT AATTGCTATG TTTTATATAT TATATATTTA TGTAGATATA -1155
TAATGCTAT GTTTTATATA TTATATATTT ACATAAATAC ATAATTGCTA
TTGCTTTATA ATAAATTTGT ATATCTTATA GGGCATCTTC CATTATACTC -1055
TTCTCTAAA TTGTTAGCTA TTCTTGCCAT CCGGGCGATT ATATTCATTT
TCACAACAAC CCTCAGATTA AGCAATTGCC CAAGTCCAA AAATCAGCAA -955
GAGGACTTG GAACCCAGGT CTGTCCGAGG CCAAAGCTCT TTTCATTACT
TCTTGAGGGT GGTTTCTAG GCATGGAGAA GCAGAGGTCA GGAATCAAG
TGTGGCGAGA GAGAGAAGAG AAGTGAAGA AGAARGGCAG GTGTCAGCTT -855
GGTGTGGGT TGGTCTCTGG GATATAGACT TTGCCAGCCA ATAATTGGAG
CTTGAACTTA GCCGGCAGAA CTGGAACAG AAGATTGTAA GGAAAGGGAC -755
TGGGATCAGT GTTCTTCTC CAGGACGGAT TACCACAGC TGTCCACGGG
CAGGCACTTG TTACTTCTG GCTGAGCAGG GCAGTGTGGC CGACGGCTGA -655
AAGGGGAAGC TGCGGCTGCT TTTGCGCAGG GGTGGTGGTG ATGAGGGTGA
TGTGGGGGGC TGGAAGGCAT GGAGGGGAAA GGATCTGGCT GACTACCTGG -555
AAGCCAGGAC AGATCCCACC CCAGAAAGGC GCAGTAGGGG CTCTCATCCT

CBE1
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CCACTAGCCC GCCCTCCCT ACCTAATTAA GGACCCTAAT CAGCTTTGGG -455
GAGATTAAGG GCTCTGGCC GCTGTACCcA cgccCCCGCC CTGGCCTGGG
CTGGCAAGGA AGACCTGTGG GCGGGCGTCA AAAGGGGGAC CGGCCCTGTG -355
ACCCCTCACC GGGCCGTGG GCCCGAGCCC CGGACTTCCC TGTAAGTGTG
AGAGCCCCCT CCGCTGGGAT AGGGTCGGTC TGAGGGCGCA GGCGAGTCCC -255
TGCTGACCCC TGACGCCTC GACGGGGGGA GGGCAGGCC GGGTGGGAGC
GGGAAGCCGG GCGGCAGAA GGGGGCTTCG GGGCGGTGTC CTTGGCCCCA -155
GTTAGTCTTC CCAGCTCCG GAGGGGGCGG TAGCAGCAGA ATCATCCAT

GGGTTACTCG GGCTTGAGA AACTCGGGGT TACGGGGAGA ACCCTAGGGG -55
AGGCCGGGGT CTCAGTCGT CAGCCTGCTC CGTCTGTGTT CGCAGAAAGCC
+1 PmeI nlacZ
| -----
GGCAATGACC GTTTAAACTT AAGCTTCCAC CATGCCTAAG AAGAAACGAA

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Figure 1. Schematics and sequence of the human retGC1 promoter-nlacZ transgenes used in the lentiviral vectors. **A:** Schematics showing the exon-intron structure and location of putative cis-elements in the human retGC1 5' flanking sequence and the lentiviral vector transgene constructs that were used to generate virus. Exons are shown as red rectangles surrounding intron 1 (bent line). The nlacZ open reading frame is shown as a blue rectangle. ATG represents the translation start site (arrow denotes the position of A = +1); "?tsp" marks a putative transcription start point (arrow denotes the position of first "A" that aligns with the "A" in the bovine GC1 tsp; see text and **B**, below); brown rectangles illustrate putative inverted cone-rod homeobox (CRX) protein binding sites; orange diamonds refer to putative head to tail CRX binding elements (CBEs); the green rectangle represents a putative Ret-1 binding site. The color coding and nucleotide positions are uniform across panels and figures. **B:** Sequence showing the entire GCE7 promoter fragment (which also contains the GCE1 and GCE8 fragment sequences) and the point of fusion with the nlacZ gene in the lentiviral vector constructs. The **turquoise A** at position -425 indicates the putative tsp identified by aligning the human and bovine retGC1 promoter sequences (GenBank accession number U77096). The Inr-like sequence that is also found in the bovine retGC1 5' flanking region is shown in lowercase [28]. The putative inverted CRX binding elements and Ret-1 binding element are highlighted in **brown** and **green**, respectively. The AT rich repeats are shown in **purple**. Exon 1 and exon 2 sequences flanking intron 1 are shown in **red**. The red line (|) indicates the translation start point of human retGC1 (ATG, where the A is defined as position +1); the sequence at the start of the nlacZ open reading frame is in **blue**. Vector and restriction enzyme cloning sequences are in **dark purple**.

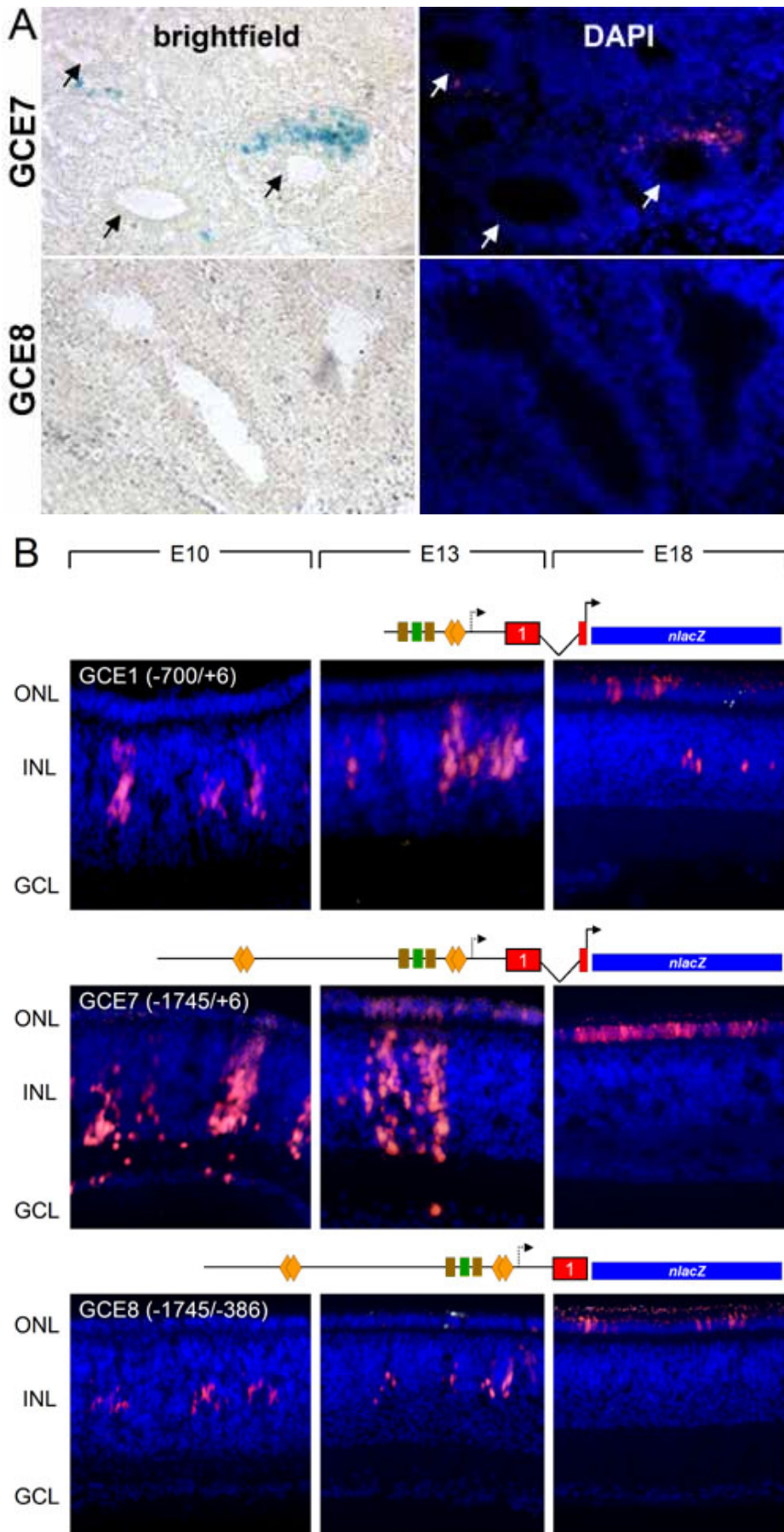


Figure 2. Cell specific and developmental activity of human retGC1 promoters. **A:** Brightfield (left panels) and brightfield-DAPI merged images (right panels) of cross sections of embryonic age (E) 18 pineal glands transduced with either the GCE7-*nlacZ* (GCE7) or the GCE8-*nlacZ* (GCE8) lentiviral vector. Arrows indicate lumens in the field of view with pinealocytes positioned around the perimeter. X-gal staining is shown as blue under brightfield optics (left panels) or red in the negative image overlaid with the DAPI image to show cell positions (right panels). **B:** Brightfield-DAPI merged images showing cross sections of retinas taken from stage 10-13 embryos injected with lentiviral vectors containing the GCE1, GCE7 or GCE8 promoter-*nlacZ* transgenes (shown schematically above the image panels) and harvested at E10, E13, or E18. X-gal staining is red (*nlacZ* positive cells) and DAPI staining is blue (cell nuclei). The outer nuclear layer (ONL), inner nuclear layer (INL), and ganglion cell layer (GCL) are labeled. The vector diagrams are described in Figure 1A.

the pineal gland even though integrated GCE1-nlacZ and GCE8-nlacZ transgenes were present in these tissues.

Upon visual inspection of the retinas, the intensity of X-gal staining of cells transduced by the GCE7 vector (6 retinas) appeared to be greater than the intensity of cells transduced with the GCE8 promoter (6 retinas; Figure 2B).

Temporal dynamics and cell specificity of nlacZ expression in developing retina: In this series of experiments we characterized the developmental regulation and cell specificity of the activities of the GCE1, GCE7 and GCE8 retGC1 promoter fragments. We have previously shown that the expression of retGC1 in developing chicken retina begins at approximately E13-E14 [24]. Using this time frame as a guide, retinas of injected embryos were collected at times prior to (E10), at (E13), or following (E18) the onset of retGC1 expression in vivo to examine the activities of the retGC1 promoter fragments over the course of retinal development.

A small number of nlacZ positive cells were occasionally detected in the inner nuclear layer (INL) of retinas of E10 and E13 embryos that had been injected with the GCE1-nlacZ vector (Figure 2B, top panel). By E18, a significant number of nlacZ positive cells were also detected in the outer nuclear layer (ONL) of these embryos. These data show that GCE1 is active in photoreceptor cells at E18 and retains its ability to drive transgene expression in cells located in the INL at this late stage in development.

In contrast to the GCE1 results, several nlacZ positive cells were detected in the retinas of E10 and E13 embryos that had been injected with GCE7-nlacZ vector (Figure 2B, middle panel). At these early stages of development, GCE7 exhibited

activity in all cell layers of the retina. By E18, nlacZ expression driven by GCE7 was restricted to the photoreceptor cells, a pattern of expression resembling that of the native retGC1 promoter.

In retinas of E10 and E13 embryos that had been injected with GCE8-nlacZ vector, the expression of nlacZ was restricted to cells located within the central region of the INL and to the cells within the ONL (Figure 2B, bottom panel). The pattern of expression resembled that of GCE7 at E18, but the number of nlacZ stained cells in the ONL was much lower.

Silencing of retGC1 promoter activity in vivo: We used genomic PCR in order to verify that the absence of GCE7 expression in non-photoreceptor cells was not merely due to the absence of the transgene in these cells. The ONL of E18 GCE7-nlacZ retinas was separated from the INL and GCL using microdissection techniques described in the Methods section. Genomic DNA was prepared from the cells in these layers and PCR was used to detect the presence of the integrated nlacZ transgene. The results of these analyses showed that integrated GCE7-nlacZ transgene was present in both nlacZ positive and nlacZ negative cell layers (Figure 3). These results, together with the nlacZ staining pattern, support the conclusion that GCE7 is only active in photoreceptor cells at E18 and that it is actively silenced in the INL and GCL at this stage of development.

DISCUSSION

In this study, we have characterized the in vivo activity profiles of selected fragments of the human retGC1 5' flanking region using a viral vector mediated gene transfer method that we have successfully used in studies of mouse and chicken photoreceptor gene promoters [24]. The results of our analyses show that a 1.4 kb fragment of the retGC1 promoter contains distinct elements that drive its activity, control its expression during retinal development, and limit its expression to specific retinal cell types in vivo.

The chicken embryo is an excellent model system for developmental analyses of gene expression in the retina. Its superiority as a model system is easily appreciated if one considers the availability of fertile eggs, the accessibility of the developing embryo, and the striking parallels between the morphogenesis of avian embryos and those of higher vertebrates including humans. Additionally, the recent completion of the chicken genome [32] and the successful use of viral vectors to facilitate overexpression or misexpression of various genes in the chick, the chicken embryo continues to remain on the forefront of the field of developmental biology. In chicken retina, photoreceptor cells are fully developed and functional by E18, a time just prior to hatching. The results of our in vivo experiments show that the activities of the GCE7 and GCE8 retGC1 promoter fragments are limited to the photoreceptor cells in the functionally mature retina (E18), a pattern identical to that observed for retGC1 in mature human and chicken retinas [9]. The presence of a second putative CBE in the distal promoter region (-1539 to -1549, relative to the ATG) of the longer promoter fragments could contribute to the increased cell specificity exhibited by these fragments. The CBE is found in other

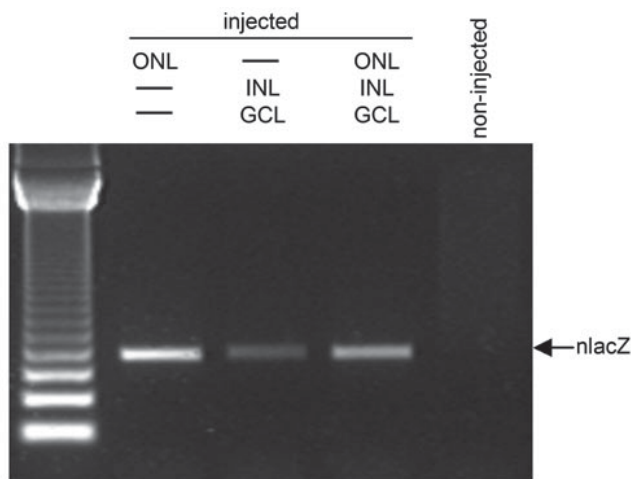


Figure 3. GCE7 promoter activity is silenced in non-photoreceptor cells at E18. PCR amplification of a 456 bp region of the nlacZ gene from genomic DNA extracted from the separated outer nuclear layer (ONL), the remaining inner nuclear layer (INL) and ganglion cell layer (GCL), or an intact retinal section containing all three layers. The cell layers included in the DNA extraction are indicated above the gel lanes. A 123 bp DNA ladder was run in the leftmost lane of the gel and amplified DNA from a non-injected control retina was run in the rightmost lane.

photoreceptor gene promoters [26] and serves as a binding site for CRX, a photoreceptor specific transcription factor that plays an important role in the transactivation of multiple photoreceptor specific and pineal specific genes, including itself [26,33]. The presence of additional Ret-1 and multiple, inverted CRX/OTX-like binding sites in the proximal promoter region suggests that cooperative binding of several retina specific transcription factors may be involved in the regulation of human retGC1 gene expression. Deletion analyses of one or combinations of these cis-elements may elucidate the putative role of CRX and other factors in regulating retGC1 expression. The inclusion of Intron 1 appears to augment the activity of the retGC1 promoter and it is possible that its positive effect is related to general cellular mRNA processing and export functions.

Interestingly, our data suggest that the expression pattern in mature retina represents the culmination of a series of developmental changes in the regulation of these promoters that effectively restrict their activation to specific cell types. Both GCE7 and GCE8 exhibited activity in the developing retina as early as E10, the activity being highest in non-photoreceptor cells. The onset of expression of these promoters was slightly earlier than that observed for the retGC1 gene in normal developing retina (about E13) [24]. One possible explanation for this observation is that the GCE7 and GCE8 promoter fragments do not contain cis-elements present in the native retGC1 promoter that actively suppress expression of this gene in non-photoreceptor cells during development. It is also possible that the ubiquitous cellular expression pattern exhibited by the GCE7 fragment at E13 reflects the normal expression pattern of retGC1 in developing retina, which is not yet known. If this scenario was found to be true by immunohistochemical studies of developing retina, our data would suggest that elements within GCE7 and GCE8 interact with trans-acting factors as the retina matures to actively silence transcriptional activity in non-photoreceptor cells.

Our genomic PCR data from nlacZ positive and nlacZ negative cells in the E18 retinas are consistent with the notion that silencing mechanisms play a role in establishing the retGC1 expression pattern that is observed in fully developed retina. The absence of GCE7 activity in the INL and GCL at E18 could be due to silencing effects mediated either by the onset of expression of trans-acting inhibitory factors or by the suppression of expression of activating factors in these cells (Figure 3). Tissue restrictive cis-elements such as the neuron restrictive silencer element were not found in the human retGC1 promoter, but such elements have been identified in the promoters of several neural specific genes [34,35]. Our observations that the human retGC1 promoter fragments functions as expected in the fully developed chicken retina and that the bovine and human retGC1 5' flanking sequences are highly homologous suggest that the cis-elements that are required for the cell specific expression of this gene are highly conserved across vertebrates. The results of our studies support further investigation of the mechanisms controlling expression of retGC1 in normal developing and mature retina, the results of which may reveal a previously unappreciated

pattern of gene regulation. Overall, our study lays the groundwork for targeting the expression of retGC1 transgenes in animal models of this disease [20,21] and should enhance our efforts to develop an effective, long term therapy for LCA1.

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REFERENCES

- Lucas KA, Pitari GM, Kazerounian S, Ruiz-Stewart I, Park J, Schulz S, Chepenik KP, Waldman SA. Guanylyl cyclases and signaling by cyclic GMP. *Pharmacol Rev* 2000; 52:375-414.
- Arshavsky VY, Lamb TD, Pugh EN Jr. G proteins and phototransduction. *Annu Rev Physiol* 2002; 64:153-87.
- Stryer L. Cyclic GMP cascade of vision. *Annu Rev Neurosci* 1986; 9:87-119.
- Dizhoor AM. Regulation of cGMP synthesis in photoreceptors: role in signal transduction and congenital diseases of the retina. *Cell Signal* 2000; 12:711-9.
- Polans A, Baehr W, Palczewski K. Turned on by Ca²⁺! The physiology and pathology of Ca(2+)-binding proteins in the retina. *Trends Neurosci* 1996; 19:547-54.
- Liu X, Seno K, Nishizawa Y, Hayashi F, Yamazaki A, Matsumoto H, Wakabayashi T, Usukura J. Ultrastructural localization of retinal guanylate cyclase in human and monkey retinas. *Exp Eye Res* 1994; 59:761-8.
- Lowe DG, Dizhoor AM, Liu K, Gu Q, Spencer M, Laura R, Lu L, Hurley JB. Cloning and expression of a second photoreceptor-specific membrane retina guanylyl cyclase (RetGC), RetGC-2. *Proc Natl Acad Sci U S A* 1995; 92:5535-9.
- Yang RB, Foster DC, Garbers DL, Fulle HJ. Two membrane forms of guanylyl cyclase found in the eye. *Proc Natl Acad Sci U S A* 1995; 92:602-6.
- Cooper N, Liu L, Yoshida A, Pozdnyakov N, Margulis A, Sitaramayya A. The bovine rod outer segment guanylate cyclase, ROS-GC, is present in both outer segment and synaptic layers of the retina. *J Mol Neurosci* 1995; 6:211-22.
- Dizhoor AM, Lowe DG, Olshevskaya EV, Laura RP, Hurley JB. The human photoreceptor membrane guanylyl cyclase, RetGC, is present in outer segments and is regulated by calcium and a soluble activator. *Neuron* 1994; 12:1345-52.
- Yang RB, Garbers DL. Two eye guanylyl cyclases are expressed in the same photoreceptor cells and form homomers in preference to heteromers. *J Biol Chem* 1997; 272:13738-42.
- Semple-Rowland SL, Larkin P, Bronson JD, Nykamp K, Streit WJ, Baehr W. Characterization of the chicken GCAP gene array and analyses of GCAP1, GCAP2, and GC1 gene expression in normal and rd chicken pineal. *Mol Vis* 1999; 5:14 .

13. Venkataraman V, Nagele R, Duda T, Sharma RK. Rod outer segment membrane guanylate cyclase type 1-linked stimulatory and inhibitory calcium signaling systems in the pineal gland: biochemical, molecular, and immunohistochemical evidence. *Biochemistry* 2000; 39:6042-52.
14. Yang RB, Fulle HJ, Garbers DL. Chromosomal localization and genomic organization of genes encoding guanylyl cyclase receptors expressed in olfactory sensory neurons and retina. *Genomics* 1996; 31:367-72.
15. Perrault I, Rozet JM, Calvas P, Gerber S, Camuzat A, Dollfus H, Chatelin S, Souied E, Ghazi I, Leowski C, Bonnemaïson M, Le Paslier D, Frezal J, Dufier JL, Pittler S, Munnich A, Kaplan J. Retinal-specific guanylate cyclase gene mutations in Leber's congenital amaurosis. *Nat Genet* 1996; 14:461-4.
16. Perrault I, Rozet JM, Gerber S, Ghazi I, Ducroq D, Souied E, Leowski C, Bonnemaïson M, Dufier JL, Munnich A, Kaplan J. Spectrum of retGC1 mutations in Leber's congenital amaurosis. *Eur J Hum Genet* 2000; 8:578-82.
17. Duda T, Krishnan A, Venkataraman V, Lange C, Koch KW, Sharma RK. Mutations in the rod outer segment membrane guanylate cyclase in a cone-rod dystrophy cause defects in calcium signaling. *Biochemistry* 1999; 38:13912-9.
18. Duda T, Venkataraman V, Jankowska A, Lange C, Koch KW, Sharma RK. Impairment of the rod outer segment membrane guanylate cyclase dimerization in a cone-rod dystrophy results in defective calcium signaling. *Biochemistry* 2000; 39:12522-33.
19. Kelsell RE, Gregory-Evans K, Payne AM, Perrault I, Kaplan J, Yang RB, Garbers DL, Bird AC, Moore AT, Hunt DM. Mutations in the retinal guanylate cyclase (RETGC-1) gene in dominant cone-rod dystrophy. *Hum Mol Genet* 1998; 7:1179-84.
20. Semple-Rowland SL, Lee NR, Van Hooser JP, Palczewski K, Baehr W. A null mutation in the photoreceptor guanylate cyclase gene causes the retinal degeneration chicken phenotype. *Proc Natl Acad Sci U S A* 1998; 95:1271-6.
21. Yang RB, Robinson SW, Xiong WH, Yau KW, Birch DG, Garbers DL. Disruption of a retinal guanylyl cyclase gene leads to cone-specific dystrophy and paradoxical rod behavior. *J Neurosci* 1999; 19:5889-97.
22. Coleman JE, Huentelman MJ, Kasparov S, Metcalfe BL, Paton JF, Katovich MJ, Semple-Rowland SL, Raizada MK. Efficient large-scale production and concentration of HIV-1-based lentiviral vectors for use in vivo. *Physiol Genomics* 2003; 12:221-8.
23. Hamburger V, Hamilton HL. 1951. A series of normal stages in the development of the chick embryo. *J Morphol* 88: 49-92.
24. Coleman JE, Fuchs GE, Semple-Rowland SL. Analyses of the guanylate cyclase activating protein-1 gene promoter in the developing retina. *Invest Ophthalmol Vis Sci* 2002; 43:1335-43.
25. Semple-Rowland SL, Lee NR. Avian models of inherited retinal disease. *Methods Enzymol* 2000; 316:526-36.
26. Livesey FJ, Furukawa T, Steffen MA, Church GM, Cepko CL. Microarray analysis of the transcriptional network controlled by the photoreceptor homeobox gene Crx. *Curr Biol* 2000; 10:301-10.
27. Morabito MA, Yu X, Barnstable CJ. Characterization of developmentally regulated and retina-specific nuclear protein binding to a site in the upstream region of the rat opsin gene. *J Biol Chem* 1991; 266:9667-72.
28. Johnston JP, Farhangfar F, Aparicio JG, Nam SH, Applebury ML. The bovine guanylate cyclase GC-E gene and 5' flanking region. *Gene* 1997; 193:219-27.
29. Ginn SL, Fleming J, Rowe PB, Alexander IE. Promoter interference mediated by the U3 region in early-generation HIV-1-derived lentivirus vectors can influence detection of transgene expression in a cell-type and species-specific manner. *Hum Gene Ther* 2003; 14:1127-37.
30. Zaiss AK, Son S, Chang LJ. RNA 3' readthrough of oncoretrovirus and lentivirus: implications for vector safety and efficacy. *J Virol* 2002; 76:7209-19.
31. Fülle H-J, Gallardo EM. Organization of two human retinal guanylyl cyclase genes: isolation analysis and comparison of upstream regulatory elements. *Invest Ophthalmol Vis Sci* 2001; 42:S353.
32. Burt DW. The chicken genome and the developmental biologist. *Mech Dev* 2004; 121:1129-35.
33. Furukawa A, Koike C, Lippincott P, Cepko CL, Furukawa T. The mouse Crx 5'-upstream transgene sequence directs cell-specific and developmentally regulated expression in retinal photoreceptor cells. *J Neurosci* 2002; 22:1640-7.
34. Mori N, Schoenherr C, Vandenberg DJ, Anderson DJ. A common silencer element in the SCG10 and type II Na⁺ channel genes binds a factor present in nonneuronal cells but not in neuronal cells. *Neuron* 1992; 9:45-54.
35. Schoenherr CJ, Paquette AJ, Anderson DJ. Identification of potential target genes for the neuron-restrictive silencer factor. *Proc Natl Acad Sci U S A* 1996; 93:9881-6.