Analyses of the Guanylate Cyclase Activating Protein-1 Gene Promoter in the Developing Retina

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PURPOSE. To determine the activity, cell specificity, and developmental expression profiles of fragments of the chicken guanylate cyclase activating protein (GCAP)-1 promoter.

METHODS. The intrinsic activities of five GCAP1 promoter-luciferase constructs were measured in transiently transfected primary chicken embryonic retinal cultures. Lentivirus vectors carrying GCAP1 promoter-nlacz transgenes were used to examine the cell specificities and temporal expression characteristics of selected promoter fragments in developing retina.

RESULTS. Three of the five GCAP1 promoter fragments exhibited significant activity in vitro. The expression characteristics of the promoter fragments in vivo varied as a function of promoter length. Expression of nlacz driven by the 0.6- and 1.7-kb GCAP1 promoter fragments was first observed on embryonic day (E)12 and was restricted to the inner nuclear layer (INL). By E16, nlacz staining was also detected in the outer nuclear layer (ONL). Expression of nlacz driven by the 4.2-kb GCAP1 promoter fragment was not observed until E16 and was restricted to the ONL.

CONCLUSIONS. The general organization of regulatory cis elements within the GCAP1 promoter is different from other photoreceptor-specific gene promoters. Elements located within 0.3 kb upstream of the transcription start point are capable of producing efficient gene expression; however, additional elements located within 4.0 kb upstream of the transcription start point are necessary to confer on the fragment the cell specificity and developmental expression characteristics of the native GCAP1 promoter. The results of the current study show that the lentiviral vector system is a useful tool for the characterization of the promoters of genes expressed in neural retina. (Invest Ophthalmol Vis Sci. 2002;43:1335–1343)

Guanylate cyclase activating protein (GCAP)-1 is an EF-hand calcium-binding protein that activates photoreceptor guanylate cyclase (GC)-1 under low intracellular calcium conditions, thereby hastening the recovery phase of phototransduction.1–3 The expression of GCAP1 and GC1 in vertebrate retina is limited to cone and rod photoreceptor cells, a distribution that is consistent with their roles in phototransduction.4–10 Within photoreceptor cells, GCAP1 is localized to the inner and outer segments and synaptic regions and appears to be expressed at higher levels in the cone cells of human, monkey, and bovine retinas.11,12 The expression of GCAP1 has also been detected in the pineal glands of cow and chicken.13,14 Studies of the interactions of GCAP1 with GC1 suggest that these proteins exist in photoreceptors as a stable complex independent of intracellular calcium concentrations and that activation of GC1 occurs as a result of a calcium-dependent conformational change in the complex.8,15–18 Although at least three variants of GCAP are expressed in the retina (GCAP1–3),5,10,19,20 recent studies of GCAP1/2-knockout mice suggest that only GCAP1 is capable of restoring normal light response kinetics to photoreceptor cells (W. Baehr, written communication, December 2001, and Ref. 21). The current view that GCAP1 is essential for normal phototransduction is supported by the observation that missense mutations in the GCAP1 gene (Y99C and E155G) have been linked to autosomal dominant cone dystrophy in humans.22–24 These mutations, which interfere with the binding of calcium to GCAP1, lead to persistent activation of GC1, even under high-calcium conditions.24–26 These results clearly indicate that GCAP1 plays a pivotal role in phototransduction and retinal disease. Therefore, it is of interest to understand how the expression of GCAP1 is regulated in developing and mature retina.

In retinal photoreceptors, the magnitudes, cellular specificities, and temporal dynamics of expression of several photoreceptor-specific genes are regulated at the transcriptional level. The intrinsic activities and cellular specificities of these genes can be attributed to complex interactions between cis-acting regulatory elements within their promoters and the cell-specific transcription factors that interact with them. The onset of expression of these genes in developing retina has also been shown to be dependent on the interactions between promoter cis elements and transcription factors and is often linked temporally to the differentiation and maturation of the photoreceptor cells.27–35 Few studies have been undertaken to examine the activities of photoreceptor-specific promoters in developing retina in vivo.30,33–36 Recently, the importance of correct temporal regulation of gene expression in developing retina has been clearly demonstrated in studies of cone–rod homeobox (CRX)34,37 and neural retina leucine zipper58 knockout mice. The results of these studies show that the absence of expression of these key trans-acting factors in retina results in the downregulation of expression of several photoreceptor-specific genes and abnormal development and function of the photoreceptor cells.

In this series of experiments, we examined the expression characteristics of fragments of the chicken GCAP1 promoter, both in vitro and in vivo, with the purpose of identifying regions of the promoter that play a role in regulating the activity, cell specificity, and developmental expression of GCAP1. Our previous analyses of the sequence of the 5′ flank region of this gene15 served as a guide for the selection of the GCAP1 promoter fragments that were analyzed in these experiments. The intrinsic activities of these fragments were determined by measuring the expression levels of GCAP1-luciferase fusion constructs in transiently transfected primary embryonic chicken retinal cultures, which have been used to characterize the activities and cell specificities of the promoters of photoreceptor-specific genes obtained from a variety of species.39–41 We used lentiviral vectors as a novel tool to...
extend the in vitro analyses of promoter function to the in vivo environment of the developing chicken retina. Lentiviruses pseudotyped with vesicular stomatitis virus glycoprotein (VSV-G) are ideal vectors for this type of analysis, because they are capable of transducing several different cell types, exhibit rapid integration and expression of transgenes in transduced cells,12 and have a large cargo capacity (>18 kb).13 The cell specificity and the onset of the activity of selected GCAP1 promoter fragments in developing retina was assessed in vivo by monitoring the activity of GCAP1 promoter-nlacZ transgenes in the retinas of animals that had received injections of lentivirus carrying these transgenes before the development of the neural retina. The onset of expression of each of the GCAP1 promoter-nlacZ transgenes in developing retina was compared with the expression profiles of the GCAP1 and GC1 genes in normal, developing chicken retina.

METHODS

Northern Blot Analyses

Embryonic retina, pigmented epithelium, and choroid tissues were isolated using an RNA extraction kit (RNeasy Total RNA; Qiagen, Valencia, CA). Samples containing 10 µg RNA were electro-phoresed on a 1.1% formaldehyde gel and transferred to a nylon transfer membrane (Micron Separation, Inc., Westborough, MA). Northern blots were hybridized consecutively with radiolabeled cDNA probes specific for GCAP1, GC1, and iodopsin, as previously described.33 The GCAP1 and iodopsin results were confirmed by repeating the analyses on a second series of independent samples. Blots were exposed to film (BioMax; Eastman Kodak, Rochester, NY) for 12 to 16 hours at −80°C, and the resultant hybridization signals were imaged using a commercial system (Gel Doc 1000; Bio-Rad, Hercules, CA). The 18S rRNA was visualized by staining the blot with methylene blue.

Preparation of Constructs

The GCAP1 promoter fragments were amplified from appropriate regions of the GCAP1 cosmid clones, ccos16 and ccos24,17 using polymerase chain reaction and Pfu DNA polymerase (Stratagene, La Jolla, CA). For each of the GCAP1 promoter fragments, unique upstream primers containing a NotI site were used in combination with three different sets of downstream primers containing a Pmel site to generate the following fragments (transcription start point, +1) fragment 1, −292/−302; fragment 2, −1436/+302; fragment 3, −3121/+222; fragment 4, −4009/+222; and fragment 5, −1434/+29 (see Fig. 2A). In this study, these fragments were referred to as 292, 1436, 3121, 4009, and 1434, based on the position of the 5′ nucleotide. The sequence-specific (GenBank AF172707; GenBank is provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD, and is available at http://www.ncbi.nlm.nih.gov/genbank) primers used to amplify the fragments were as follows: 292 (sense, 5′-ACC CGT GGT CTT TTC; antisense, 5′-GCT GCA GTC ACT CTG-3′); 1436 (sense, 5′-ACC CGA CTC CTT CAA; antisense, same as 292); 3121 (sense, 5′-AAT CCT GCC CAT CAC TGC CCT ATC; antisense, same as 292); 4009 (sense, 5′-GGG CGA AAT GTA AAA-3′); and 1434 (sense, 5′-AAT CCT GCC CAT CAC TGC CCT ATC; antisense, same as 3121); and 1434 (sense, 5′-AAT CCT GCC CAT CAC TGC CCT ATC; antisense, same as 3121) and 1434 (sense, 5′-ACC CGA CTC CTT CAA; antisense, 5′-CGG GCA AAT GTA AAA GC). Products from the polymerase chain reaction were subcloned into a vector (pCR-TOPO-blunt II; Invitrogen, Carlsbad, CA) and the GCAP1 promoter fragments were excised from the pCR-TOPO-blunt II clones using NotI and Pmel and were ligated into the appropriate vectors. For the in vitro activity assay constructs, the multiple cloning site (MCS) of the PGGL vector (Promega, Madison, WI) was modified by ligating the SacI/XboI fragment of the MCS of pBluescript II SK (Stratagene) vector into the MCS of the PGGL vector. The modified vector was then digested with XhoI, blunt ended, and digested with NotI, and the NotI-Pmel GCAP1 promoter fragments were ligated into the vector. For the in vivo lentiviral constructs, NotI-Pmel fragments were ligated into pTY-nlacZ digested with NotI and Pmel. The marine interphotoreceptor retinoid-binding protein (IRBP) promoter (mIRBP1783), which included nucleotides −1783 to +101, was amplified from the pIRBP1783-EGFP plasmid vector, by PCR. The same cloning strategy described for the GCAP1 promoter constructs was used to generate PGGL and lentivirus pTY-nlacZ plasmid vectors containing the mIRBP1783 promoter. Transfection-grade DNA was prepared for each construct (Endotoxin-free Plasmid Maxiprep; Qiagen).

Cell Cultures and Transfections

Dispersed embryonic day (E)12 chick retinal cultures were prepared and transiently transfected, essentially as previously described.39–41 Isolated neural retina was incubated in 0.25% trypsin at 37°C for 20 minutes, dispersed by trituration with a flame-narrowed glass pipette, and plated at a density of 2 × 10⁵ cells/well in 24-well culture plates that had been coated with poly-l-ornithine (Sigma, St. Louis, MO). Cultures were maintained in basal medium of eagle (Life Technologies, Rockville, MD) supplemented with 5 g/L glucose, 10% fetal bovine serum, and antibiotics at 37°C in 5% CO₂. Cells were transfected the day after seeding by the calcium phosphate method. Briefly, 10 µg of promoter vector DNA and 0.5 µg of control vector DNA containing the nlacZ reporter gene driven by the CMV promoter were added to 125 µL 0.2 M CaCl₂. Next, 125 µL 2× HEPES-buffered saline was added in drops to the DNA/CaCl₂ mixture. The transfection mixture was allowed to incubate for 20 minutes at room temperature and then 62.5 µL of the transfection mixture was added to each well. Cells were incubated overnight at 37°C in 5% CO₂ and rinsed three times with PBS the following day. The transfection experiments were replicated four times, and a new preparation of cultured cells was used for each experiment (n = 4). Within each experiment, transfection of each promoter construct was performed in duplicate, with the same transfection mixture. The photoreceptor-specific mIRBP1783 promoter was used as a positive control in all experiments.40,45–50

Luciferase and β-Galactosidase Assays

Cell lysates from the E12 primary retinal cultures were prepared 40 to 48 hours after transfection by adding 200 µL lysis buffer (provided in the assay kits described later) to each well, scraping the cells using a rubber policeman, and processing the lysates for the luciferase or β-galactosidase chemiluminescent assays according to the manufacturer’s protocols (Galacto-star or Luciferase Assay Kits; Tropix, Bedford, MA). Luciferase and β-galactosidase activities were measured in 20 and 40 µL aliquots of each lysate, respectively. Assays were run in duplicate and quantified using a luminometer (TD20/20; Turner Designs, Sunnyvale, CA) with an integration time of 10 seconds. Activity data were corrected for transfection efficiency across experiments by normalizing luciferase levels to β-galactosidase levels. Promoter activity was expressed as the fold activity over the promoterless PGGL vector. Data were analyzed using one-way repeated-measures ANOVA, and post hoc pair-wise comparisons were performed using the Student-Newman-Keuls post hoc test (SigmaStat; SPSS Sciences, Chicago, IL).

Lentivirus Production

Viruses pseudotyped with VSV-G were prepared using a self-inactivating lentiviral vector system.31 Packaging cells (293T) were plated in 10-cm culture dishes at a density of 6 × 10⁶ cells/dish in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum, and antibiotics (Life Technologies). The 293T cells were grown to 80% to 90% confluence and were then transiently transfected with 6 µg pTY-nlacZ (a transgene-carrying vector), 12 µg pHP (a packaging vector), 5.5 µg pHEF (encodes the VSV-envelope), and 0.5 µg pCEP4-tat (encodes tat protein) per dish, using transfection reagent (Superfect; Qiagen). All four of the plasmids were added to 300 µL DMEM, and the DNA mixture was vortexed and incubated at room temperature for 5 minutes. The transfection reagent (50 µL) was added
to the DNA mixture, which was then vortexed and incubated at room temperature for an additional 5 minutes. During this time, the medium was removed from the cells and replaced with 4.5 mL fresh medium. The transfection mixture was added in drops to the cultures, which were incubated at 37°C in 5% CO₂ for 3 to 4 hours. After the incubation period, the cells were rinsed one time with medium. Fresh medium (6 mL) was again added to the cells, and the cells were incubated overnight. The next day, the medium was removed and 6 mL fresh medium was added to the cells. The medium containing the virus was harvested 48 and 72 hours after transfection and frozen at −80°C until concentration. To concentrate the virus, the medium was rapidly thawed and passed through a 0.45-μm low-protein binding filter (Du-rapore; Millipore, Bedford, MA) to remove cell debris. The filtered medium containing the virus was concentrated 140-fold by ultracentrifugation at 20,000g for 2.5 hours at 4°C. The virus pellet was resuspended by gentle shaking at 4°C for 4 hours, aliquoted, and stored at −80°C until use. Infectious titers of virus were determined by infecting 4 × 10⁶ T5671 cells seeded in 24-well plates with limiting dilutions of TY-EF1α-nlacZ virus in the presence of 8 μg/mL of infection-transfection reagent (Polybrene; Sigma). After 3 to 4 hours of infection, fresh medium was added to the cells. After 48 hours, the cultures were stained with 5-bromo-4-chloro-3-indolyl-β-d-galactosidase (X-gal) substrate, as previously described.⁵² Virus titer was determined by counting the number of blue-nucleated cells and infectious titers were expressed as the number of transducing units per milliliter (TU/mL). Particle titers were determined using a p24 ELISA kit obtained from SAIC (Frederick, MD), according to the protocols provided. Infectious titers of virus carrying tissue-specific promoters were estimated by multiplying the particle titers of the tissue-specific promoter-containing viruses by the infectious titer-to-particle titer ratio obtained for the TY-EF1α-nlacZ virus. The titers of all virus preparations were approximately 1 × 10⁷ TU/mL.

**Embryonic Injections**

All animals were handled according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Chicken eggs were set on day 0 and incubated on their sides without rotation at 37.5°C in 60% humidity. Viral injections were performed on Hamberger-Hamilton stage 10 to 12 embryos (~E2). A small opening was made in the eggshell overlying the embryo, the position of which was determined using an egg-candling light. With the aid of a dissecting microscope, injection of the virus into the ventricular space of the neural tube was performed with the use of a micromanipulator (Sutter Instrument Company, Novato, CA) fitted with a pulled glass capillary needle that was connected to a manual microinjector (Sutter). The virus was mixed with fast green (1.0 μL 0.3% fast green in PBS per 20 μL virus) to assist in the visualization of the injected virus. After the embryo was penetrated, 0.5 to 1.0 μL virus was slowly injected into the neural tube. The egg was then sealed with parafilm, and incubation was continued until the embryo reached the desired age for analysis.

**Histochemistry and In Vivo Promoter Analyses**

Neural retina was dissected from the eyes of injected embryos at selected ages, and dispase was used as necessary to aid in the removal of the pigmented epithelium. Retina whole mounts were prepared by placing the tissue photoreceptor side down on a filter insert (Millicell; Millipore) containing PBS and flattening the specimen using fine tipped glass rods. To detect expression of nlacZ, retinas were fixed in 4% paraformaldehyde for 15 minutes. The retinas 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 X-gal substrate at 37°C for 3 to 4 hours. After this incubation, the retinas were rinsed three times in PBS. Retinas were cryoprotected with 30% sucrose and mounted in optimal cutting temperature (OCT) medium. Sixteen- to 20-μm-thick serial sections were cut with a cryostat through areas positive for X-gal staining, mounted on slides, and counterstained with 4',6'-diamino-2-phenylindole (DAPI). Thirty to 80 sections, taken from the retinas of at least two animals injected with TY-GCAP1 promoter-nlacZ virus were analyzed for each time point. In some cases, the pineal glands and brains of E20 embryos that had been injected with virus were removed and fixed in 4% paraformaldehyde. Pineal glands were stained in toto with X-gal and processed for cryosectioning, as described for retinal sections. The brains of E20 embryos were cut into four regions using a microtome blade as follows: cerebellum-brainstem, optic tectum, anterior forebrain, and posterior forebrain. The brain regions were stained in toto with X-gal, rinsed in PBS, and viewed under a dissecting microscope (Carl Zeiss, Thornwood, NY). In some cases 16- to 20-μm-thick sections were cut through the various regions of the brain using a cryostat, and the sections were stained with X-gal. Bright-field and fluorescence microscopy was performed with microscope (Axioplan 2; Carl Zeiss) fitted with a digital camera system (SPOT 2 Enhanced Digital Camera System; Diagnostics Instruments Inc., Sterling Heights, MI) for imaging. The DAPI nuclear stain was visualized with a long-pass DAPI filter. The images of the X-gal-stained sections were produced by creating a negative image of the stained section and then overlaying it with the DAPI-stained image of that same section, using the digital camera imaging software.

**RESULTS**

**Expression of GCAP1 in Developing Chicken Retina**

Northern blot analyses were performed to determine the onset and relative level of expression of the gene encoding GCAP1 in developing embryonic chicken retina (Fig. 1). Because the functional relationship between GCAP1 and GC1 is closely linked, analyses of GC1 expression were included for comparison. The expression of the gene encoding iodopsin was also included in our analyses as a control. GCAP1 and GC1 transcripts were first detected in developing chicken retina between E14 and E15 and between E13 and E14, respectively. The relative levels of GCAP1 and GC1 transcripts, which were comparable at each developmental stage, increased gradually as a function of embryonic age, reaching maximum levels between E19 and E20. Iodopsin transcripts were first detected...
at E14, a result that agrees with previous studies of the expression of iodopsin in developing chicken retina. The onset of transcription of all these genes in retina coincides with the onset of photoreceptor outer segment development and cGMP synthesis in developing chicken retina, which occur at approximately E15 and E18, respectively.

**In Vitro GCAP1 Promoter Activity**

The activity of each promoter fragment was measured in primary retinal cultures transiently transfected with the promoter-reporter constructs. Cultures were prepared from the retinas of E12 embryos in our experiments, because preliminary studies showed that the promoters of the GCAP1 and IRBP genes are active in these cultures. The five GCAP1-luciferase fusion constructs tested in this series of experiments are shown in Figure 2A. A comparison of the activities of the GCAP1 promoter fragments using ANOVA revealed that they were significantly different from each other (F = 9.79, df = 4, P < 0.001; Fig. 2B). The activities of the 292, 1436, and 4009 fragments, which were comparable to each other, were significantly greater than the activities of either the 1434 or the 3121 fragments (P < 0.05). The activity of the 1434 promoter fragment was significantly greater than that exhibited by the 3121 fragment (P < 0.05). A comparison of the activities of the 292, 1436, and 4009 GCAP1 promoter fragments with that of the mIRBP1783 fragment revealed that the activities of the GCAP1 promoter fragments were approximately one half that of the IRBP promoter fragment assayed under identical conditions (Fig. 2B). Comparable results were obtained in another series of experiments in which cultures were transiently transfected with the pTY-based GCAP1 and IRBP promoter-nlacZ constructs that were used to generate the lentiviral vectors (data not shown).

**Lentiviral Transduction of Avian Tissues**

The goals of this experiment were to determine whether lentivirus pseudotyped with VSV-G could transduce chicken retinal progenitor cells and whether lentivirus could be used as a tool to examine the expression characteristics of promoters in vivo. To address these questions, we examined the expression and cellular distribution of EF1α-nlacZ and mIRBP1783-nlacZ lentiviral transgenes in the retinas of E6 (EF1α-nlacZ) and E20 (EF1α-nlacZ and mIRBP1783-nlacZ) embryos that had received injections of lentiviruses carrying either of these transgenes early in development (~E2). Examination of wholemounts of retinas taken from E6 and E20 embryos that had been injected with TY-EF1α-nlacZ virus and stained with X-gal revealed the presence of several discrete clusters of blue-nucleated cells that were distributed through the entire focal plane of the retina (Fig. 3A). Cross-sectional analyses of these retinas revealed that the nlacZ reporter gene was expressed in all cell layers of the retina. The staining intensities of the cells in E6 and E20 retinas were comparable, suggesting that the activity of the EF1α promoter was similar at both of these stages of development (Figs. 3B–D). The columnlike staining pattern that we observed in the retinas of the embryos injected with TY-EF1α-nlacZ virus closely resembled the staining pattern that has been reported in retroviral studies of cell lineage in devel-

![Figure 2](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932914/ on 06/04/2017)
retinata 1,47 This result suggests that the TY-EF1α-nlacZ transgene carried by the lentivirus was integrated into the DNA of retinal progenitor cells and passed to subsequent clones. In contrast to the clustered, columnlike nlacZ staining pattern observed in the retinas of embryos injected with TY-EF1α-nlacZ lentivirus, nlacZ staining in the retinas of embryos injected with TY-mIRBP1783-nlacZ lentivirus was limited to cells on the surface of the retina (Fig. 3E). Cross-sectional analyses of these retinas revealed that expression of the mIRBP1783 promoter was restricted to photoreceptor cells (Fig. 3F), a result that corroborates previous studies of the cell specificity of this promoter fragment. 10,40,49 An analysis of selected pineal glands and brains taken from E20 embryos showed that transduction of these tissues was minimal or undetectable after neural tube injection of lentivirus (data not shown). Together, these results indicate that lentivirus could be used to examine the expression characteristics of promoters in vivo.

### Analyses of GCAP1 Promoter Fragments In Vivo

The three GCAP1 promoter fragments that showed comparable levels of activity in our in vitro assays (Fig. 2B) were analyzed in vivo. Lentiviral vectors containing the 292, 1436, and 4009 GCAP1 promoter fragments driving the nlacZ reporter gene were generated and injected into the neural tubes and brains taken from E20 embryos showed that transduction of these tissues was minimal or undetectable after neural tube injection of lentivirus (data not shown). Together, these results indicate that lentivirus could be used to examine the expression characteristics of promoters in vivo.

Analyses of GCAP1 Promoter Fragments In Vivo

The three GCAP1 promoter fragments that showed comparable levels of activity in our in vitro assays (Fig. 2B) were analyzed in vivo. Lentiviral vectors containing the 292, 1436, and 4009 GCAP1 promoter fragments driving the nlacZ reporter gene were generated and injected into the neural tubes of E2 embryos to obtain an estimate of the onset of expression of these fragments in developing retina and their cell specificities. GCAP1 promoter-driven nlacZ expression was examined in the retinas of E12, E16, and E20 embryos—stages of development that were selected based on the results of our analyses of the onset of normal GCAP1 expression in developing retina (Fig. 1) and on the milestones of photoreceptor development in chicken. These stages correspond to time points that precede the onset of GCAP1 expression in vivo (E12), that approximate the onset of GCAP1 expression and the development of photoreceptor outer segments in vivo (E16), and that include the period when GCAP1 expression has reached maximal levels in vivo, just before hatching (E20).

### Onset of Expression in Developing Retina

X-gal-stained retinal cells were detected as early as E12 in wholemounts of retinas from embryos that had been injected with either the 292 or the 1436 promoter-nlacZ lentiviral vector. The overall number of cells expressing nlacZ driven by either of these promoter fragments was much lower in the retinas of E12 embryos (292, n = 2; 1436, n = 3) than in the retinas of E16 (292, n = 3; 1436, n = 2) and E20 (292, n = 5; 1436, n = 3) embryos (data not shown). No detectable X-gal staining was observed in retinas of E12 embryos that had received injections of the 4009 promoter-nlacZ lentiviral vector (n = 6). The first evidence of X-gal staining resulting from 4009 promoter-driven expression of nlacZ was observed at E16 (1 positive retina of 6). By E20, the X-gal staining in these embryos had increased sufficiently so that positively stained cells could be detected in all retinas examined (n = 5).

### Cell-Specificity of Expression

The cell-specificity of the activity of each promoter-nlacZ transgene was determined by examining cross sections cut from wholemounts of the retinas that had been removed from embryos injected with the various lentiviral vectors (Fig. 4A). Both the 292 and 1436 promoter-reporter transgenes were expressed in cells located within the inner nuclear layer (INL) at E12. A few nlacZ-positive cells were also observed within the ganglion cell layer (GCL) in these retinas at this time. In E16 and E20 retinas, X-gal-stained cells were also detected within the outer nuclear layer (ONL). In these retinas, the number of stained cells observed in the ONL was generally higher than that observed in the INL. In contrast to the rather nonspecific cellular staining pattern observed in retinas transduced with either the 292 or 1436 promoter-nlacZ transgene, cross-sectional analyses of E16 and E20 retinas transduced with virus carrying the 4009 promoter-nlacZ transgene revealed that only photoreceptor cells within the ONL were stained in these retinas.

### Discussion

The results of our in vitro and in vivo analyses of various fragments of the GCAP1 promoter suggest that cis-elements regulating the activity, developmental expression, and cell-specific expression of the GCAP1 promoter are located in distinct regions of the promoter. The 292, 1436, and 4009 fragments all exhibited similar activity levels in vivo, a result that suggests that the cis-elements essential for conferring activity to these fragments are located within the 292 fragment. In our analyses, we noted that removal of the 25-bp repeated sequence, which comprises approximately 50% of the 5′ untranslated region (UTR), resulted in a significant reduction in the activity of the 1436 promoter fragment. Inclusion of the sequence between nucleotides −1437 and −3121 also produced a significant reduction in promoter activity that could be ameliorated by addition of the sequence between nucleotides −3122 and −4009 to the fragment. It is possible that the observed decrease in activity in the 1436 promoter with the truncated 5′ UTR is due to a reduction in the efficiency of translation of the transcripts produced from this promoter-reporter transgene and that interactions between cis-elements located within the −1437 to −3121 and the −3122 to −4009 regions are necessary to confer significant levels of activity to the longer GCAP1 promoter fragments. To test these possibilities, it is necessary to assay the transcription levels and func-

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**Figure 3.** Retinal wholemounts and cross sections prepared from embryos that received injections of TY-EF1α-nlacZ. (A–D) or TY-mIRBP1783-nlacZ lentivirus. (E, F). (A) Area from a wholemount of an E6 retina showing a cluster of infected clones intensely stained with X-gal. (B) Cross section through retina shown in (A). (C) Cross section through retina of an E20 embryo showing presence of cells stained with Xgal in all retinal cell layers. (D) Inverted bright-field image shown in (C) (nlacZ-positive cells in red) overlaid with the DAPI-stained image (blue) to show the retinal cell layers clearly. (E) Area from a wholemount of an E20 retina of an embryo injected with TY-mIRBP1783-nlacZ lentivirus and stained with X-gal. (F) Cross section through the retina from (E) showing that the mIRBP1783 promoter limited expression of the nlacZ reporter gene to photoreceptor cells within the outer nuclear layer (ONL). PR, photoreceptor side; V, vitreous side; INL, inner nuclear layer; GCL, ganglion cell layer.
In vivo analyses of the expression characteristics of the GCAP1 promoter fragments were performed to identify regions within the GCAP1 promoter that control the cell specificity and developmental onset of expression of the native GCAP1 gene. Analyses of embryonic retinas transduced with lentiviral vectors carrying the nlacZ reporter gene driven by the 292, 1436, or 4009 GCAP1 promoter fragments revealed that the 292 and 1436 GCAP1 promoter fragments, both of
which exhibited activity in vitro and in vivo, did not possess the cis elements required to restrict their activities to photoreceptor cells. Furthermore, the 292 and 1436 promoter fragments exhibited activity in vivo before the normal onset of expression of the GCAP1 gene during development. By including additional upstream sequence in the 4009 GCAP1 promoter fragment, we obtained a fragment that exhibited the expression characteristics of the endogenous GCAP1 gene. These results suggest that the general organization of the GCAP1 promoter differs from those of previously characterized photoreceptor gene promoters, such as IRBP and rhodopsin, in which many of the cis elements in these promoters that are responsible for restricting promoter activity to photoreceptor cells are located within 1 kb of the transcription start point. Based on the in vivo expression characteristics of the 1436 and 4009 promoter fragments, it appears that cis elements located in the distal promoter region are needed to delay the onset of expression, a result similar to that reported in recent in vivo studies of the Xenopus rhodopsin promoter. The GCAP1 promoter contains a cluster of putative cis elements between nucleotides −143 and −838 that include binding sites for transcription factors that have been shown to regulate the expression of retina- and photoreceptor-specific genes (Fig. 4B). We have reported that at least two putative CRX-like binding sites (C/TAATC/T) are present within the first 1 kb upstream of the transcription start point in the 5′ flanking region of the chicken GCAP1 gene. In addition, one Ret-1-like element (−187 to −184), two OTX-like binding elements (−196 to −202 and −832 to −838), and one PCE-1/Ret-1-like element (−818 to −825) and one CRX-like binding sites (−818 to −825) are also located within this region (Fig. 4B). Our analyses show that the shorter GCAP1 promoter fragments that contain these elements (292 and 1436) do not exhibit the expression characteristics of the native GCAP1 promoter in developing retina. Clearly, additional cis-acting elements located in the distal GCAP1 promoter region (−1437 to −4009) are required to produce the cell specificity and developmental expression characteristics of the native GCAP1 gene. As mentioned earlier, our in vitro data indicate that sequence located in the region between nucleotides −1437 and −3121 suppresses GCAP1 promoter activity in retinal cells. Silencing mechanisms similar to those reported for the regulation of neuron-specific gene promoters could play a role in suppressing GCAP1 promoter activity in nonphotoreceptor cells and in producing the temporal expression characteristics of this promoter in developing retina. Similar mechanisms have been postulated for other photoreceptor gene promoters, such as the murine IRBP promoter where a −70/+101 fragment of this promoter containing cis elements that are highly conserved in retina- and photoreceptor-specific promoters exhibits significant activity in vitro, but additional sequence located between nucleotides −70 and −156 is necessary to restrict its activity to photoreceptor cells in vivo. Recent studies of other photoreceptor gene promoters suggest that specific combinations of regulatory factors expressed in photoreceptor cells that bind to and transactivate these promoters are needed for photoreceptor-specific gene expression. Examination of the sequence located upstream of the 1436 GCAP1 promoter fragment revealed the presence of additional putative homeodomain protein-binding elements (see Fig. 4B). The region between nucleotides −2413 and −2423 contains a head-to-tail arrangement of two CRX-like binding elements (consensus CTAATNNGATT), which is similar to that recently identified in several putative CRX-regulated photoreceptor genes. Additional CRX-like (−3305 to −3310) and OTX-like (−3556 to −3362) DNA binding elements are located within the −3122 to −4009 region of the GCAP1 promoter—elements that could influence the expression characteristics of the 4009 promoter fragment (see Fig. 4B). The results of these experiments provide a rough blueprint of the structural and functional organization of the chicken GCAP1 promoter. Additional studies are needed to confirm that the putative cis elements identified within the GCAP1 promoter bind trans-acting factors and that these interactions serve to shape the activity characteristics of this promoter.

In establishing the usefulness of lentiviral-mediated gene transfer as a tool for analyses of promoter function in the developing retina, we demonstrated that lentivirus can transduce chicken retinal progenitor cells. In addition, we showed that the expression of transgenes carried by lentivirus, which transduces both progenitor and terminally differentiated retinal cells, can be targeted to specific cell types by selecting appropriate internal promoters. The experimental paradigm presented herein should be amenable for studies of photoreceptor gene promoters from other species that exhibit activity in primary cultures of chicken retinal cells and, thus, should have broad appeal for in vivo analyses of promoter function. Furthermore, we showed that the lentivirus vector system used in this study is capable of carrying and expressing transgenes up to 7.4 kb in size, a cargo well below the recently demonstrated capacity of this vector system of over 18 kb. The large cargo capacity of this vector is an important feature of this system that will make it useful in studies of the expression characteristics of large promoter fragments in vivo.

Finally, it is important to note that the utility of this method is not compromised by the experimental variability due to differences in viral titer or injection procedure. In experiments in which only small populations of progenitor cells were transduced by the virus, it was possible to obtain data concerning the expression characteristics of the internal promoters carried by these viruses by examining the expression of the reporter gene in the clones derived from transduced cells. We have recently achieved a dramatic increase in transduction efficiency of retina (>80% cells transduced) by modifying the lentiviral transducing vector and the methods for packaging the viral vector. These modifications should greatly simplify and enhance the analyses of the tissue specificity of promoter fragments using lentiviral vectors.

Acknowledgments
The authors thank Lung-Ji Chang (University of Florida, Gainesville, FL) for kindly providing the lentivirus plasmid vectors. Jeffrey Boatright (Emory University, Atlanta, GA) for kindly providing the pIRBP1783-EGFP plasmid vector and Wolfgang Baehr (University of Utah Moran Eye Center, Salt Lake City, UT) for assistance with the DNA sequencing.

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