

EFFICIENT TRANSDUCTION AND TARGETED EXPRESSION OF LENTIVIRAL
VECTOR TRANSGENES IN THE DEVELOPING RETINA

By

JASON EDWARD COLEMAN

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by

Jason Edward Coleman

I dedicate this work to my parents, brothers, sisters, and friends who have all been a great source of encouragement and support throughout this endeavor and to the inspiring and loving memory of my grandfather, Dr. Joseph Edward Coleman.

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Abstract of Dissertation Presented to the Graduate School
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By

Jason Edward Coleman

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Chair: Susan L. Semple-Rowland, PhD
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Gene therapy holds great promise as an effective treatment for genetic diseases. Retinal diseases caused by genetic mutations are among the leading causes of blindness and are an excellent place to begin studying the basic principles of gene transfer-based treatments. In addition to understanding the molecular basis of a target disease, perhaps the most difficult steps in the development of somatic gene therapies are engineering a suitable method to deliver therapeutic transgenes to the diseased cells and achieving appropriate levels of expression for extended periods. The primary goal of this study was to develop a lentivirus-based gene delivery vector that can be used to target the expression of a functional, therapeutic transgene to photoreceptor cells in the retina.

Lentiviral vectors derived from the human immunodeficiency virus type 1 are emerging as the vectors of choice for long-term, stable *in vitro* and *in vivo* gene transfer. Several inherited retinal diseases are caused by mutations in genes that are expressed in photoreceptor cells and are required for normal function of these cells. Cell-specific

promoters can be incorporated into viral vector gene expression constructs and are used to direct expression of transgenes to specific cell types, and the level of expression of these transgenes is controlled by selecting promoters that possess different intrinsic activity levels.

A self-inactivating lentiviral vector system was used in a novel manner to study the intrinsic activity profiles of promoters that regulate the expression of photoreceptor-specific genes. Using these methods, we were able to identify regions of these promoters that are capable of targeting gene expression to retinal cells during development. Data from these studies provide clues regarding the *cis*-acting elements that are important for regulating photoreceptor-specific genes *in vivo*. Furthermore, we have improved the design of and methods of producing lentiviral vectors that will facilitate use of this system for delivering a normal, functional copy of a therapeutic transgene to retinal cells. The results of these studies lay the foundation for future experiments aimed at studying the potential use of lentiviral vector therapies for treating autosomal recessive retinal diseases such as Leber congenital amaurosis type 1 (LCA1).

CHAPTER 1 INTRODUCTION

Retinal Disease as a Model for Gene Therapy Efficacy

The basic premise of gene therapy is to transfer (permanently or transiently) genetic material to genetically defective cells, enabling these cells to function normally without further treatment. Researchers within the field of gene therapy have recently made promising advances toward realizing this goal, but we are still in the early stages of gene therapy research. Thus, it is imperative that initial gene therapy studies be conducted using animal models of well-defined genetic diseases that will provide the framework for the development of treatments for more complex diseases. For example, genetic diseases that affect tissues such as the retina could contribute greatly to the basic principles and applications of gene therapy.

One practical advantage of studying the efficacy of gene therapy for retinal diseases is that the eye is an easily accessible, immune-privileged organ. Another advantage is that many of the genetic mutations affecting retinal function occur in genes that encode proteins critically involved in the phototransduction cascade and the visual cycle – two processes that are well understood in retina. Consequently, the etiology of several retinal diseases has been defined at the molecular level (www.sph.uth.tmc.edu/RetNet). Therefore, this and the fact that there are several well-defined animal models available of these diseases (Lin et al. 2002; Petersen-Jones 1998; Semple-Rowland et al. 1998), strongly support a focus on retinal disease in efforts to define principles of gene therapy. Within the past 5 years, results from several studies showed that the expression of various

transgenes in cells transduced with viral vectors can successfully slow retinal degeneration in animal models of primary retinal disease (Acland et al. 2001; Ali et al. 2000; Bennett et al. 1998; LaVail et al. 2000; Lewin et al. 1998; McGee Sanftner et al. 2001; Takahashi et al. 1999). One notable advance has been the demonstration that functional vision can be restored in a canine model of a congenital retinal dystrophy, Leber congenital amaurosis (LCA), by transferring a normal copy of the RPE65 gene to cells within the retina (Acland et al. 2001).

In the remaining portion of this chapter, I will focus on discussing LCA and recent progress toward development of gene therapies for retinal disease. Specific topics will include descriptions of animal models of LCA1, the rationale for selection and use of specific viral vectors for the development of therapies, and the importance and development of strategies to target gene expression to specific cell types affected by retinal disease.

Leber Congenital Amaurosis

Clinical Phenotype and Genetics

Leber first described the condition known as LCA in 1869 (Leber 1869). LCA is a family of clinically and genetically heterogeneous inherited retinal diseases that produce the earliest and most severe forms of congenital blindness (Perrault et al. 1999). It is generally assumed that LCA accounts for 5% of all cases of retinal dystrophies, but may be even more frequent due to the high rate of consanguinity among LCA families (Cremers et al. 2002; Foxman et al. 1985; Perrault et al. 1999). Visual deficits are usually detected by the age of 6 months in infants and LCA patients rarely present with a visual acuity better than 20/400 through life (Cremers et al. 2002). The electroretinographic responses of LCA patients are severely attenuated or non-existent at birth and, based on

some published criteria, the electroretinogram (ERG) should be extinguished before the age of 1 year (Foxman et al. 1985).

Uncomplicated LCA is inherited in an autosomal recessive mode. By 1996, Perrault and her colleagues had identified the first gene linked to LCA, the gene encoding the enzyme retinal guanylate cyclase-1 (GC1; designated LCA1) (Perrault et al. 1996). Since this discovery, mutations in six additional genes expressed in retina have been linked to LCA. These genes, which encode proteins that are involved in several aspects of rod and cone cell function, include RPE65 (Marlhens et al. 1997), CRX (Freund et al. 1998), AIPL1 (Sohocki et al. 2000a;Sohocki et al. 2000b), CRB1 (den Hollander et al. 2001;Lotery et al. 2001) and RPGRIP1 (Dryja et al. 2001).

Several GC1 mutations have been identified and most of these are frameshift and missense mutations (Perrault et al. 2000). The frameshift mutations generate premature translation termination codons that are predicted to lead to the absence of GC1 protein (Perrault et al. 1996;Perrault et al. 2000;Rozet et al. 2001). The missense mutations, many of which occur in the catalytic domain, have been shown to severely compromise or abolish GC1 activity (Rozet et al. 2001). Functional consequences of an F589S missense mutation in GC1 show that the mutation reduces basal GC1 activity by 80% and disrupts the ability of GCAP1 to stimulate GC1 under low-calcium conditions (Duda et al. 1999b). Most GC1 mutations identified so far are assumed to result in significant reductions in the intracellular levels of cGMP in photoreceptor cells, reductions that could lead to a situation equivalent to constant light exposure during retinal development (Perrault et al. 2000).

Clinicopathology of LCA1

Ophthalmological examinations of LCA1 patients reveal that the fundus appears normal early in life, but abnormalities such as salt-and-pepper pigmentation and the attenuation of retinal vessels begin to appear after several years (Edwards et al. 1971). Most of the reported histopathologic studies of LCA1 retinas have revealed that the rods and cones degenerate late in life (Francois and Hanssens 1969; Mizuno et al. 1977; Noble and Carr 1978). Immunohistochemical analyses performed in a postmortem eye obtained from a young LCA1 patient (11.5 years old) revealed that substantial numbers of rods and cones were retained in the macula and far periphery; however there was an overall reduction in the labeling of cone outer segment proteins (Milam et al. 2003). From a therapeutic standpoint, the results of these analyses are encouraging and suggest that the retinal circuitry was intact and functional. Further insight into the pathophysiological and cellular consequences of the GC1 mutations linked to LCA1 have been obtained primarily from studies of two animal models of this disease, the GUCY1*B chicken and the GC1-knockout mouse.

Animal Models of LCA1

There are currently two animal models of LCA1, the GC1-knockout mouse and the GUCY1*B chicken. The phenotypes of these two animal models are strikingly different. Comparisons of the two models provide important clues about the consequences of GC1 null mutations on the development, function and health of cone and rod photoreceptor cells.

GC1-knockout mouse

The retinas of GC1-knockout mice are morphologically normal at birth, but exhibit reductions in the amplitudes of both the rod and cone cell responses to light stimulation

(Yang et al. 1999). By one month of age, cone responses to light are barely detectable and the ERGs of both the rod a- and b-waves are dramatically reduced (Yang et al. 1999). These mice do not display any detectable visual deficits despite the changes in rod function that progress until 5 months of age. The first signs of photoreceptor degeneration occur between 4 and 5 weeks of age and is marked by a rapid and specific loss of cones, leaving a normal population of rod cells (Yang et al. 1999). This pattern of photoreceptor degeneration differs significantly from that observed in the GUCY1*B chicken, which is described below.

GUCY1*B chicken

The GUCY1*B chicken, formerly known as the retinal degeneration or *rd* chicken (Semple-Rowland and Cheng 1999), is recognized as a naturally occurring model of human LCA1 and is the only animal model of inherited retinal disease that possesses a cone-dominant retina (Semple-Rowland et al. 1998; Semple-Rowland and Lee 2000). A deletion-rearrangement of the GC1 gene results in loss of the transmembrane domain of GC1, destabilization of the transcript, and a total absence of the GC1 enzyme in the GUCY1*B retina.

The retinas of GUCY1*B animals are morphologically indistinguishable from normal retinas at hatching. Early signs of retinal pathology appear 7 to 10 days post-hatch in the photoreceptor layer of the central retina. Degeneration of the photoreceptor layer is progressive so that by 21 days of age, marked degeneration of the photoreceptor outer segments is apparent. By 60 days of age, the mid-peripheral retina shows signs of degeneration and at 115 days of age, loss of photoreceptors from the central retina is complete. By 6 to 8 months of age, cell loss from the inner retina is also apparent. The amplitudes of the ERGs recorded from GUCY1*B chickens under photopic and scotopic

conditions are absent or less than 7% of those recorded from normal chickens (Ulshafer et al. 1984). The levels of cGMP in the photoreceptor layer of 1-2 day old GUCY1*B chicken retinas are only 10 to 20% of those measured in age-matched normal retinas (Semple-Rowland et al. 1998). These results have led to the hypothesis that decreased levels of cGMP may result in a state of constitutive hyperpolarization of the photoreceptor cells, a condition that could mimic the degenerative events associated with constant light exposure (Fain and Lisman 1999; Hao et al. 2002).

Upon comparison of the mouse and chicken models, it is evident that the only common feature of the progressive retinal degenerations is that cone function and survival are severely compromised by the absence of GC1. Differences in the spatial organization and composition of photoreceptor cells in mouse and chicken retinas may provide a likely explanation as to why the phenotypes and pathologies are so contrasting. The retina of the chicken is cone dominant (80% cones; 20% rods), whereas that of the mouse is rod dominant (3-5% cones; 95-97% rods). Therefore, the effects of cone degeneration on rod survival and function in a rod-dominant retina appear to be different than in a cone-dominant retina. Furthermore, the cone cells in mouse are evenly distributed among the rod cell population throughout the retina. The distribution of cone cells in the chicken is more analogous to that found in the macula/fovea region of central retina in humans. The finding that visual function is severely compromised in both LCA1 patients and GUCY1*B chickens is consistent with the central role of cones in vision in humans and chickens. Therefore, the data obtained from animal models and LCA1 patients suggest that cone cells should be the primary targets for gene therapies aimed at treating this disease.

Gene Therapy Vectors

In general, vectors or gene delivery vehicles that facilitate the transfer of genetic material to cells can be grouped into two broad categories: non-viral vectors and viral-based vectors. Several studies have shown that genetic material can gain entry into cells by forming complexes with liposomal or other cationic molecules. However, while the development of effective non-viral vectors is rapidly progressing, the technology is still in its infancy (Brisson and Huang 1999; Johnson-Saliba and Jans 2001; Lechardeur and Lukacs 2002). Many of the recent advances in gene therapy have been facilitated by the use of viral-based vectors. These vector systems take advantage of the natural capabilities of viruses to deliver genetic material to cells. In particular, gene transfer vectors based on viruses from the *Parvoviridae*, *Adenoviridae* and *Retroviridae* families have shown the most promise in this regard. The unique characteristics of the different viruses and the vectors derived from them must be considered when choosing a vector for use in developing viral vector-based therapies. For example, host immune response, longevity and/or kinetics of transgene expression, cargo capacity (size limit of a particular gene of interest), vector tropism and the performance of gene regulatory sequences within the context of vector-mediated gene expression can vary widely with each vector system.

***Parvoviridae*- and *Adenoviridae*-based Vectors**

Vectors based on adeno-associated virus (AAV), a non-pathogenic member of *Parvoviridae*, have been widely used in retinal gene therapy research. Recombinant AAV (rAAV) vectors have been shown to efficiently transduce retinal cells of several species and are capable of long-term expression of transduced genes (Acland et al. 2001; Ali et al. 1998; Bennett et al. 1997; Bennett et al. 1999; Flannery et al. 1997; Grant et al. 1997).

Some limitations are encountered with the use of rAAV vectors. Traditional versions of rAAV vectors have a cargo capacity of less than 5 kb, which can pose problems in applications where large cDNAs and regulatory sequences are required. To increase the rAAV payload, some groups are exploring the possibility of using a trans-splicing strategy to assemble a complete transgene from two different vectors after dual-transduction of cells (Reich et al. 2003). Another feature of rAAV that could be problematic in the treatment of rapidly advancing retinal diseases is that it may take up to 4 weeks to achieve maximal expression of transgenes after infection (Sarraf et al. 2002). Despite these limitations, rAAV vectors carrying normal copies of diseased genes (Acland et al. 2001), genes encoding growth factors (Lau et al. 2000) and genes encoding ribozymes specifically targeted to cleave mutated genes (Hauswirth et al. 2000; LaVail et al. 2000; Lewin et al. 1998) have been successfully used to restore visual function or slow retinal degeneration in animal models of inherited retinal disease.

Adenovirus (Ad) has also been developed as a gene transfer vector. As with rAAV, Ad vectors have been shown to transduce photoreceptors *in vivo* after subretinal injection (Akimoto et al. 1999; Bennett et al. 1994; Bennett et al. 1996b; Bennett et al. 1998). Although Ad vectors have been routinely generated to high titer and although Ad vectors exhibit efficient transduction of retinal cells, some caveats and limitations exist. For example, Ad is only effective in situations where transient expression of a gene is required since the viral DNA does not integrate into the host genome. The short-lived expression of Ad vectors is due, in part, to the induction of a host immune response triggered by expression of the adenoviral genes in the target cells (Bennett et al. 1996a; Hoffman et al. 1997; Reichel et al. 1998). Gutless Ad vectors have been developed

to circumvent this problem, to increase cargo capacity and to allow for stable integration of the transgene (Kochanek et al. 2001; Mitani and Kubo 2002; Yant et al. 2002).

Subretinal injections of adenoviral vectors carrying normal copies of either the phosphodiesterase β -subunit, neurotrophic factors, or anti-apoptotic factors have been shown to delay photoreceptor degeneration in the *rd* mouse model of retinal degeneration (Bennett et al. 1996b; Bennett et al. 1998; Cayouette and Gravel 1997).

***Retroviridae*-based Vectors**

Retroviral vectors were among the first virus-based systems used to develop gene transfer therapies (Buchsacher, Jr. and Wong-Staal 2000). Retroviruses can integrate foreign genes into the host genome and sustain long-term expression. These viruses consist of a diploid RNA genome surrounded by an enveloped capsid and can be divided into two major taxonomic groups: simple and complex. The simple and complex retroviral genomes consist of the conserved *gag*, *pol* and *env* genes flanked by *cis*-acting long terminal repeat sequences (LTRs), and contain a packaging signal (ψ) adjacent to the 5' LTR. The 5' LTR comprises a viral promoter-enhancer region and transcription start site; and the 3' LTR contains sequences required for efficient polyadenylation of viral transcripts.

Simple retroviruses, such as murine leukemia virus (MLV), are well characterized. Vectors based on MLV were among the earliest developed and have been at the forefront of clinical gene transfer technology. Early versions contained a nearly complete viral genome. Over the years, these vectors have been streamlined to contain only the genes necessary to transduce cells and stably integrate genetic material into the host genome. MLV vectors are now engineered to be replication defective by removing viral genes and

leaving only the *cis*-elements necessary for a single round of replication (Brenner and Malech 2003).

MLV-based vectors have recently been used in human gene therapy trials designed to treat X-linked severe combined immune deficiency (Aiuti et al. 2002; Cavazzana-Calvo et al. 2000; Hacein-Bey-Abina et al. 2002). While the therapeutic outcome of this experimental treatment has proven to be beneficial and promising, researchers have recently identified two patients in the trial that have developed cases of a rare leukemia (Brenner and Malech 2003; Hacein-Bey-Abina et al. 2003). These results suggest that the MLV-based retroviral vectors have oncogenic potential in humans (Fox 2003). The adverse effects observed in these cases could be linked to an intrinsic property of the oncoretrovirus-based vector system or insertional mutagenesis (Brenner and Malech 2003). Regardless of the cause, these results point to a need for additional studies aimed at developing safer gene transfer vectors for future use in humans.

Lentiviruses are complex retroviruses that can transduce dividing and non-dividing cells. For this reason, these viruses have been the focus of intense research efforts aimed at developing more efficient and versatile gene transfer vectors. In recent years, several groups have described the generation of gene transfer vectors derived from the human immunodeficiency virus type 1 (HIV-1), a lentivirus that holds great promise as a basis for gene transfer vectors (Iwakuma et al. 1999; Quinonez and Sutton 2002; Zufferey et al. 1998).

HIV-1 is the most well studied lentivirus. In addition to the essential retroviral *gag*, *pol*, and *env* genes that make up the HIV-1 genome, several accessory proteins are encoded by the genome. The so-called non-essential accessory proteins include *vif*, *vpu*,

vpr, and nef. The essential regulatory proteins, tat and rev, also unique to lentiviruses, interact with viral genomic *cis*-elements to promote transcription elongation and to facilitate nuclear export of viral RNAs, respectively.

Since HIV-1 is a lethal human pathogen, concerns about the biosafety of HIV-1-derived vectors have been a primary focus of research efforts directed toward the development of these vectors for clinical use in humans. Several steps have been taken to diminish the possibility of generating wild-type virus during packaging and following administration of these vectors. Firstly, the genome has been divided among three bacterial expression plasmids in first- and second-generation lentiviral vector systems: (1) a transducing vector that carries *cis*-elements and the promoter/gene of interest, (2) a packaging plasmid that carries an attenuated viral genome and modified LTRs, and (3) an envelope glycoprotein expression plasmid. Co-transfecting these plasmids in a producer cell line generates a replication-incompetent lentiviral vector. Secondly, modifications have been made to the transducing and packaging vectors by minimizing the amount of homologous sequence between the two vectors, thereby greatly reducing the likelihood of recombination. Finally, the latest versions of the transducing vectors are self-inactivating (SIN) (Iwakuma et al. 1999; Zufferey et al. 1998). In SIN vectors, nearly all sequence has been deleted in the 3' LTR, which is used as a template to generate both LTRs in the integrated proviral form of the vector. Therefore, both LTRs are inactivated (i.e. do not activate transcription) following integration and transcription of the inserted transgene is activated by a heterologous internal promoter.

In light of the problems encountered during recent human gene therapy trials with oncoretroviral vectors (Hacein-Bey-Abina et al. 2003), it will be important to continue

improving the biosafety of future lentiviral vector systems for use in humans. Lentiviral vectors have the potential to initiate oncogenic events because they integrate transgenes into host cell DNA. While the specific target sites for lentiviral vector transgene integration have not been elucidated, studies have shown that integrated HIV-1 DNA is primarily detected in non-coding regions of human DNA in blood lymphocytes (Lyn et al. 2001). Two possible ways to circumvent the problem of insertional mutagenesis due to random integration events are (1) to develop vectors that integrate transgenes at specific, non-oncogenic sites and (2) to develop vectors that do not interfere with endogenous gene expression. A mechanism for site-specific integration could be incorporated into the lentiviral vector system by manipulating integrase activity or other “forces” that influence target site selection (Belteki et al. 2003; Bushman 2002). The incorporation of elements such as DNA insulator sequences (Chung et al. 1997; Pannell and Ellis 2001) into future vector systems is likely to significantly improve the performance of lentiviral vectors and to improve their biosafety by isolating the effects of any enhancer/promoter elements contained in integrated transgenes.

In regard to the performance of lentiviral vectors in retina, recent studies have shown that these vectors are capable of transducing photoreceptors following subretinal injection, resulting in the long-term expression of transgenes (Auricchio et al. 2001; Cheng et al. 2002; Lotery et al. 2002; Miyoshi et al. 1998). Lentiviral vector-mediated expression of the β -phosphodiesterase gene in photoreceptors in the *rd* mouse attenuates photoreceptor degeneration for up to 4 months after injection (Takahashi et al. 1999). We have chosen to utilize an HIV-1-derived lentiviral vector system for gene

rescue studies in the GUCY1*B chicken model for LCA1 because of the large cargo capacity and the rapid kinetics of transduction/transgene expression.

Regulation of Vector-Mediated Gene Expression

The ability to regulate gene expression in a physiological manner and to target expression to specific cell types such as photoreceptors is crucial to the development of successful somatic gene rescue strategies. Several factors are known to influence gene expression (e.g. chromatin organization, gene copy number, and gene methylation). Regulation of transcription initiation is the most straightforward mechanism of gene regulation. The interactions of cellular *trans*-acting transcription factors with *cis*-acting DNA elements found in the proximal promoter region play a central role in regulating transcription initiation. The proximal promoter region of some photoreceptor genes has been shown to be sufficient to confer tissue-specific gene expression (Flannery et al. 1997; Liou et al. 1991; Mani et al. 1999), although additional *cis*-elements located in distal promoter regions or in the gene itself may act to enhance or repress levels of gene expression (DesJardin and Hauswirth 1996; Wang et al. 1992).

Gene Regulation in Retinal Photoreceptors

Examination of the transcription factors and promoters involved in the regulation of photoreceptor genes has begun to reveal the molecular mechanisms that control gene expression in these cells. NRL and CRX proteins are two photoreceptor-specific transcription factors that are crucial for the expression of several photoreceptor genes and for photoreceptor development (Chen et al. 1997; Furukawa et al. 1997; Mears et al. 2001; Rehemtulla et al. 1996; Swaroop et al. 1992). Significant progress has been made in identifying common *cis*-acting DNA elements that regulate the expression of a number of photoreceptor-specific genes. Regulatory regions conserved in the proximal promoters of

the photoreceptor genes encoding arrestin (Boatright et al. 1997b;Kikuchi et al. 1993;Mani et al. 1999), β -phosphodiesterase (Di Polo et al. 1996;Mohamed et al. 1998), interphotoreceptor retinoid-binding protein (IRBP) (Boatright et al. 2001;Bobola et al. 1995;Liou et al. 1991), rod opsin (Chen and Zack 1996;Gouras et al. 1994;Nie et al. 1996), and cone opsins (Chen et al. 1994;Wang et al. 1992) have been identified and characterized using transgenic and *in vitro* analyses.

Truncated murine opsin promoters have been used successfully to target viral transgene expression to photoreceptor cells (Flannery et al. 1997). Both opsin and β -phosphodiesterase promoters have been used to drive expression of ribozymes and the β -phosphodiesterase gene, respectively, in photoreceptor cells (LaVail et al. 2000;Lewin et al. 1998;Takahashi et al. 1999).

As mentioned previously, one of the main objectives of my dissertation research is to develop a lentiviral-based vector system that can be used to rescue the retinal degeneration phenotype in the GUCY1*B chicken. In constructing this system, I have focused much of my effort on identifying and utilizing promoters that limit expression of GC1 transgenes to photoreceptor cells. The 5' flanking regions from IRBP, guanylate cyclase activating protein-1 and GC1, all of which are known photoreceptor-specific genes, were characterized and tested *in vivo* to accomplish this objective. These studies are presented in Chapters 2 and 3.

CHAPTER 2
A 4.0 KB FRAGMENT OF THE GUANYLATE CYCLASE ACTIVATING PROTEIN-
1 (GCAP1) PROMOTER TARGETS GENE EXPRESSION TO PHOTORECEPTOR
CELLS IN THE DEVELOPING RETINA

Note

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Introduction

Guanylate cyclase activating protein 1 (GCAP1) is an EF-hand calcium-binding protein that activates photoreceptor guanylate cyclase 1 (GC1) under low intracellular calcium conditions, thereby hastening the recovery phase of phototransduction (Dizhoor and Hurley 1999; Palczewski et al. 2000; Polans et al. 1996). 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 (Cooper et al. 1995; Dizhoor et al. 1994; Dizhoor et al. 1995; Frins et al. 1996; Gorczyca et al. 1995; Howes et al. 1998; Palczewski et al. 1994). 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 (Cuenca et al. 1998; Kachi et al. 1999). The expression of GCAP1 has also been detected in the pineal glands of bovine and chicken (Semple-Rowland et al. 1999; Venkataraman et al. 2000). 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 (Duda et al. 1996; Gorczyca et al. 1995; Otto-Bruc et al. 1997; Rudnicka-Nawrot et al. 1998; Tucker et al. 1997). While at least three variants of GCAP are expressed in retina (GCAP1-3) (Dizhoor et al. 1995; Gorczyca et al. 1994; Haeseleer et al. 1999; Palczewski et al. 1994), recent studies of GCAP1/2 knockout mice suggest that only GCAP1 is capable of restoring normal light response kinetics to photoreceptor cells (personal communication with W. Baehr and cited reference) (Mendez et al. 2001). 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 (Downes et al. 2001; Payne et al. 1998; Wilkie et al. 2001). These mutations, which interfere with the binding of calcium to GCAP1, lead to persistent activation of GC1 even under high calcium conditions (Dizhoor et al. 1998; Sokal et al. 1999; Wilkie et al. 2001). 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 upon the interactions between promoter *cis*-elements and transcription factors, and is often

linked temporally to the differentiation and maturation of the photoreceptor cells (e.g. see cited references) (Hauswirth et al. 1992;He et al. 1998;Johnson et al. 2001;Kennedy et al. 2001;Livesey et al. 2000;Morrow et al. 1998;van Ginkel and Hauswirth 1994). Relatively few studies have been carried out to examine the activities of photoreceptor-specific promoters in developing retina *in vivo* (Chen et al. 1994;Kennedy et al. 2001;Lem et al. 1991;Mani et al. 2001;van Ginkel and Hauswirth 1994). Recently, the importance of correct temporal regulation of gene expression in developing retina has been clearly demonstrated in studies of cone-rod homeobox (CRX) (Furukawa et al. 1999;Livesey et al. 2000) and neural retina leucine zipper (Mears et al. 2001) knockout mice. The results of these studies show that the absence of expression of these key trans-acting factors in retina results in the down-regulation of expression of several photoreceptor-specific genes and abnormal development and function of the photoreceptor cells.

In this series of experiments, we have 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' flanking region of this gene (Semple-Rowland et al. 1999) 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, an *in vitro* system that has been used to characterize the activities of the promoters of photoreceptor-specific genes obtained from a variety of species (Boatright et al. 1997b;Boatright et al. 1997a;Chen et al. 1997). We

utilized 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 (Vigna and Naldini 2000), and have a large cargo capacity (>18 kb) (Kumar et al. 2001). 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 prior to the development of the neural retina. The onset of expression of each GCAP1 promoter-nlacZ transgene in developing retina was compared to the expression profiles of the GCAP1 and GC1 genes in normal, developing chicken retina.

Methods

Northern Blot Analyses

Embryonic retina-pigmented epithelium-choroid tissues were removed from both eyes of each embryo and total RNA from these tissues was isolated using an RNeasy total RNA kit (Qiagen). Samples containing 10 µg of RNA were electrophoresed on a 1.1% formaldehyde gel and transferred to a nylon transfer membrane (Micron Separations Incorporated). Northern blots were hybridized consecutively with radiolabeled cDNA probes specific for GCAP1, GC1, and iodopsin as previously described (Semple-Rowland and van der 1992). The GCAP1 and iodopsin results were confirmed by repeating the analyses on a second series of independent samples. Blots were exposed to Kodak BioMax film (Eastman Kodak) for 12-16 hours at -80°C and the resulting

hybridization signals were imaged using a BioRad Gel Doc 1000 system. 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 GCAP1 cosmid clones, ccos16 and ccos24 (Semple-Rowland et al. 1999) using the polymerase chain reaction and *Pfu* DNA polymerase (Stratagene). 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 *PmeI* site to generate the following fragments (transcription start point = +1): (1) -292/+302, (2) -1436/+302, (3) -3121/+222, (4) -4009/+222, and (5) -1434/+29 (Fig. 2-2A). In this study, these fragments will be referred to as 292, 1436, 3121, 4009 and 1434, names based on the position of the 5' nucleotide. The sequence-specific (GenBank AF172707) primers used to amplify the fragments were as follows: 292 (sense - 5' ACC CGT GTG CTT TTC; antisense - 5' GCT CCA GTC ACT CT), 1436 (sense - 5' ACC CGA CTC CTT CAA; antisense - same as 292), 3121 (sense - 5' AAT CCT GCC CAT CAC TGC CCT ATC; antisense - 5' AGT TTT GAG GTC GGT GGG TGA GTC), 4009 (sense - 5' GGG CGA TTG GCA GGG AGG AG; antisense - same as 3121), 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 the pCR-TOPO-blunt II vector (Invitrogen) and the DNA sequences of positive clones were verified by sequence analyses. GCAP1 promoter fragments were excised from the pCR-TOPO-blunt II clones using *NotI* and *PmeI* and ligated into the appropriate vectors. For the *in vitro* activity assay constructs, the multiple cloning site (MCS) of the pGL2 vector (Promega) was modified by ligating the *SacI/XhoI* fragment of the MCS of the pBluescript II SK vector

into the MCS of the pGL2 vector. The modified vector was then digested with *Xho*I, blunt-ended, digested with *Not*I and the *Not*I-*Pme*I GCAP1 promoter fragments were ligated into the vector. For the *in vivo* lentiviral constructs, *Not*I-*Pme*I fragments were ligated into pTY-nlacZ digested with *Not*I and *Pme*I. The murine IRBP promoter (mIRBP1783), which included nucleotides -1783 to +101, was amplified from the pIRBP1783-EGFP plasmid vector using the polymerase chain reaction. The same cloning strategy described for the GCAP1 promoter constructs was used to generate pGL2 and lentivirus pTY-nlacZ plasmid vectors containing the mIRBP1783 promoter. Transfection-grade DNA was prepared for each construct using an endotoxin-free DNA maxiprep kit (Qiagen).

Cell Cultures and Transfections

Dispersed embryonic day 12 (E12) chick retinal cultures were prepared and transiently transfected essentially as previously described (Adler et al. 1982; Ameixa and Brickell 2000; Boatright et al. 1997b; Kumar et al. 1996; Politi and Adler 1986). Isolated neural retina was incubated in 0.25% trypsin at 37°C for 20 minutes, dispersed by trituration using a flame-narrowed glass pipette, and plated at a density of 2×10^6 cells / well in 24-well culture plates that had been coated with poly-L-ornithine (Sigma). Cultures were maintained in basal medium of eagle (Life Technologies) 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 using 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 of 0.2 M CaCl₂. Next, 125 µl of 2x HEPES-buffered saline was added dropwise to the DNA/CaCl₂ mixture. The

transfection mixture was allowed to incubate for 20 minutes at room temperature and then 62.5 μ l of the transfection mixture was added to each well. Cells were incubated overnight at 37°C in 5% CO₂ and rinsed 3 times with PBS the following day. The transfection experiments were replicated 4 times and a new preparation of cultured cells was used for each experiment ($n = 4$). Within each experiment, transfection of each promoter construct was carried out in duplicate using the same transfection mixture. The photoreceptor-specific mIRBP1783 promoter was used as a positive control in all experiments (Boatright et al. 1997a; Boatright et al. 2001; Chang et al. 2000).

Luciferase and β -galactosidase Assays

Cell lysates from the E12 primary retinal cultures were prepared 40-48 hours post-transfection by adding 200 μ l lysis buffer (provided in the assay kits, see below) 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, Tropic). Luciferase and β -galactosidase activities were measured in 20 and 40 μ l aliquots of each lysate, respectively. Assays were run in duplicate and quantified using a TD20/20 luminometer (Turner Designs) with an integration time of 10 seconds. Activity values were corrected for transfection efficiency across experiments by normalizing luciferase values to β -galactosidase values. Promoter activity was expressed as fold-activity over the promoterless pGL2 vector. Data were analyzed using one-way repeated measures ANOVA and post-hoc pairwise comparisons were performed using the Student-Newman-Keuls post-hoc test (SigmaStat).

Lentivirus Production

Viruses pseudotyped with VSV-G were prepared using a self-inactivating lentiviral vector system (Iwakuma et al. 1999). Packaging cells (293T) were plated in 10 cm culture dishes at a density of 6×10^6 cells / dish in Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum, and antibiotics (Life Technologies). The 293T cells were grown to 80-90% confluence and were then transiently transfected with 6 μ g pTY-nlacZ (transgene-carrying vector), 12 μ g pHP (packaging vector), 5.5 μ g pHEF (encodes VSV-G envelope) and 0.5 μ g pCEP4-tat (encodes tat protein) per dish using Superfect transfection reagent (Qiagen). All four of the plasmids were added to 300 μ l DMEM and the DNA mixture was vortexed and incubated at room temperature for 5 minutes. Superfect (50 μ l) 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 of fresh medium. The transfection mixture was then added dropwise to the cultures which were then incubated at 37°C in 5% CO₂ for 3-4 hours. Following the incubation period, the cells were rinsed one time with medium. Fresh medium (6 ml) was added back to the cells and the cells were incubated overnight. The next day, the medium was removed and 6 ml of fresh medium was added to the cells. The medium containing the virus was harvested 48 and 72 hours post-transfection and frozen at -80°C until concentration. To concentrate the virus, the medium was rapidly thawed and passed through a 0.45 μ m low-protein binding Durapore filter (Millipore, Bedford, MA) to remove cell debris. The filtered medium containing the virus was concentrated 140-fold by ultracentrifugation at 20,000 x g 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 4×10^4 TE671 cells seeded in 24-well plates with limiting dilutions of TY-EF1 α -nlacZ virus in the presence of 8 $\mu\text{g/ml}$ polybrene. After 3-4 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 (Chang et al. 1999). Virus titer was determined by counting the number of blue-nucleated cells and infectious titers were expressed as the number of transducing units per ml (TU/ml). Particle titers were determined using a p24 ELISA kit obtained from BD Biosciences following the protocols provided therein. Dilutions of 1×10^{-6} and 1×10^{-7} of lysed viral particles were assayed to obtain the mean ng p24/ml of each sample. The average infectivity of virus using the methods described above was determined for TY-EF1 α -nlacZ virus. Infectious titers of virus carrying tissue-specific promoters were estimated by multiplying the particle titers of the GCAP1/IRBP promoter-containing viruses by the infectious titer to particle titer ratio obtained for the virus. The titers of all virus preparations were approximately 1×10^7 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 and 60% humidity. Viral injections were performed on Hamberger-Hamilton stage 10-12 embryos ($\sim\text{E2}$). 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 carried out using a micromanipulator (Sutter

Instrument Company) fitted with a pulled glass capillary needle that was connected to a Sutter manual microinjector. The virus was mixed with fast green (1.0 μ l 0.3% fast green in PBS per 20 μ l virus) to assist in the visualization of the injected virus. Upon penetration of the embryo, 0.5-1.0 μ l of 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 Millipore-Millicell insert containing PBS and flattened 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 3-4 hours. Following this incubation, the retinas were rinsed three times in PBS. Retinas were cryoprotected with 30% sucrose and mounted in OCT medium. Sixteen to twenty micron thick serial sections were cut through areas positive for X-gal staining using a cryostat, mounted on slides, and counterstained with DAPI. Thirty to eighty sections, taken from the retinas of at least two different 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 with X-gal *in toto* and processed for

cryosectioning as described above. 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 Zeiss dissecting microscope; in some cases 16-20 μm thick sections were cut through the various regions of the brain using a cryostat and the sections were stained with X-gal as described above. Brightfield and fluorescence microscopy was performed using a Zeiss Axioplan 2 microscope (Carl Zeiss Incorporated) fitted with a SPOT 2 Enhanced Digital Camera System (Diagnostics Imaging Incorporated) for imaging. The DAPI nuclear stain was visualized using a longpass DAPI filter. The images of the X-gal stained sections were produced by creating a negative image of the stained section that was then overlaid with the DAPI-stained image of that same section using the SPOT camera imaging software.

Results

Expression of GCAP1 in Developing Chicken Retina

Northern blot analyses were carried out to determine the onset and relative level of expression of the gene encoding GCAP1 in developing embryonic chicken retina (Fig. 2-1). Since 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 on E14-15 and E13-14, 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 (Adler et al. 2001; Bruhn and Cepko 1996). The onset of transcription of all of these genes in retina coincides with the onset of photoreceptor outer segment development and cGMP synthesis in developing chicken retina which occur around E15 (Meller and Tetzlaff 1976) and E18 (Semple-Rowland et al. 1998), respectively.

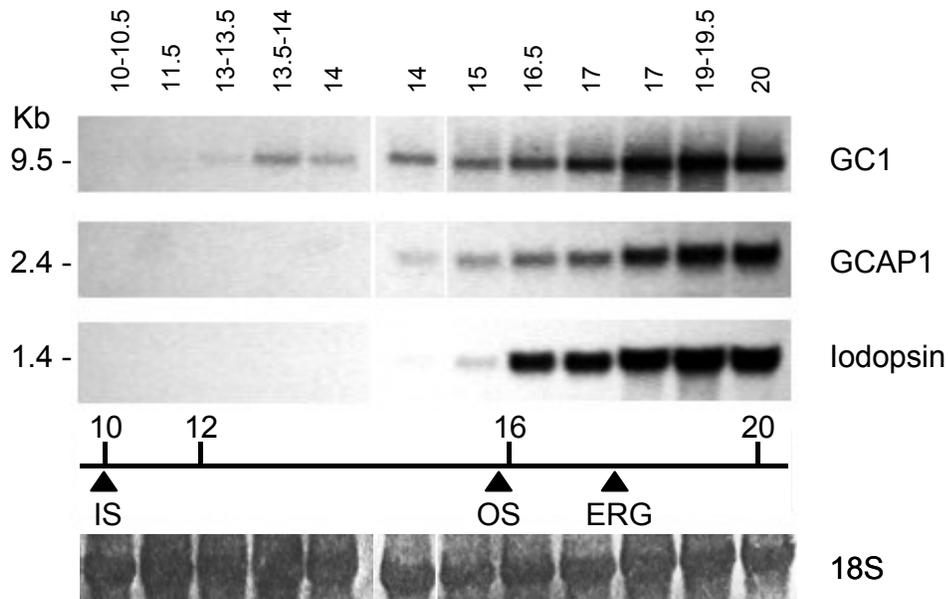


Figure 2–1. Expression of GCAP1, GC1 and iodopsin genes in developing chicken retina. Northern blot hybridized consecutively with GCAP1, GC1 and iodopsin cDNA probes and then stained with methylene blue to show 18S rRNA (RNA loading control) at selected developmental stages. The 18S rRNA was used as a loading control. The numbers across the top of the figure correspond to embryonic age. The time line at the bottom of the figure indicates the developmental ages at which the photoreceptor inner (IS) and outer segments (OS) first appear and the earliest age at which electroretinograms (ERG) can be recorded. The GCAP1 and iodopsin results were confirmed by repeating the northern analyses on a second series of independent samples.

***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 Fig. 2-2A. 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. 2-2B). 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 to 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. 2-2B). 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 if lentivirus pseudotyped with VSV-G could transduce chicken retinal progenitor cells and if 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 EF1 α -nlacZ and mIRBP1783-nlacZ lentiviral transgenes in the retinas of E6 (EF1 α -nlacZ) and E20 (EF1 α -nlacZ and mIRBP1783-nlacZ) embryos that had received injections of lentiviruses carrying either of these transgenes early in development (~E2). Examination of whole

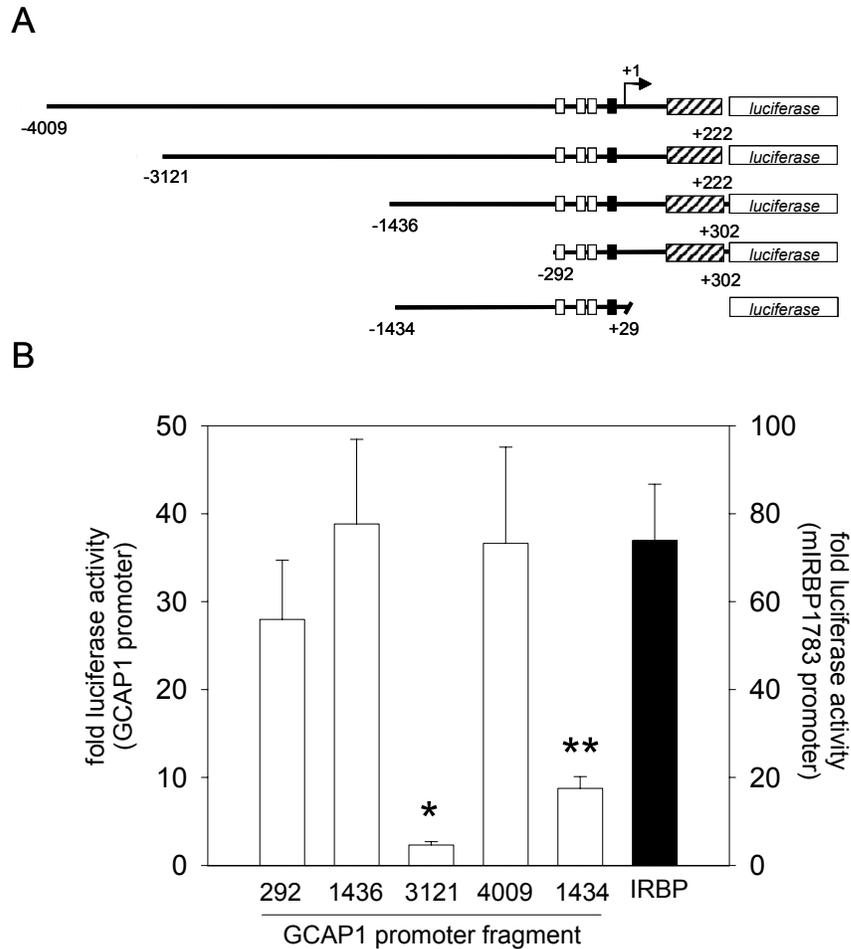


Figure 2-2. GCAP1 promoter activity in transfected E12 primary chicken embryonic retinal cultures. **A.** Diagram of the five GCAP1 promoter fragments cloned into the pGL2 vector. Each promoter fragment, except for 1434, contains a 25 bp repeated sequence (hatched bars) located within the 5' UTR and all constructs contain three proximal cone-rod homeobox (CRX)-like binding elements (white bars) and a putative TATA box region (black bars). **B.** Histogram showing levels of luciferase activity in primary retinal cultures 48 hours post-transfection. Each bar represents the mean \pm SEM activity obtained from 4 separate experiments for each promoter fragment. Open bars = GCAP1 promoter fragments; filled bar = mIRBP1783 promoter; * = activity significantly less than 292, 1436, 4009 and 1434, $p < 0.05$; ** = activity significantly less than 292, 1436 and 4009, $p < 0.05$.

mounts of retinas taken from E6 and E20 embryos that had been injected with TY-EF1 α -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. 2-3A). Cross-sectional analyses of these retinas revealed that the nlacZ reporter gene was

being 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 EF1 α promoter was similar at both of these stages of development (Fig. 2-3B, C and D). The column-like staining pattern that we observed in the retinas of the embryos injected with TY-EF1 α -nlacZ virus closely resembled the staining pattern that has been reported in retroviral studies of cell lineage in developing retina (Fekete et al. 1994). This result suggests that the TY-EF1 α -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, column-like nlacZ staining pattern observed in the retinas of embryos injected with TY-EF1 α -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. 2-3E). Cross-sectional analyses of these retinas revealed that expression of the mIRBP1783 promoter was restricted to photoreceptor cells (Fig. 2-3F), a result that corroborates previous studies of the cell-specificity of this promoter fragment (Boatright et al. 1997a; Boatright et al. 2001; Chang et al. 2000). An analysis of selected pineal glands and brains taken from E20 embryos showed that transduction of these tissues was minimal or undetectable following neural tube injection of lentivirus (data not shown). Together, these results indicated 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. 2-2B) were analyzed *in vivo*. Lentiviral vectors containing the 292, 1436 and 4009 GCAP1 promoter fragments driving the nlacZ reporter gene were

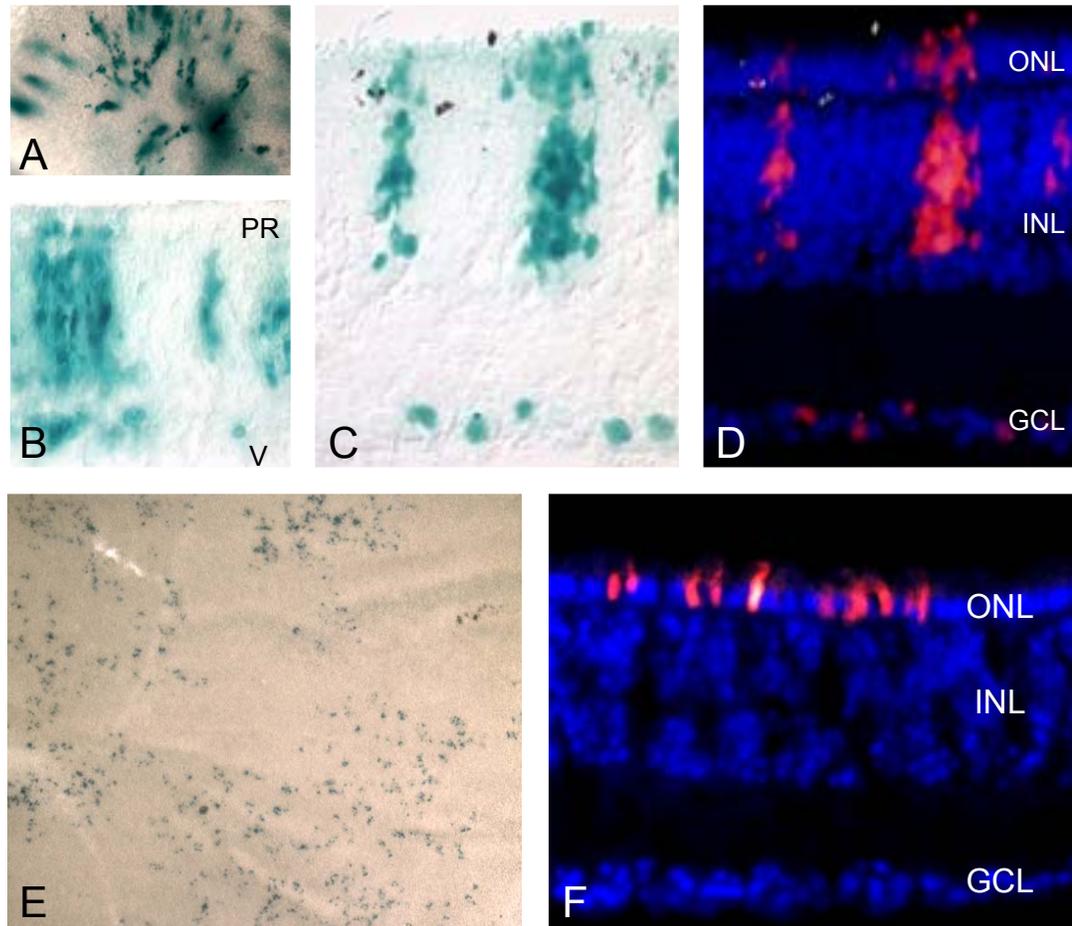


Figure 2-3. Retinal whole mounts and cross-sections prepared from embryos that received injections of TY-EF1 α -nlacZ (A-D) or TY-mIRBP1783-nlacZ lentivirus (E,F). **A.** Area from a whole mount of an E6 retina showing a cluster of infected clones intensely stained with X-gal. **B.** Cross-section through retina shown in panel A. **C.** Cross-section through a retina taken from an E20 embryo showing presence of cells stained with X-gal in all retinal cell layers. **D.** Inverted brightfield image shown in panel C (nlacZ-positive cells in red) overlaid with the DAPI image (in blue) to clearly show the retinal cell layers. **E.** Area from a whole mount of an E20 retina taken from an embryo injected with TY-mIRBP1783-nlacZ lentivirus and stained with X-gal. **F.** Cross-section through the retina from panel E showing that the mIRBP1783 promoter limits 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.

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. 2-1) 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 prior to hatching (E20).

Onset of expression in developing retina

X-gal stained retinal cells could be detected in whole mounts of retinas taken from embryos that had been injected with either the 292 or the 1436 promoter-nlacZ lentiviral vector as early as E12. 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 nlacZ expression was observed at E16 (1 positive retina out of $n = 6$). 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 whole mounts of the retinas that had been removed from embryos injected with the various lentiviral vectors (Fig. 2-4A). 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 non-specific cellular staining pattern observed in retinas transduced with either the 292 or the 1436 promoter-nlacZ transgenes, 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 vitro*, a result which 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 ~50% of the 5' 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

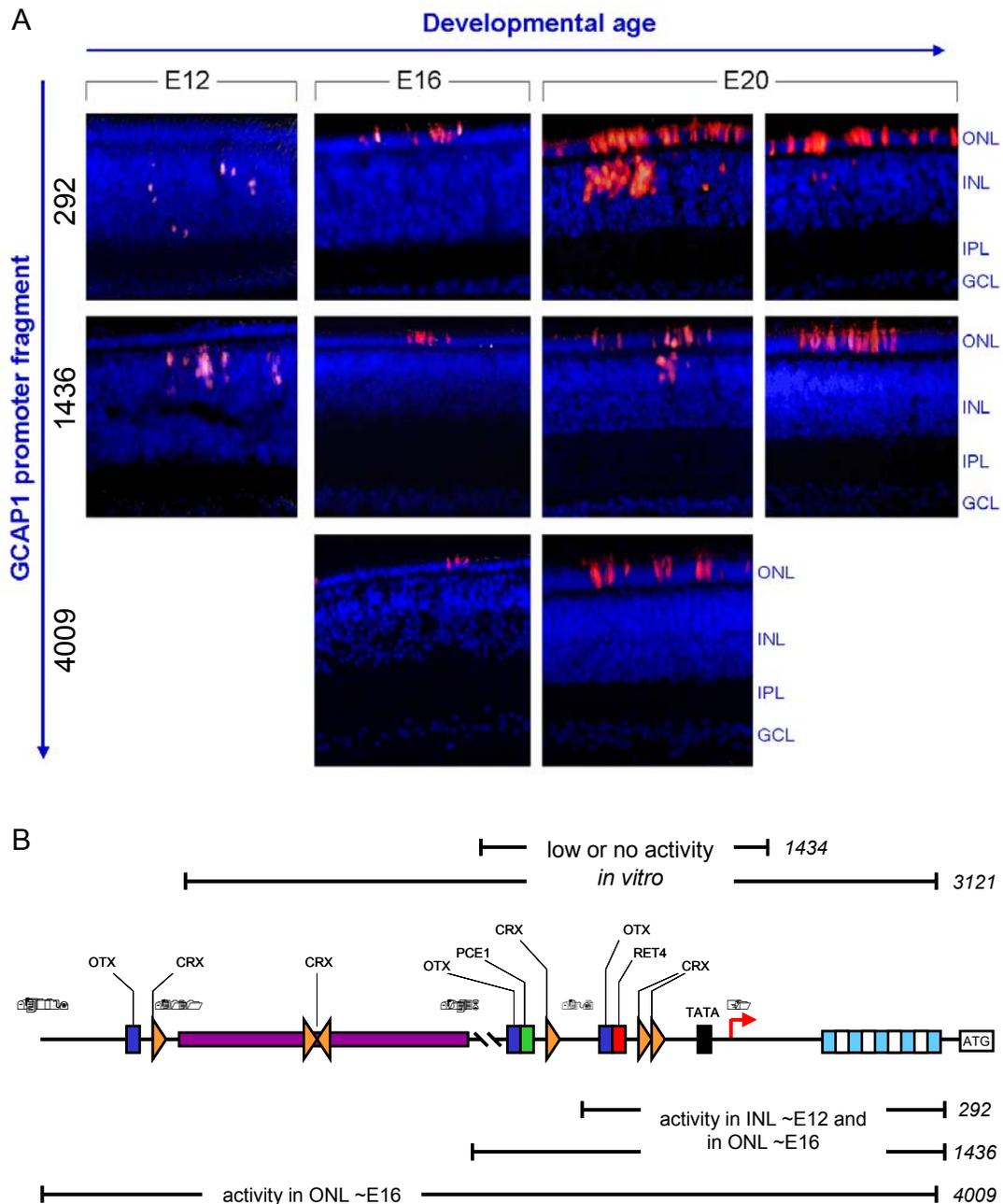


Figure 2-4. Cell specificity and temporal onset of activity of the 292, 1436 and 4009 GCAP1 promoter fragments in embryonic chicken retina. A. Examples of cross-sections through X-gal stained retinal whole mounts (nlacZ-positive cells = red; DAPI = blue). The embryonic ages are indicated along the top axis of the figure and the GCAP1 promoter fragment is indicated along the side

axis of the figure. At E12, X-gal staining was observed in the inner nuclear layer (INL) of retinas transduced with either the 292 or the 1436 promoter-nlacZ transgene. By E16 and E20, X-gal staining was observed predominately in the outer nuclear layer (ONL) and to a lesser extent in the INL. For the 292 and 1436 promoter fragments, two panels containing images from different regions of the same E20 retinas are shown to illustrate the fact that in some areas, X-gal staining was restricted to the ONL and that in other areas, staining was present in both the ONL and the INL. No detectable X-gal staining was observed in retinas of E12 embryos injected with the 4009 promoter-nlacZ lentiviral vector and only light staining was observed in the ONL at E16. By E20, the level of staining in these retinas had increased sufficiently to allow easy identification of the X-gal positive cells. Thirty to eighty cross-sections cut from two to six retinas were analyzed for each promoter fragment at the different developmental ages (see the Methods and Results sections for details). IPL = inner plexiform layer; GCL = ganglion cell layer. **B.** Schematic of the chicken GCAP1 promoter showing putative cis-DNA binding elements for retina- and photoreceptor-specific transcription factors (not to scale). The bracket bars highlight the different regions of the GCAP1 promoter containing included in the various fragments that were examined. Violet box = region between nucleotides -1437 and -3121 that negatively affected promoter activity; red arrow = transcription start point; blue and white-striped box = 25 bp repeated sequence within 5' UTR; ATG = translation start codon.

nucleotides -3122 and -4009 to the fragment. It is possible that the observed decrease in activity of 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 required to confer significant levels of activity to the longer GCAP1 promoter fragments. To test these possibilities, it will be necessary to assay the transcription levels and functional activities of additional GCAP1 promoter/reporter constructs.

In vivo analyses of the expression characteristics of the GCAP1 promoter fragments were performed in order 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* prior to the normal onset of expression of the GCAP1 gene during developmental. 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 (Boatright et al. 2001; Bobola et al. 1995; Fei et al. 1999; Kennedy et al. 2001; Mani et al. 2001; Yokoyama et al. 1992; Zack et al. 1991). 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 required to delay the onset of expression, a result similar to that reported in recent *in vivo* studies of the *Xenopus* rhodopsin promoter (Kennedy et al. 2001).

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. 2-4B). We have previously reported that at least two putative CRX-like binding sites (C/TTAATC/T) are present within the first 1kb upstream of the transcription start point in the 5' flanking region of the chicken GCAP1 gene (Semple-Rowland et al. 1999). In

addition, one Ret-4-like element (-187 to -184) (Chen and Zack 1996), two OTX-like binding elements (-196 to -202 and -832 to -838) (Chen and Zack 1996; Kimura et al. 2000) and one PCE-1/Ret-1-like element (-818 to -825) (Kikuchi et al. 1993; Morabito et al. 1991; Yu and Barnstable 1994) are also located within this region (see Fig. 2-4B). 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 above, our *in vitro* data indicates 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 (Bessis et al. 1997; Schoenherr et al. 1996; Weber and Skene 1997; Weber and Skene 1998) could play a role in suppressing GCAP1 promoter activity in non-photoreceptor 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 required to restrict its activity to photoreceptor cells *in vivo* (Boatright et al. 2001).

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 required for photoreceptor-specific gene expression (Boatright et al. 1997a; Bobola et al. 1995; Fei et al. 1999; Kimura et al. 2000). Examination of the sequence located upstream of the 1436 promoter fragment revealed the presence of additional putative homeodomain protein-binding elements (see Fig. 2-4B). 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 (Livesey et al. 2000). Additional CRX-like (-3305 to -3310) and OTX-like (-3356 to -3362) DNA binding elements are located within the -3122 to -4009 region of the GCAP1 promoter, elements that could potentially influence the expression characteristics of the 4009 promoter fragment (see Fig. 2-4B). The results of these experiments provide a rough blueprint of the structural and functional organization of the chicken GCAP1 promoter. Additional studies will be required 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 have demonstrated that lentivirus can transduce chicken retinal progenitor cells. In addition, we show 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 here 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 show 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 (Kumar et al. 2001). The large cargo capacity of this vector is an important feature of this system that will make it useful for 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.

CHAPTER 3
IN VIVO ANALYSES OF THE DEVELOPMENTAL AND CELL-SPECIFIC
ACTIVITY OF THE HUMAN RETINAL GUANYLATE CYCLASE-1 (GC1)
PROMOTER

Introduction

Retinal guanylate cyclase (GC)-1 and GC2 are two particulate GC enzymes that catalyze the conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP), a key second messenger molecule in the phototransduction cascade (Pugh, Jr. and Lamb 1990). The synthesis of cGMP is essential for recovery of the dark state following photoexcitation of photoreceptor cells (Pugh, Jr. and Lamb 1990). The activities of retinal GCs are modulated by guanylate cyclase activating proteins (GCAPs), a family of EF-hand calcium binding proteins that inhibit or stimulate enzyme activity under high or low intracellular calcium conditions, respectively (Mendez et al. 2001).

The mature GC1 protein is localized to photoreceptor outer segment membranes and the results from some studies suggest that it is expressed at higher levels in cone cells than in rod cells (Cooper et al. 1995; Dizhoor et al. 1994; Liu et al. 1994). GC1 is also expressed in the pineal gland, indicating that GC1 may also play a role in pinealocyte phototransduction (Venkataraman et al. 2000).

Mutations in the GC1 gene have been linked to specific types of inherited retinal dystrophies including autosomal recessive Leber congenital amaurosis type 1 (LCA1) and autosomal dominant cone-rod dystrophy (ADCRD) (Kelsell et al. 1998; Perrault et al. 1998). Most of the LCA1 mutations are frameshift and missense mutations that lead to

the absence of GC1 or to the abolition of its activity in photoreceptor cells (Rozet et al. 2001). All ADCRD mutations occur in a three-codon sequence located within the region of the GC1 gene encoding the dimerization domain of the enzyme. These mutations are predicted to alter the function of GC1 by enhancing or decreasing its ability to respond to GCAP1 stimulation (Duda et al. 1999; Tucker et al. 1999; Wilkie et al. 2000).

One of our research goals is to determine if photoreceptor function and vision can be restored in the avian model of LCA1, the GUCY1*B chicken (Semple-Rowland et al. 1998; Semple-Rowland and Lee 2000). The recent demonstration that viral vector-mediated gene therapy can be used to restore functional vision in a canine model of LCA2 (a subtype of LCA caused by a mutation in the RPE65 gene) (Acland et al. 2001) support the use of viral vectors for the study and treatment of LCA. We are currently conducting studies to examine the feasibility of using a lentiviral vector to deliver a functional GC1 transgene to the retinal progenitor cells of these animals.

The ability to target viral transgene expression to specific cell types and to control expression levels of the transgene are important factors that must be addressed when developing gene therapy strategies. Currently, cell-specific promoters are used in viral vectors to direct expression of transgenes to specific cell types, and the level of expression of these transgenes is controlled by selecting promoters that possess different intrinsic activity levels (Dejneka et al. 2001; Harvey and Caskey 1998; Kafri et al. 2000; Reiser 2000; Takahashi et al. 1999).

In the previous chapter, we showed that a 4.0 kb fragment of the chicken GCAP1 promoter fulfills many of the requirements that we deem important for appropriate expression of a GC1 transgene in chicken retina. Shortly after the completion of these

experiments, we initiated a collaboration with Dr. Hans-Jurgen Fülle's laboratory to examine the expression characteristics of the human GC1 promoter *in vivo* using the experimental paradigm presented in Chapter 2. The primary impetus for conducting these analyses was our goal to identify a promoter fragment that most closely mimics the expression characteristics of the native GC1 promoter and could be used to drive GC1 transgene expression in our vectors. In addition, previous efforts to clone the chicken GC1 gene and 5' flanking region were unsuccessful and the human promoter was a viable alternative. Human GC1 promoter-nlacZ transgenes were packaged into lentiviral vectors and their expression characteristics were examined *in vivo* using the experimental paradigm described in Chapter 2.

Methods

Preparation of Constructs

Three fragments of the human GC1 promoter (named GCE1, GCE7 and GCE8) were selected for use in this study based on the results of previous analyses showing that they exhibited significant levels of activity in human retinoblastoma cells (Fulle and Gallardo 2001). All cloning of the promoter fragments into the pTYF transducing vector (modified pTY vector that is described in Chapter 4) of the lentiviral vector system were carried out in the laboratory of Dr. Hans-Jurgen Fülle as described below. The three promoter fragments were amplified using the polymerase chain reaction (PCR) and *Pfu* DNA polymerase (Stratagene). The core sequences of the primers for the designated GC1 promoter fragments were as follows: GCE1 (sense = 5' CAC TTG TTA CTT TCT GGC TGA; antisense = 5' GGT CAT TGC CGG CCG GCT T); GCE7 (sense = 5' TCT GCT CCT CAT CCA ACA TTT C; antisense = same as GCE1); GCE8 (sense = same as GCE7; antisense = 5' CAC AGG TCT TCC TTG CCA G). *NotI* and *PmeI* restriction

enzyme recognition sequences were added to the sense and antisense primers, respectively. The CMV promoter was excised from the pTYF.CMV.nlacZ vector using *NotI* and *PmeI* and replaced with PCR products digested with *NotI* and *PmeI* from the aforementioned reactions to generate the GC1 promoter-nlacZ expression vectors depicted in Fig. 3-1. Transfection-grade plasmid DNA was prepared using Qiagen endotoxin-free MaxiPrep kits.

Production of Lentiviral Vector and Titters

The production, concentration and titering of viruses used for experiments in this study were performed as described in the Chapter 4 Methods section. Briefly, final, infectious titers were estimated by multiplying the concentration of p24 antigen (ng/ml) in vector stocks by the average specific transducing activity (TU/ng p24) of vector standard that was produced using the methods described in Chapter 4 (6.1×10^3 TU/ng p24). Each virus preparation yielded stocks with estimated infectious titers that were between $0.1-1.0 \times 10^{10}$ TU/ml ($0.5-1.0 \times 10^4$ ng p24/ml).

Embryonic Injections

Neural tube injections of stage 10-12 embryos were performed as described in the Chapter 2 Methods section.

Tissue Preparation, Histochemistry and Microscopy

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. Retinal whole mounts were prepared by placing the tissue photoreceptor side down on a Millipore-Millicell insert containing PBS and flattened 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 16-18 hours. Following this incubation, the retinas were rinsed three times in PBS, cryoprotected with 30% sucrose and mounted in optimal cutting temperature (OCT) medium for cryosectioning. Serial sections (20 µm) were cut through areas positive for X-gal staining using a cryostat, mounted on slides, and counterstained with 4,6-diamidino-2-phenylindole (DAPI; Molecular Probes). The sections were then coverslipped using an aqueous-based mounting medium (Gel Mount, BioMedia). Thirty to eighty sections, taken from the retinas of at least three different animals injected with the human GC1 promoter-nlacZ viral vectors were analyzed for each time point.

In some cases the pineal glands and brains of E18.5 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-thick sections using a vibratome and placed in the wells of a 12-well tissue culture plate. The sections of brain were stained with X-gal for 16-18 hours, rinsed in PBS and viewed under a Zeiss dissecting microscope. Brightfield and fluorescence microscopy were performed as described in Chapter 2.

Results

Primary Sequence Analyses

The general structure and organization of the human GC1 gene has been described previously (Yang et al. 1995; Yang et al. 1996). Recent analyses of the GC1 5' flanking region revealed that the 5' UTR is comprised of a 110 bp non-coding exon and a 304 bp intron and that the signal peptide and translation start codon of GC1 are located in exon 2

as illustrated in (Fig. 3-1; (Fulle and Gallardo 2001)). In the present study, *in silico* analyses were performed using the web-based versions of TRANSFAC (v4.0, TESS; <http://www.cbil.upenn.edu/tess/>) and the Eukaryotic Neural Network Promoter Prediction (http://www.fruitfly.org/seq_tools/promoter.html) programs. Fig. 3-1A shows a summary of the results obtained from these analyses, which revealed that a putative transcription start point (tsp) of GC1 lies 1.338 kb upstream of the ATG and that a strong TATA-box consensus sequence (TATAA/tAa/t) lies ~20 bp upstream of this site between nucleotides –1320 and –1327 (the first nucleotide of the GC1 translation start site [ATG]= +1). However, in a previous study of the bovine GC1 5' flanking region, the tsp was experimentally shown to be located within exon 1 (equivalent to nucleotide -425 in Fig. 3-1A) and was associated with an initiator (Inr) consensus site (Johnston et al. 1997). Since the overall homology of the bovine and human 5' flanking sequences is high and the results of these analyses do not concur, additional studies will be required to determine the precise location of the tsp in the human GC1 gene. Two cone-rod homeobox protein (CRX)-binding elements (CBEs; consensus CTAATNAGCTY) organized in a head-to-tail arrangement were identified at positions –459 to –469 and –1539 to –1549 relative to the translation start site. A 12-bp AT-rich sequence (TATATAATTGCT) that is repeated five times was identified between nucleotides –1195 and –1327; the significance of this repeat is unknown, but it harbors near-consensus sequences for binding of core promoter constituents such as TATA-binding protein and TFIID. The sequence and location of the –459/–469 CBE is conserved in the bovine GC1 promoter (data not shown). Overall, these results suggest that the core promoter region of

the human GC1 gene may be located near the non-coding exon 1 and contains putative CBEs that could contribute to the restricted expression pattern of GC1 *in vivo*.

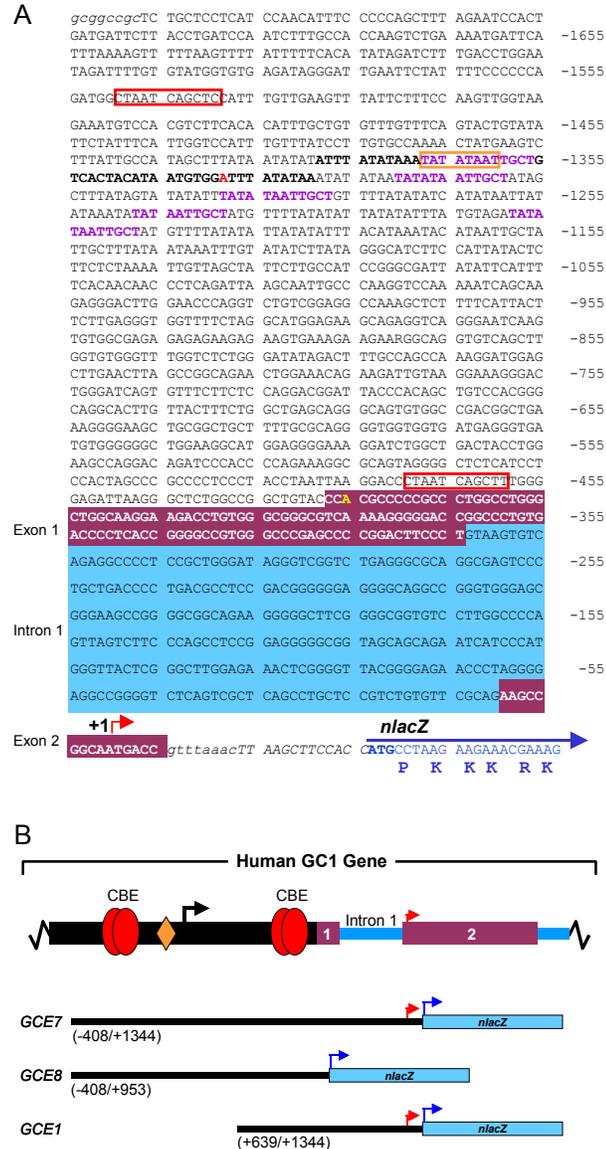


Figure 3-1. Sequence and schematic of the retinal GC1 5' flanking region-nlacZ fusion constructs. **A.** Partial sequence showing the intron-containing promoter-nlacZ fusion region of the pTYF-based constructs. *CBE* = CRX-binding element; *red boxes* = CBEs; *orange box* = putative TATA box; *purple text* = unique AT-rich repeated sequence; *purple boxes* = exons; *light blue box* = intron; *red A* = putative GC1 *tsp* identified using *in silico* analyses; *yellow A* = bovine GC1 *tsp*; *red arrow* = GC1 start codon (+1); *blue text* = nlacZ ORF with peptide sequence of the nuclear-localizing sequence of the SV40 large T-antigen; *italics text* = vector sequence. **B.** Diagram of the human GC1 promoter and schemes of the nlacZ constructs. *Orange diamond* = TATA box; *red arrow* = GC1 ATG; *blue arrow* = nlacZ ATG; *purple box* = exon.

Tissue Specificity of nlacZ Expression

All of the GC1 promoters tested drove expression of nlacZ in the retinas, but not the brains, of E18.5 embryos that had been injected with lentivirus at developmental stage 12. No nlacZ-positive cells were detected in the pineal glands of embryos that had been injected with the GCE1- or GCE8-nlacZ lentiviruses. One out of the two pineal glands examined from embryos injected with GCE7-nlacZ lentivirus contained nlacZ-positive cells. These cells were positioned near the lumen suggesting that they were pinealocytes (Fig. 3-2).

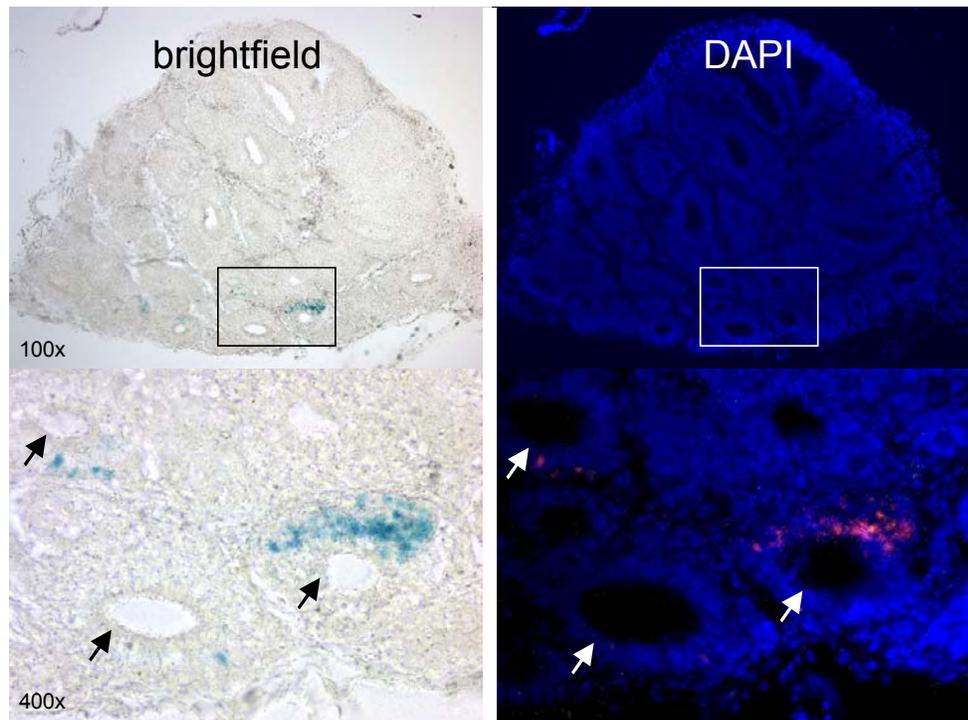


Figure 3-2. Cross-sections of pineal gland from E18.5 embryo that was injected with the TYF-GCE7-nlacZ virus. Arrows indicate lumen with pinealocytes positioned around the perimeter. X-gal staining is shown as blue (left panel) or red (right panel). The right panel shows the overlay of the negative brightfield image shown on the left and the DAPI image.

Cell Specificity and Developmental Expression of nlacZ

In Chapter 2, we showed that expression of the GC1 gene in developing chicken retina begins at approximately E14. Based on this observation, we chose to examine

retinas from injected embryos at times prior to (E10), equivalent to (E13) and several days after (E18.5) the onset of GC1 expression in order to examine the developmental activities of the GC1 promoter fragments. Very few or no nlacZ-positive cells were detected in the retinas of E10 and E13 embryos that were injected with GCE1-nlacZ lentivirus. By E18.5, a significant number of nlacZ-positive cells were detected in both the outer nuclear layer (ONL) and inner nuclear layer (INL) of these animals (Fig. 3-3, top panel). In contrast, several nlacZ-positive cells that were distributed throughout all cell layers in the retinas of E10 and E13 embryos that had been injected with GCE7-nlacZ lentivirus. By E18.5, the expression of nlacZ was restricted to photoreceptor cells in the ONL (Fig. 3-3, middle panel). Finally, nlacZ positive cells were restricted to cells located in the central band of the INL and to cells within the ONL in the retinas of E10 and E13 embryos that had been injected with GCE8-nlacZ lentivirus (Fig. 3-3, bottom panel). By E18.5, the expression of nlacZ was restricted to the ONL, a pattern resembling that generated by the GCE7 promoter.

Discussion

In Chapter 2, we established the usefulness of lentiviral-mediated gene transfer for studying promoter function *in vivo*. In the present study, we used a similar experimental paradigm to examine the expression characteristics of human GC1 promoter-nlacZ transgenes in the developing retina. The results of this study demonstrate that a 1.0 kb region of the human GC1 promoter located between nucleotides -386 and -1745 is sufficient to direct gene expression specifically to photoreceptor cells and to the pineal gland *in vivo*. Furthermore, our results show that the cellular specificity of the activity of this region of the GC1 promoter increases as a function of retinal development. Between E10 and E13, promoter activity was observed in all retinal cell layers, but by E18, this

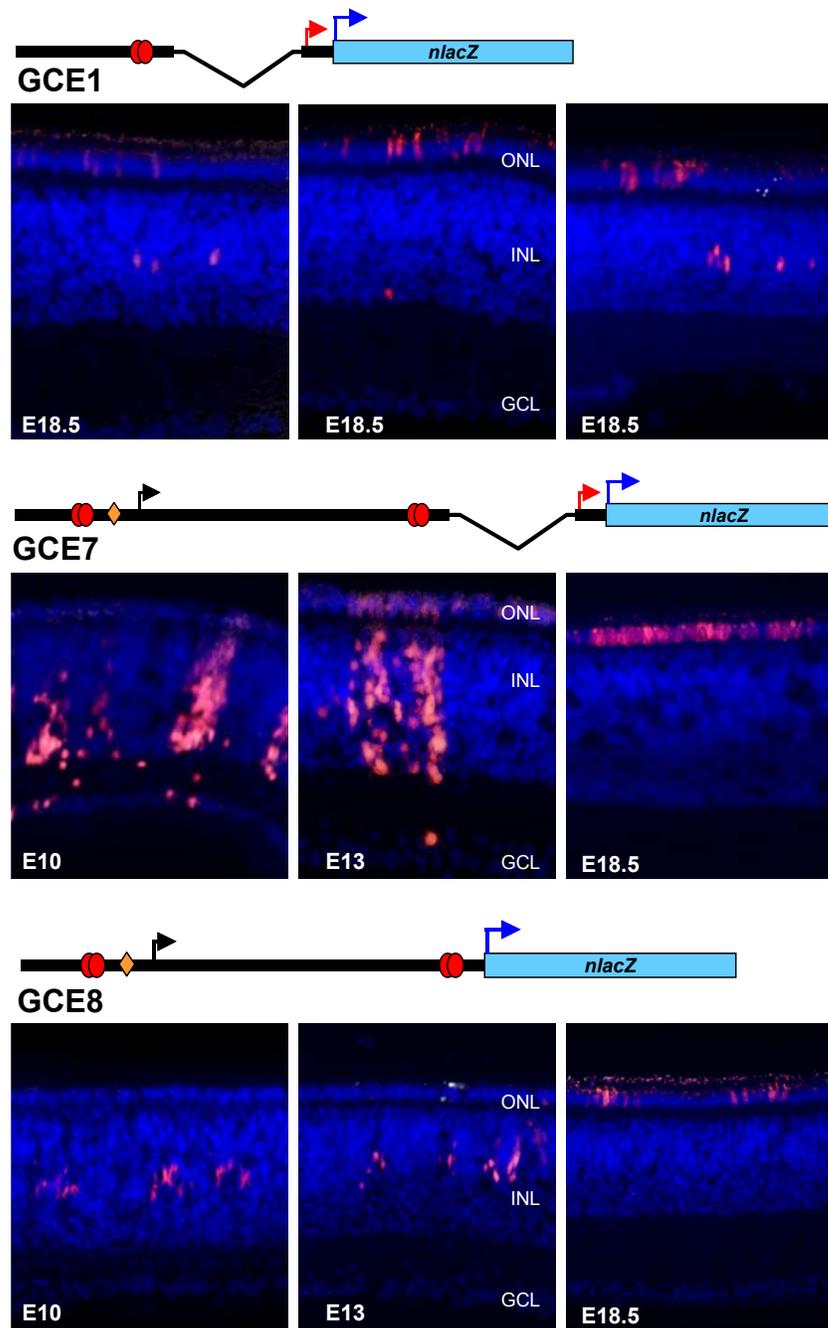


Figure 3-3. Cross-sections of retinas containing human GC1 promoter-nlacZ transgenes. A schematic of the lentiviral transgenes used for injections is depicted above each panel (refer to Fig. 3-1B for details). X-gal staining is red (nlacZ-positive cells) and DAPI staining is blue (cell nuclei). ONL = outer nuclear layer; INL = inner nuclear layer; GCL = ganglion cell layer; red ellipses = cone-rod homeobox protein binding element; orange triangle = putative TATA box; red arrow = GC1 translation start site; blue arrow = nlacZ translation start site.

activity was restricted to the ONL. Overall, the intensity of X-gal staining was significantly lower in GCE8 retinas than in GCE7 retinas. These results suggest that expression driven by the -386/-1745 region of the GC1 promoter is augmented by the presence of intron 1 (compare GCE7 and GCE8, Fig. 3-3).

CRX is a photoreceptor-specific transcription factor that plays an important role in regulating the expression of several photoreceptor-specific and pineal-specific genes, including itself (Furukawa et al. 2002; Livesey et al. 2000). It also appears to have an important role in regulating photoreceptor development (Chen et al. 1997; Furukawa et al. 2002; Furukawa et al. 1997; Furukawa et al. 1999). Comparisons of the results obtained for all three GC1 promoter fragments suggest that the two consensus CBEs identified in the proximal and distal regions of the promoter may be required for directing expression of GC1 to the photoreceptor cells *in vivo*. These results are consistent with studies showing that the promoters of several photoreceptor-specific genes contain multiple copies of CBEs (Livesey et al. 2000). Further experiments will be required to confirm the importance of these and other *cis*-acting elements in regulating the activity of the GC1 promoter.

The temporal expression profiles of the GCE7 and GCE8 promoter fragments revealed that these fragments did not possess the same temporal expression pattern as that exhibited by the intact GC1 gene in developing retina. One plausible explanation for our observations is that the endogenous GC1 transcription onset in developing retina occurs earlier than E14. Use of more sensitive detection methods, such as RT-PCR, may show that GC1 gene transcription does begin at an earlier stage of development. It is unclear why the activities of the GCE7 and GCE8 promoter fragments are restricted to the ONL

at E18.5, but not at earlier stages of development. The cellular specificity of GC1 expression has not been examined in the developing retina. One possible explanation for our observations is that the *cis*-elements required to silence GC1 expression in non-photoreceptor cells early in development are not present in these fragments. Another possibility is that the expression of the human GC1 promoter is not regulated in a normal manner in the avian retina. Finally, it is possible that the GC1 gene is expressed in all retinal cells early in development of the retina and its expression becomes more restricted over the course of development so that its expression is limited to photoreceptor cells in the late stages of development.

In summary, the results from these analyses show that the GC1 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*. By conducting these analyses *in vivo*, we have identified a GC1 promoter fragment, GCE7, which possesses expression characteristics that should be suitable for use in our future efforts to drive express lentiviral GC1 transgenes in chicken retina.

CHAPTER 4
IMPROVEMENTS IN THE DESIGN AND PRODUCTION OF HIV-1-BASED
LENTIVIRAL VECTORS RESULTS IN HIGH TRANSDUCTION EFFICIENCY IN
RETINA AND THE EFFICIENT EXPRESSION OF A RETINAL GUANYLATE
CYCLASE-1 (GC1) TRANSGENE

Note

The work presented in this chapter was published as part of a research article that appeared in *Physiological Genomics* **12**, 221-228 (2002). The guanylate cyclase activity assays were performed by Izabela Sokal in the laboratory of Dr. Krzysztof Palczewski.

Introduction

Lentiviral vectors derived from the human immunodeficiency virus type 1 (HIV-1) are emerging as the vectors of choice for long-term, stable *in vitro* and *in vivo* gene transfer. These vectors are attractive because they can carry large transgenes (up to 18 kb in size) (Kumar et al. 2001) and they are capable of stably transducing both dividing and quiescent cells (Iwakuma et al. 1999; Miyoshi et al. 1998; Zufferey et al. 1998).

The increase in interest in these vectors has given rise to a need for efficient and reproducible methods to produce large quantities of high-titer lentiviral vector.

Traditionally, lentiviral vectors are produced by co-transfecting human cell lines with plasmid DNAs that encode the viral components required for viral packaging. Transient transfection of these cell lines is often accomplished using the conventional calcium phosphate co-precipitation technique (Naldini et al. 1996). Disadvantages of this method include: (1) the large amount of plasmid DNA that is required for transfection; (2) the difficulties associated with scaling up the precipitation reaction; and (3) the high degree

of variability observed in transfection efficiency and viral production. Recently, several groups have developed packaging cell lines that facilitate the production of lentiviral vectors by reducing the need for multi-plasmid transfections (Farson et al. 2001; Klages et al. 2000; Pacchia et al. 2001; Xu et al. 2001). Although the use of packaging cell lines has streamlined the packaging procedure, the resulting viral titers have not been significantly higher than those obtained using transient co-transfection methods. In addition, the advantages of these new cell lines are often offset by the need to develop new lines for each generation of improved lentiviral vector.

To achieve large-scale production of high-titer lentiviral vector it is critical that transfection of the virus-producing cell cultures be both efficient and reproducible; however, little effort has been made to optimize this step in vector production. The results from the experiments presented in Chapters 2 and 3 demonstrate that lentiviral vectors transduce cells in developing retina, but improvements to the vector system and production methods would make this system more suitable for our future studies in the GUCY1*B chicken model of LCA1. The goals of the experiments described here were (1) to design and produce lentiviral vectors that exhibit high transduction efficiency in developing chicken retina and (2) to construct a vector that produces active guanylate cyclase-1 (GC1) and can be used as a base vector to develop therapeutic vectors for gene therapy studies aimed at treating LCA1. We were able to accomplish our first goal by combining a transfection method that utilizes the activated dendrimer-based transfection reagent, Superfect, with a novel vector concentration protocol. By using our new method, we were able to reproducibly generate lentiviral vector stocks with titers greater than 1×10^{10} transducing units per ml (TU/ml) using less than one-third of the total amount of

plasmid DNA that is commonly required for production of this vector To achieve our second goal, we constructed a modular lentiviral vector system that encodes functional GC1 and carries a multiple cloning site that facilitates the interchange of transgene components into the vector.

Materials and Methods

Lentiviral Vector Constructs

The transducing vector used in our experiments was derived from a previously described self-inactivating vector (Cui et al. 1999;Iwakuma et al. 1999). The pTY vector was modified by inserting a cPPT-DNA FLAP element upstream of the multiple cloning site, an element that has been shown to significantly improve the transduction efficiency of recombinant lentiviral vectors *in vitro* and *in vivo* (Follenzi et al. 2000;Zennou et al. 2001). All polymerase chain reaction (PCR) products used for cloning as described below were amplified using *Pfu* high-fidelity DNA polymerase and cloned into intermediate pTOPO-BluntII vectors (Invitrogen) for use in subsequent steps. ***pTYF.linker***: A 186-bp fragment containing the cPPT-DNA FLAP sequence was amplified from the pNHP vector using core primers that have been previously described (Zennou et al. 2000). *EagI* and *NotI* linkers were added to the sense and antisense primers, respectively. The resulting fragment was excised with *NotI* and *EagI* and cloned into the *NotI* site of the pTY vector in the sense orientation, creating the pTYF.linker vector (Fig. 4-1 A). The integrity of this modification was verified by DNA sequencing. ***pTYF.EF1 α .linker***: The human elongation factor-1 α (EF1 α) promoter was amplified from pTY.EFGFP (Zaiss et al. 2002) using sense and antisense primers containing *NotI* and *NheI* linkers, respectively. The EF1 α promoter was excised using *NotI* and *NheI* and then cloned into

the *NotI* and *NheI* sites of pTYF-linker thereby generating the pTYF.EF1 α .linker vector (Fig. 4-1 B). ***pTYF.EF1 α .PLAP***: The placental alkaline phosphatase (PLAP) reporter gene was amplified from pRISAP (Chen et al. 1999) (gift from C. Cepko) with sense and antisense primers containing *PmeI* and *KpnI* linkers, respectively. The PLAP *PmeI/KpnI* fragment was then cloned into the *SmaI/KpnI* sites of pTYF.EF1 α .linker to make the pTYF.EF1 α .PLAP vector (Fig. 4-2). ***pTYF.EF1 α _IRES.EGFP***: The polio virus internal ribosome entry site (IRES) was obtained from pTYAT.CBA_IRES.EGFP (gift from A. Timmers) using sense and antisense primers containing *SmaI-ClaI* and *MluI* linkers, respectively. The cDNA encoding enhanced green fluorescent protein (EGFP) was amplified from pEGFP-N1 (Clontech) using sense and antisense primers with *KpnI-EcoRV* and *MluI* linkers, respectively. The IRES and EGFP fragments were excised using *SmaI/MluI* and *MluI/KpnI* and ligated into the *SmaI* and *KpnI* sites of pTYF.EF1 α .linker, resulting in the pTYF.EF1 α _IRES.EGFP cloning vector (Fig. 4-1 C).

pTYF.mIRBP1783.bGC1: First, a pTYF-mIRBP1783-linker vector was generated by excising the mIRBP1783 promoter from pTYF-mIRBP1783-nlacZ with *NotI* and *PmeI*. The fragment was then cloned into the *NotI/SmaI* sites of the pTYF.linker vector. The cDNA encoding bovine GC1 was amplified from the pSVL GC1 clone using the following primers: sense – 5'-CCA TCG ATA GTT TAA ACG AGC CCC GGA CTT; antisense – 5'-CCA TCG ATG ACC CAG CCT CAC TTC C. The resulting fragment was cloned into pTYF-mIRBP1783-linker using *ClaI*. The amplified bovine cDNA, which included the entire open reading frame (ORF), extends from nucleotide 26 to nucleotide 3393 (GenBank L37089).

pTYF.EF1 α .bGC1-IRES-EGFP: The bGC1 ORF was excised with *ClaI* and cloned into the *ClaI* site of the pTYF.EF1 α _IRES-EGFP vector. ***pTYF.GCE7.bGC1-IRES-***

EGFP: The GCE7 promoter was excised from pTYF-GCE7-nlacZ (see Chapter 3) using *NotI* and *PmeI* and cloned into the *NotI/PmeI* site of pTYF.EF1 α .bGC1-IRES-EGFP. Transfection-grade DNA was prepared using endotoxin-free DNA mega- or maxiprep kits (Qiagen).

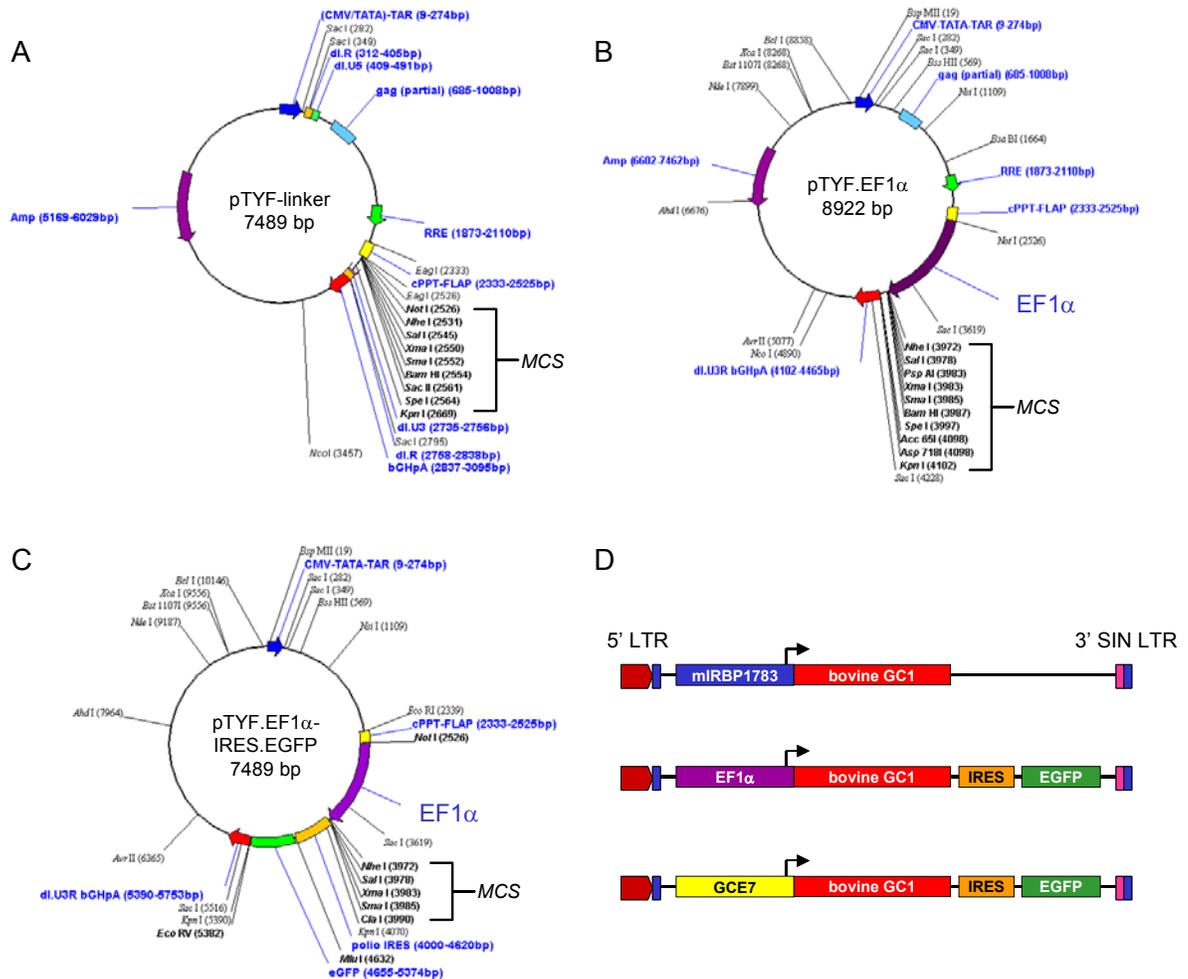


Figure 4-1. A – C. Maps of the modular cloning plasmid vectors constructed for the SIN lentiviral vector system used in this study. Note the extensive listing of unique cloning sites. **D.** Schematics of bovine GC1 expression cassettes cloned into pTYF-based vectors. The black arrow indicates the transcription start point.

Lentiviral Vector Production, Concentration and Titters

VSV-G-pseudotyped lentiviruses carrying an EF1 α -PLAP transgene were prepared using the lentiviral vector system illustrated in Fig. 2. 293T cells (Invitrogen Corporation,

#R70007) were seeded in 75 cm² (T-75) culture flasks at a density of 1 x 10⁷ cells per flask and grown in Dulbecco's modified Eagle medium (DMEM; Gibco) containing 10% fetal bovine serum and antibiotics (130 U/ml penicillin and 130 µg/ml streptomycin; growth medium). The cultures were maintained at 37°C in 5% CO₂ throughout the virus production period. On the following day, when the cultures reached 90-95% confluency, the growth medium was replaced with 5.0 ml of fresh medium.

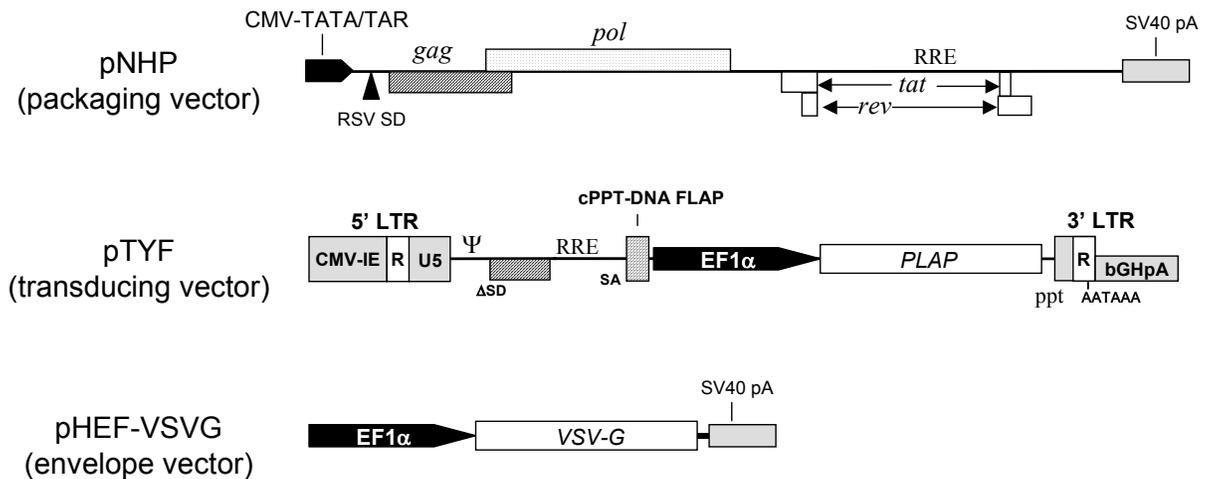


Figure 4-2. The HIV-1-based self-inactivating lentiviral vector system. The helper construct, pNHP, contains deletions in the regions encoding the accessory proteins *vif*, *vpr*, *vpu* and *nef* and has been previously described (Zaiss et al. 2002). The self-inactivating transducing construct, pTYF, has a central polypurine tract (cPPT)-DNA flap element located just upstream of the multiple cloning site and carries an EF1 α -PLAP transgene. The packaging construct, pHEF.VSVG, encodes the vesicular stomatitis virus G (VSV-G) glycoprotein for pseudotyping (Chang et al. 1999). The pTYF.EF1 α .PLAP construct was used to produce vector for the in vitro and in vivo experiments unless stated otherwise.

For one large-scale preparation of virus, 20 T-75 flasks of 293T cells were transfected as follows: Transfection mixture for all 20 flasks was prepared by gently mixing 142 µg pNHP, 70 µg pTYF and 56 µg pHEF.VSVG plasmid DNA and 8.0 ml DMEM in one 50 ml polystyrene tube. After mixing, 560 µl of Superfect was added to

the DNA solution. The contents of the tube were gently mixed and incubated at room temperature for 10 min. Next, 430 μ l of the Superfect-DNA mixture was added dropwise to the T-75 flask (transfection start point) and the flask was incubated for 4-5 h.

Following the incubation period, the medium containing the transfection mixture was replaced with 7.0 ml of fresh growth medium. The next day, the media containing the first batch of virus was harvested from each flask and 6.5 ml of fresh growth medium was added to the cells. Upon collection, all virus-containing media was filtered through a 0.45 μ m low protein-binding Durapore filter (Millipore) to remove cell debris. To prepare transfection mixture sufficient for one T-75 flask, the amounts of DNA, DMEM and Superfect were each divided by 20 to scale the reaction down. We have also found that viral vector can be produced in larger or smaller cell culture flasks or plates by simply scaling cell numbers and the amount of DNA, DMEM and Superfect linearly with respect to the cell growth area.

For some experiments, virus-containing media was concentrated using ultrafiltration and centrifugation as outlined in Diagram 1. For ultrafiltration, the virus stock collected from 20 T-75 flasks at 30 h post-transfection (~120 ml) was divided into two 60 ml aliquots and centrifuged through Centricon-80 ultrafiltration columns (Millipore) for 1 h in 4°C at 2,500 x g. The retentate was retrieved by centrifuging the inverted column for 1 min in 4°C at 990 x g and was stored at 4°C until further processing. On the following day, the virus-containing retentate was added to the ~120 ml of virus-containing media collected at 45 h post-transfection. Four 30 ml conical-bottom tubes (polyallomer Konical tubes; Beckman), each containing a 220 μ l cushion of 60% iodixanol solution (used directly from the Optiprep stock solution obtained from

Axis-Shield) were prepared. Iodixanol was used because of its demonstrated safety in human clinical trials (Jorgensen et al. 1992). Media containing virus (30 ml) was gently pipetted into each tube, taking care not to disturb the iodixanol, and the samples were centrifuged at 50,000 x *g* for 2.5 h at 4°C using a Beckman SW-28 swinging bucket rotor. The media just above the media/iodixanol interface was carefully removed from each tube and discarded, leaving ~750 µl of the solution in each tube (220 µl of iodixanol plus ~500 µl of media). The residual media containing virus and the iodixanol were mixed gently by shaking at 200 r.p.m for 2-3 h at 4°C. The resulting mixtures were pooled into one 3 ml conical-bottom tube (polyallomer Konical Tubes; Beckman) and centrifuged at 6100 x *g* for 22-24 h at 4°C using a Beckman SW-50.1 swinging bucket rotor. The resulting supernatant was removed and discarded and the remaining pellet was resuspended in 50 µl of PBS or artificial cerebrospinal fluid by incubating the virus at 4°C for 10-14 h. The final viral vector was gently mixed by pipetting, aliquoted and stored at -80°C until use.

Infectious titers of the TYF.EF1 α .PLAP virus were determined by incubating 1.75 x 10⁵ TE671 cells seeded in 12-well plates with limiting dilutions of the viral stock (1/10, 1/100 and 1/1000) in the presence of 8 µg/ml polybrene. After a 4-5 h incubation period, fresh medium was added directly to the cells and, after 48 h, cultures were fixed, rinsed in PBS, heated in PBS at 65°C for 30 min and stained for PLAP activity using previously reported methods (Fekete and Cepko 1993). The number of transducing units (TU; defined as an infectious particle) was determined by estimating the number of PLAP-positive cells per well and final infectious titers were expressed as TU/ml. Estimates of the infectious titers of vectors lacking a strong promoter or the PLAP marker gene were

based on the titers of unconcentrated TYF.EF1 α .PLAP virus that was produced in parallel each time.

Delivery of EF1 α -PLAP Vector to Chicken Neural Tube

The neural tube injections and preparation of retinal flat mounts were carried out using the methods described in Chapter 2 (Coleman et al. 2002). The brains of injected embryos were fixed overnight in 4% paraformaldehyde at 4°C. The next day, the tissues were rinsed thoroughly in PBS and 100 μ m thick sections were cut using a vibratome. Floating brain sections and retinal flat mounts were subsequently processed for routine PLAP histochemistry using the techniques described above and as described at <http://genetics.med.harvard.edu/~cepko/protocol/xgalplap-stain.htm>. All tissues were collected on embryonic day 7 (E7) or 2 days post-hatch, 5 or 23 days after injections, respectively. Digital images of retinal flat mounts were captured with a Nikon Coolpix 995 camera fitted to a Zeiss Stemi V6 microscope. In some cases, the percent area of retina transduced by the vector was determined as follows: TIFF images at a resolution of 1024 x 768 pixels were reduced by 35%, converted to grayscale using Adobe Photoshop and imported into the Scion Image program (available at <http://www.scioncorp.com>). The density slice setting was used to select all of the pixels within the area of the flat mount that represented PLAP-positive areas and these were expressed as a percent of the total retinal area. Three to seven retinas were analyzed for each dose of vector.

Analyses of GC1 Expression Vectors

GC1 immunocytochemistry was performed on dispersed primary chicken retinal cultures and DF-1 cells (immortalized chicken fibroblast cells; obtained from American

Tissue Culture Company) that were transiently transfected with the pTYF-IRBP1783.bGC1 or pTYF.EF1 α .bGC1/EGFP plasmid vectors, respectively.

Primary retinal cultures

The preparation, maintenance and transient transfection of the primary retinal cultures were performed as described under the *Methods* section in Chapter 2 with the exception that the cells were grown on glass coverslips coated with poly-D-ornithine.

DF-1 cell cultures

On the day prior to transfection, DF-1 cells were seeded into wells of 12-well plates that contained tissue culture-treated glass coverslips (Fisherbrand) and maintained in culture media as described above. Briefly, 3 μ g DNA was added to 50 μ l plain DMEM and mixed with 10 μ l Superfect and incubated at RT for 10 min. While the DNA-Superfect mixture was incubating, the culture media was removed from the DF-1 cells and replaced with 0.5 ml fresh media. The transfection mixture (25 μ l) was then added to each well and incubated at 37°C and 5% CO₂ for 4-5 hrs. Following the incubation period, fresh media was added to each well and replaced one time on the following day.

Immunocytochemistry and fluorescence microscopy

Forty-eight hours after transfection, both the primary retinal cultures and DF-1 cells were fixed using 4% paraformaldehyde for 5 min at RT, rinsed three times in PBS and processed for immunocytochemistry as follows. The cells were first blocked in PBS containing 10% goat serum for 30 min at RT. The cells were then incubated overnight at 4°C with a GC1 polyclonal antibody (1/333 dilution in PBS containing 1.0% BSA and 0.3% Triton X-100; GC2, gift from A. Yamazaki). On the following day, the cells were rinsed three times for 15 min each and then incubated for 1 h at RT with a goat anti-

mouse secondary antibody (1/500 dilution in PBS) tagged with the Alexa-594 fluorophore (Molecular Probes). The cells were subsequently rinsed three times for 15 min each and counterstained with DAPI. The coverslips were carefully removed from the wells and mounted in Gel Mount (Biomedica) on glass slides. The stained cells and/or direct GFP fluorescence were viewed using the appropriate fluorescent filter sets and digital images were acquired using a SPOT2 Enhanced Digital Camera System mounted in a Zeiss Axioplan 2 fluorescence microscope.

Generation of stably transduced cell lines

TE671 cells were seeded into the wells of a 24-well culture plate and grown overnight at 37°C, 5% CO₂. On the following day, 300 ml of fresh media was added to the wells and TYF.EF1 α _IRES.EGFP, TYF.EF1 α .bGC1/EGFP or TYF.GCE7.bGC1/EGFP virus was added to the media at an MOI of ~5. After 24 hours, the cells were seeded into T-25 flasks and maintained by passaging two times a week.

GC1 activity assays

GC activity was measured in washed membrane fractions obtained from TE671 cells (~50 passages) and purified bovine rod outer segments (ROS) (150 μ g total protein). The fractions were incubated for 15 min at 30°C with 1.5 mM [α ³²P]GTP (19,000-22,000 dpm/nmol; DuPont NEN), 50 mM HEPES, pH 7.8, 60 mM KCl, 20 mM NaCl, 10 mM MgCl₂, 0.4 mM EGTA, and either 1.0 μ M or 0.030 μ M free CaCl₂ in the presence or absence of GCAP1 protein (5 μ g). The assays were repeated twice, each with similar results.

Results

Lentivirus Production and Concentration

The goals of our first series of experiments were to determine the optimum ratio of total plasmid DNA to Superfect reagent that produced the highest titer virus and the optimum time for viral harvest. This ratio was determined to be 1:2 (ratios of 1:1, 1:1.5, 1:2, 1:5, and 1:10 were tested; data not shown). The titers of virus-containing media harvested directly from transfected 293T cultures were determined 30, 45, 60, and 70 hours post-transfection to identify the timeframe during which virus production by these cultures is at maximum levels (Fig. 4-3). The average titer values were 8.0×10^6 , 6.8×10^6 , 2.6×10^6 and 0.8×10^6 TU/ml at 30, 45, 60 and 70 hours post-transfection, respectively. Therefore, we collected culture media 30 and 45 hours post-transfection for subsequent experiments. It should also be noted that 293T cells passaged between 2 and 60 times were used for transfections and that passage number did not significantly affect transfection efficiency or final vector titers.

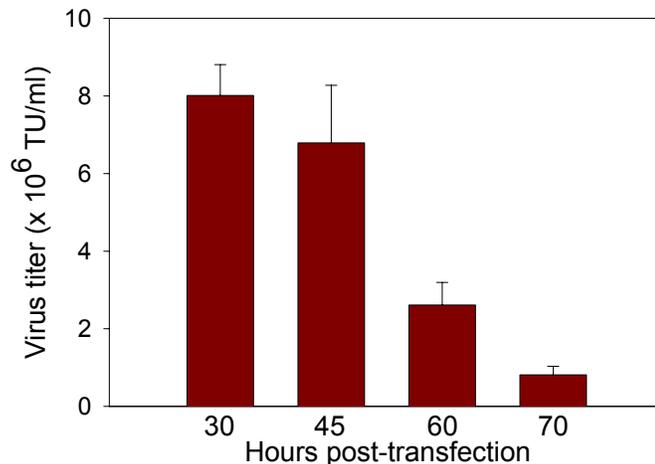
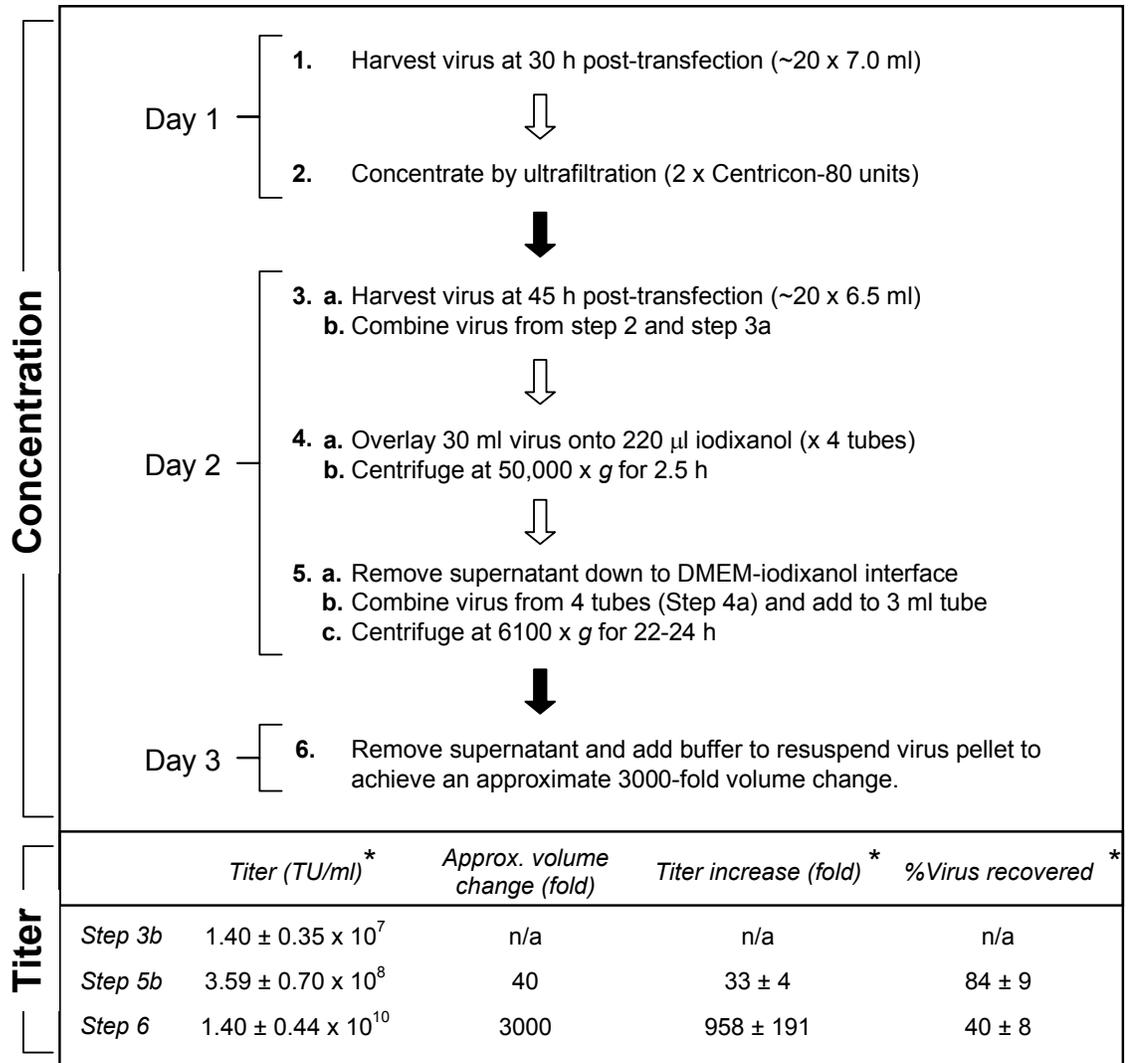


Figure 4-3. Production of lentivirus by transfected 293T cells as a function of time. VSV-G-pseudotyped lentiviruses carrying an EF1 α -PLAP transgene were prepared using the lentiviral vector system illustrated in Fig. 4-2. Each bar represents the mean titer \pm SEM of unconcentrated virus-containing medium collected at each time point (n = 3).



*Mean \pm SEM derived from 13 separate large-scale virus preparations

Figure 4-4. Outline and results of the vector production protocol. The top panel shows a simplified flow diagram of the concentration procedure that is described in detail under Methods. The bottom panel summarizes the viral titer results obtained following each step of the concentration procedure.

The goal of our second series of experiments was to develop a concentration protocol that would minimize virus loss and yield the highest titer virus in the smallest possible volume. The concentration procedure and results are summarized in Fig. 4-4. The average starting titer of the virus-containing media (Fig. 4-4, bottom panel, Steps 1-3) was $1.40 \pm 0.35 \times 10^7$ TU/ml. The next step in the concentration procedure (Fig. 4-4, bottom panel, Step 4) yielded an average titer of $3.59 \pm 0.70 \times 10^8$ TU/ml in a volume of

~3.0 ml, resulting in a 33-fold increase in titer and an average recovery of 84%. Further concentration of the virus stock by low-speed centrifugation (Fig. 4-4, bottom panel, Steps 5c and 6) yielded $1.40 \pm 0.44 \times 10^{10}$ TU/ml, a 958-fold increase over the average starting titer. The average overall percent recovery of the virus was 40%.

***In vivo* Performance of the Lentiviral Vector**

Administration of ~0.5 μ l of TYF.EF1 α .PLAP virus (1×10^{10} TU/ml) into the chicken neural tube resulted in efficient transduction of large numbers of neural progenitor cells (Fig. 4-5). Cross-sections of stained retinas revealed numerous PLAP-positive cell columns (Fig. 4-5 D, bottom panel). Columns of PLAP-positive cells were also observed throughout the developing brain (Fig. 4-5 E). We also examined the relationship between viral dose and the percent of the retina transduced by the virus and determined that the transduction efficiency of the virus in developing retina was dose-dependent (Fig. 4-5 A-C). The percent of total retinal area exhibiting PLAP expression was estimated to be 5%, 63% and 85% in embryos receiving injections of 10^8 , 10^9 and 10^{10} TU/ml vector, respectively (Fig. 4-5 D, top panel). PLAP expression was maintained in retinas from injected embryos that were examined 2 days after hatching, 21 days post-injection (Fig. 4-6). The relationship between the amount of virus injected and the extent of viral transduction was maintained in these retinas, the number of PLAP-expressing cells being significantly less in embryos injected with 10^7 TU/ml virus (Fig. 4-6, left panel) than embryos injected with 10^{10} TU/ml virus (Fig. 4-6, right panel).

GC1 Immunocytochemistry

A small percentage of cells in the primary GUCY1*B chicken retinal and DF-1 cultures transiently transfected with either the pTYF.IRBP1783.bGC1 or the pTYF.EF1 α .bGC1/EGFP vector, respectively, stained positively for the GC1 protein

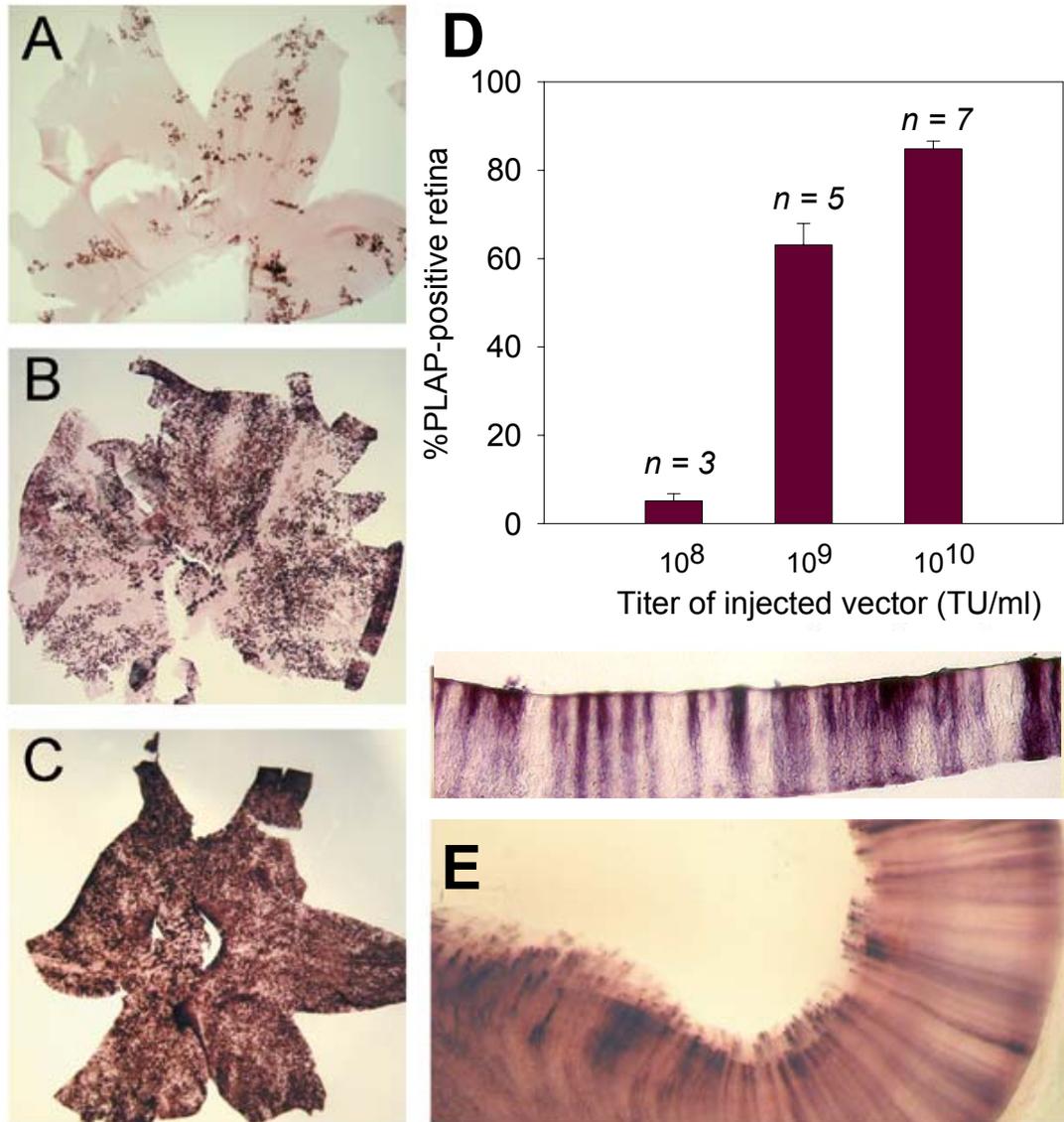


Figure 4-5. Lentiviral vector-mediated transduction of PLAP in chicken neural progenitor cells. **A - C.** PLAP expression in representative flat mounts of E7 chicken retinas from embryos receiving injections of (A) 10^8 , (B) 10^9 or (C) 10^{10} TU/ml virus. **D. top panel:** Histogram showing the quantification of the percent area of PLAP-positive retina following injections of different doses of vector. Bars represent the mean \pm SEM for each group ($n = 3-7$). **bottom panel:** Cross-section of the retina shown in C. **E.** Cross-section showing PLAP-positive cells in the lateral anterior cortex of an E7 embryo that had received a neural tube injection of 10^{10} TU/ml virus.

(Fig. 4-7). A limited number of cells stained positive for GC1 in the two culture systems, which was consistent with the respective transfection efficiencies usually achieved in these cells and suggested that the staining was specific. GC1 expression driven by the

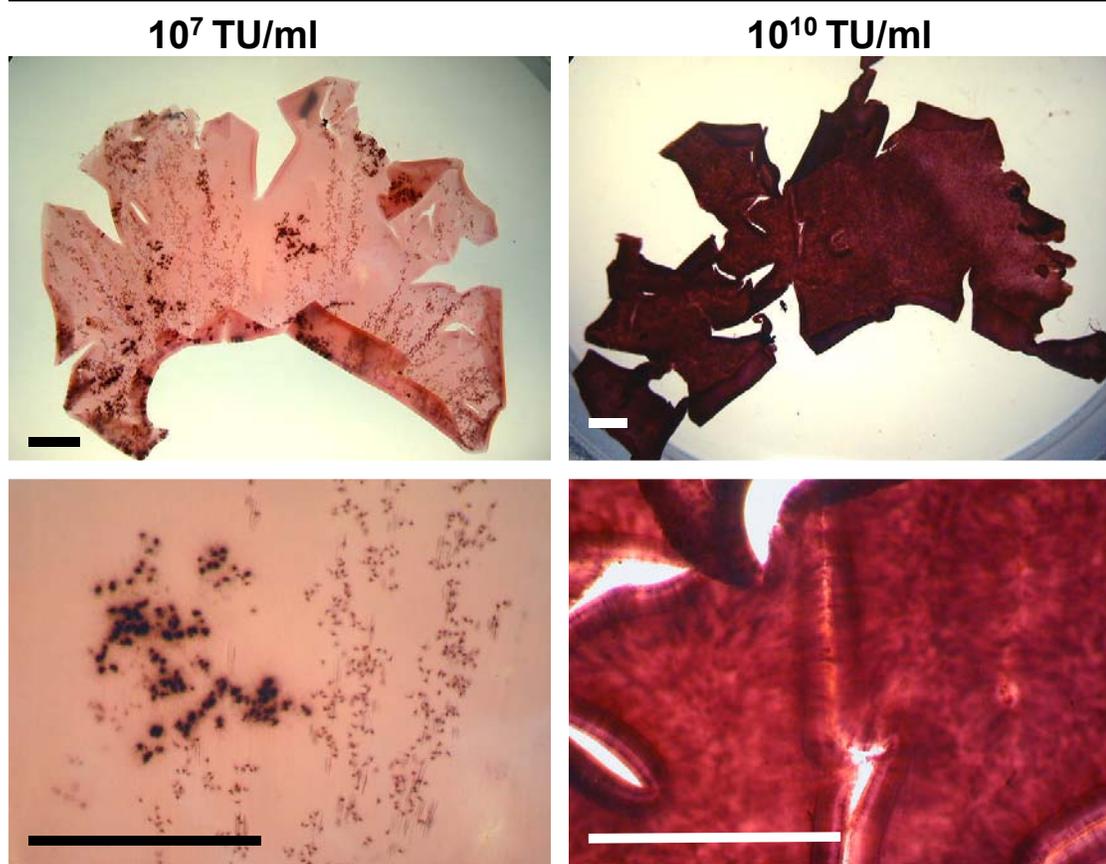
TYF.EF1 α .PLAP

Figure 4-6. PLAP expression in post-hatch chicken retinas. Embryos were injected with different doses of virus at stage 10-12 (neural tube). The retinas were processed for PLAP staining 2 days after hatching, 21 days after the injections. The bottom panels show close-ups of areas from the retinas pictured in the corresponding top panels. Scale bars = 2 mm.

IRBP1783 promoter was limited to cells exhibiting photoreceptor cell morphology in the GUCY1*B retinal cultures (Fig. 4-7, top panel) and was limited to the membrane surrounding the nucleus and to the apical ends of the cells that eventually differentiate into the outer segments. GC1 immunostaining of the DF-1 cells was present throughout the cell body and its processes and co-localized with GFP fluorescence, a result that indicates that both cistrons of the bicistronic EF1 α -bGC1-IRES-EGFP transgene were expressed (Fig. 4-7, bottom panel). We were unable to determine from these analyses if the GC1-labeled protein within the soma was associated with the cell membrane.

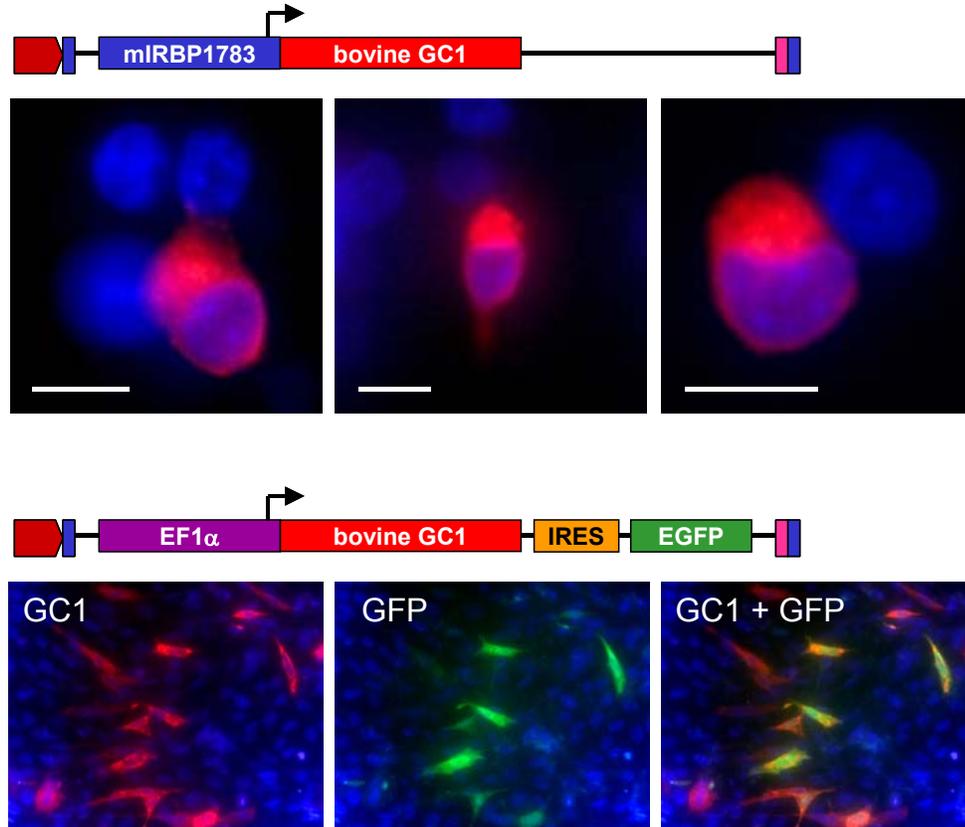


Figure 4-7. Expression of recombinant bovine GC1 in avian-derived retinal photoreceptor cells (top panel) and DF-1 fibroblast cells (bottom panel). Bovine GC1 protein is labeled red and the green in the bottom panel is intrinsic GFP fluorescence. Cells were transfected with the plasmid vectors illustrated above each panel as described in the Methods section of this chapter. Scale bars = 5 μm

GC1 Enzyme Activity

To assess the activity of the GC1 enzyme produced from the pTYF.EF1 α .bGC1/EGFP and pTYF.GCE7.bGC1/EGFP expression vectors, TE671 cells were first transiently transfected with the plasmid DNA and processed for GC1 activity analyses 48 h post-transfection. The results of these analyses are shown in Fig. 4-8 A. Three samples were analyzed in this experiment: (1) bovine ROS membranes; (2) mock-transfected TE671 cells; TE671 cells transfected with the (3) pTYF.EF1 α .bGC1/EGFP vector or the (4) pTYF.GCE7.bGC1/EGFP vector. The activity of the GC1 enzyme in

each sample was assayed under low and high calcium conditions in the presence and absence of GCAP1 protein. The results of these analyses show that the GC1 enzyme produced by our vector exhibits activity characteristics closely resembling those of the native GC1 enzyme present in bovine ROS. The activity of the GC1 enzyme in the TE671 cells dramatically increased when calcium levels were reduced in the presence of GCAP1 protein.

The function of the bicistronic transgene within the pTYF.EF1 α .bGC1/EGFP vector was also assessed in TE671 cells that were transduced with virus made from this construct. The results indicate that the virus is capable of stably expressing the GC1 transgene and that the bicistronic transgene efficiently expressed functional GC1 (Fig. 4-8A) in conjunction with the GFP marker protein (Fig. 4-8B). Together, these results show that the pTYF-based constructs are suitable for use as the backbone for the final therapeutic lentiviral vectors that will be used to express functional GC1 in the GUCY1*B retina.

Discussion

By optimizing both the DNA transfection and viral concentration steps for production of lentiviral vector, we have overcome many of the problems that we had previously encountered in our efforts to produce large volumes of high-titer lentiviral vector in a consistent manner. We found that Superfect-mediated transfection of viral packaging cells consistently yielded large-scale vector stocks (~120 ml) with starting titers averaging $>1.0 \times 10^7$ TU/ml, titers that were comparable to vector stocks prepared using other transfection reagents. Use of Superfect greatly simplified the transfection protocol and significantly reduced the amount of plasmid DNA required for the

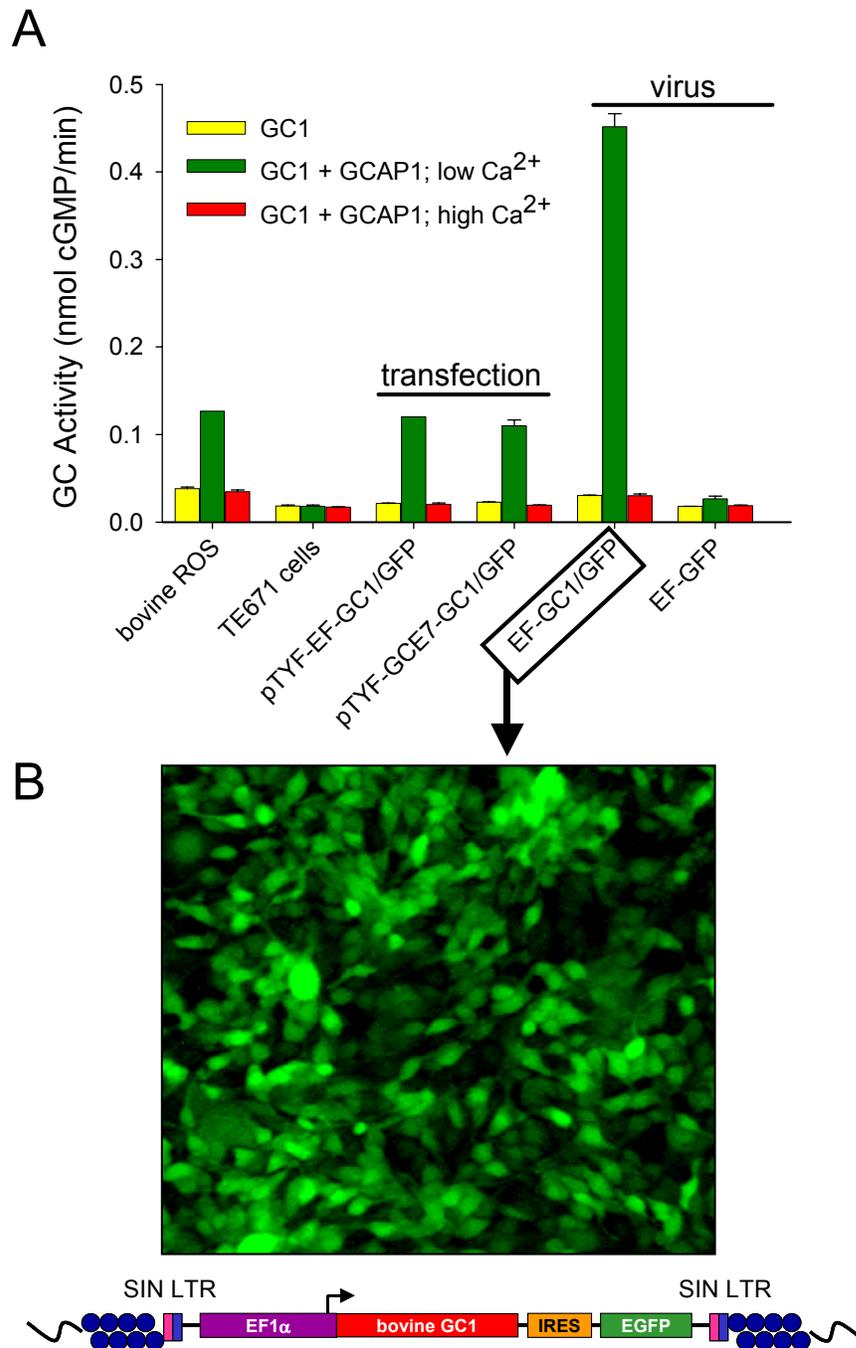


Figure 4-8. Expression of recombinant bovine GC1 from transiently transfected transgenes and transgenes packaged into the lentiviral vectors. A. Histogram showing the GCAP- and calcium-dependent enzymatic activity of GC1 *in vitro*. TE671 cells were transfected with plasmid or transduced with virus as described in the Methods section. **B.** GFP fluorescence in TE671 cells transduced with TYF.EF1 α .bGC1/EGFP virus and schematic of the integrated transgene. This image coupled with the data shown in A demonstrates that the bi-cistronic transgene is functional when packaged into the lentivirus.

procedure. The viral concentration protocol that we developed consistently increased the titers of the viruses by approximately 1000-fold ($\sim 1 \times 10^{10}$ TU/ml). Furthermore, all vectors that we produced using these methods exhibited high transduction efficiencies *in vivo*.

One of the goals of this study was to produce viral stocks that could be used to transduce a high percentage of cells in the retina following delivery of lentiviral vector into the neural tube of the developing chicken embryo. The injected virus transduced several populations of neural progenitor cells, including those fated to become the neural retina (Figs. 4-5 and 4-6). A majority of cells exposed to virus during this stage of development are mitotic and have not yet differentiated (Prada et al. 1991). By varying the concentration of the virus injected, we found that the percent of retina transduced could be controlled in a linear fashion using doses between 10^8 and 10^9 TU/ml. Injections of virus at a concentration of 10^{10} TU/ml produced maximal levels of retinal transduction. In the previous chapters, we showed that it is possible to specifically target lentiviral vector-mediated expression of transgenes to retinal photoreceptor cells by selecting appropriate promoter fragments. Together, these results illustrate the effectiveness of our vector to transduce cells within the developing nervous system and illustrate the potential use of this vector as a tool for studies of mechanisms regulating gene expression *in vivo*. We also demonstrate that efficient expression of the EF1 α -PLAP transgene persists in the fully developed retina and that the vector is well-suited for use in our future studies of GC1 expression in the GUCY1*B chicken model LCA1.

In summary, the transfection and concentration protocols outlined here allow efficient, reproducible production of high-titer lentiviral vectors that exhibit robust

transduction properties *in vivo*. The transfection protocol itself is simple and can be easily implemented by investigators interested in producing lentiviral vector in their laboratories. Furthermore, the methods can be easily adapted to large-scale lentiviral production protocols that are currently being developed for use in large animal studies or for possible use in clinical studies.

CHAPTER 5 CONCLUSIONS AND FUTURE DIRECTIONS

The results presented in Chapters 2 and 3 demonstrate that lentiviral vectors are capable of efficiently transducing avian neural progenitor cells and that the promoters of photoreceptor cell-specific genes can be incorporated into these vectors to achieve targeted transgene expression *in vivo*. The improvements made to the production and concentration protocols used to generate lentiviral vectors as described in Chapter 4 will facilitate the use of these vectors *in vivo*. In addition, we have successfully developed and tested a bicistronic lentiviral vector construct that is capable of mediating the expression of functional retinal guanylate cyclase-1 (GC1). Together, these results lay the groundwork for future studies of somatic gene therapy in the GUCY1*B chicken model of Leber congenital amaurosis type 1 (LCA1).

Targeted Gene Expression in Retina

Overall, the results from the experiments presented in Chapters 2 and 3 show that the GCE7 or GCAP4009 promoter fragments are suitable for driving the cell-specific expression of a GC1 transgene in retina. These results also lead to more specific questions regarding the mechanisms that control the onset of expression and the cell-specific regulation of these promoters in the developing retina. The experimental paradigm presented in Chapters 2 and 3 could be extended to help answer the following questions that are relevant to future studies of gene rescue in the GUCY1*B chicken: (1) Do the GCAP1 and GC1 promoters exhibit cone-specific or cone/rod-specific expression? (2) What are the intrinsic activity levels exhibited by the GCAP1 and GC1

promoters *in vivo*? (3) What cell types express GC1 during retinal development and is there a switch in the cellular specificity of its expression?

Understanding the photoreceptor subtypes in which the GCAP1 and GC1 promoters are expressed is relevant to our efforts to rescue retinal function in the GUCY1*B chicken. The results of immunohistochemical analyses show that GCAP1 and GC1 are present in higher concentrations in cone cells than in rod cells (Cooper et al. 1995;Liu et al. 1994). Furthermore, clinical studies provide evidence that cone cells may be more dependent on GCAP1 and GC1 in terms of survival and function. For example, patients diagnosed with retinal diseases that are linked to mutations in GCAP1 and GC1 exhibit phenotypes (e.g. diminished cone cell electroretinograms) and behaviors (e.g. photophobic behavior and decreased visual acuity) that are indicative of compromised cone cell function (Milam et al. 2003;Perrault et al. 1999;Wilkie et al. 2001). Thus, successful treatment of diseases like LCA1 may depend to some extent on our abilities to insure that cone cells are included in the target cell population.

The precise cellular specificities of the GCAP1 and GC1 promoters could be determined by performing co-localization studies using antibodies specific for nlacZ and for cone (iodopsin) and rod (rhodopsin) specific markers. A reasonable approach would be to perform the immunocytochemical analyses on dispersed primary retinal cultures that have been prepared from the retinas of embryos that received injections of the various promoter-nlacZ lentiviral vectors. We have found that accurate identification of rod and cone cells expressing reporter genes is facilitated by use of dispersed cultures. Preliminary data obtained from this type of analysis are shown in Fig. 5-1.

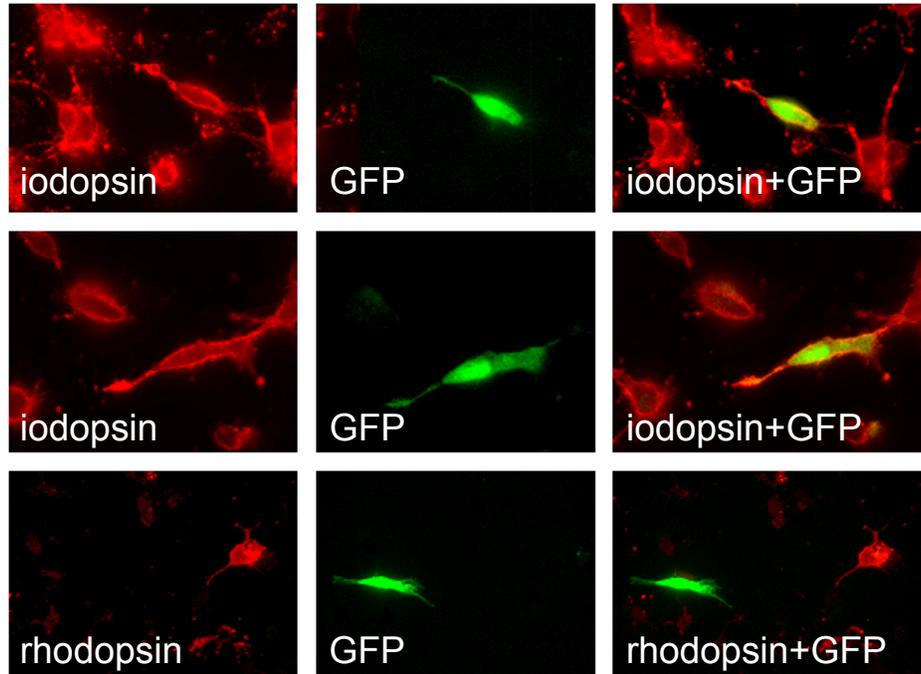


Fig. 5-1. Immunolabeling of primary embryonic chicken retinal cultures with cone (anti-iodopsin) and rod cell markers (anti-rhodopsin). Cultures were transfected with a mIRBP1783-GFP plasmid vector and the preparation and transfection of the cultures was performed as described in Chapter 2.

In addition to cell-specificity, it is also important to examine the levels of transcriptional activity exhibited by promoters when developing gene therapy strategies.. Strong, ubiquitous promoters are generally used in experimental gene delivery systems because they are readily available and usually guarantee high levels of expression in many cell types; however, abnormally high levels of expression of therapeutic genes in targeted cells may have deleterious effects on the function of these cells. . For example, it has been suggested that over expression of guanylate cyclase-1 (GC1) may result in protein aggregation and/or can interfere with proper trafficking of GC1 to its position in the membrane, both of which may be detrimental to photoreceptor cells (Rozet et al. 2001). The over expression of GC1 in photoreceptor cells could also interfere with proper regulation of the catalytic activity of this enzyme by GCAP1. Therefore, we have put

considerable effort into identification of different promoters that may provide optimal levels of expression of GC1 in photoreceptors. It should be noted that levels of GC1 as low as 50% of that present in wild-type retina are sufficient to sustain photoreceptor survival and function in the chicken retina (Semple-Rowland et al. 1998).

To assess the intrinsic activity levels of selected GCAP1 and GC1 promoter fragments, the activities of these fragments could be analyzed *in vivo* using the methods described in Chapter 2. Comparisons of the results obtained from these experiments with those obtained *in vitro* would provide a more detailed picture of the intrinsic activities of these promoters.

Finally, since we plan to introduce the virus during the early stages of embryonic development, it is important to understand the expression characteristics of the selected promoters in developing retina. In our analyses of the GCAP1 promoter, we found that inclusion of the distal region of the GCAP1 5' flanking region in the promoter fragment resulted in delayed, but specific expression of nlacZ in photoreceptor cells (Fig. 2-4). In contrast, the cell-specificity of expression of GC1 promoter fragments changed over the course of development, expression being limited to photoreceptor cells during the later stages of development. Studies are currently planned to determine if the absence of expression of the GC1 promoters in non-photoreceptor cells late in development is due to silencing of expression of the transgene in retinal cells within the inner nuclear and ganglion cell layers. Use of laser capture dissection techniques will allow us to excise groups of cells from the INL that are positioned in columns marked by nlacZ-positive cells positioned in the ONL. The cells will then be genotyped to confirm the presence or absence of the integrated transgene. The presence of the transgene in the absence of

reporter expression would be consistent with the hypothesis that the promoter is actively silenced in these cells.

Lentiviral Vector Transduction in Retina

One of the long-term research goals of this project is to determine if vision can be restored in hatchling GUCY1*B chickens by delivering a lentiviral GC1 transgene to the retinal progenitor cells of these animals. We chose to use the lentiviral vector system because it circumvents some of the limitations of other vector systems. For example, the size of the GC1 transgene that we plan to use in these studies exceeds the cargo capacity of traditional recombinant AAV (rAAV) vectors, a problem that is not one that arises when using lentiviral vectors. Another issue of importance concerns the time required for transgenes to reach maximum levels of expression. Transgenes carried by lentiviral vectors begin to express and reach maximum expression levels more rapidly (within 3 days) than those carried by rAAV (within 2-4 weeks) (Sarra et al. 2002).

To rescue the function of GC1-null photoreceptors in the retina and restore vision, it is desirable to be able to transduce a high percentage of these cells with the therapeutic vector. Our initial attempts to transduce chicken retinal progenitor cells with lentiviral vector were disappointing in this regard, the number of retinal cells being transduced representing less than 5% of the total population. In view of the potential impact that poor transduction efficiency could have on the outcome of future gene rescue experiments, much of our research effort was devoted to improving the performance of the lentiviral vector *in vivo*. As described in Chapter 4, two changes were made to the system that dramatically improved transduction efficiencies *in vitro* and *in vivo*. Because of these efforts, we were able to produce viral vectors with titers ranging from $10^9 - 10^{10}$ TU/ml (~100-fold increase over previous efforts) that were capable of transducing greater than

80% of the retinal cell population when injected into the neural tube. These modifications should significantly improve the outcome of our future efforts to rescue sight in the GUCY1*B chicken. In the future, DNA insulator elements from the chicken β -globin gene (Chung et al. 1997) could be added to the transducing vector, flanking the transgene insert. Insulators have been shown to reduce variegation effects and to significantly decrease transcriptional silencing in retroviral vector transgenes (Pannell and Ellis 2001). This addition would contribute to a further gain in the biosafety and performance of our lentiviral vector system *in vivo*.

Several recent advances in the biosafety and performance of lentiviral vector systems are beginning to assuage concerns over use of these vectors in gene therapy applications. Since I began my research program, three generations of lentiviral vectors have been developed in efforts to improve the biosafety and performance of the virus (Vigna and Naldini 2000). The third-generation vector system consists of a modified helper vector that does not contain the Tat encoding region and a fourth expression vector that encodes the Rev protein, a protein that enhances viral packaging. These vectors could be readily incorporated into the second-generation system that we used in the current studies. It may be prudent to utilize the third-generation packaging vector system in our future studies of gene rescue in the GUCY1*B chicken.

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BIOGRAPHICAL SKETCH

Jason Edward Coleman was born in Evansville, Indiana. After moving with his family to Jacksonville, Florida, he received formal training in the visual arts in high school and during the first couple years of college before pursuing a research career in biology. In the spring of 1998, he graduated from the University of Florida where he received his BS in Neurobiology. Following graduation, he worked as a research assistant in the Neuroscience Department at the University of Florida. In the fall of 1998, he entered the Interdisciplinary Program in Biomedical Sciences at the University of Florida, College of Medicine. He joined the Department of Neuroscience in May of 1999 where he pursued his doctoral degree under the supervision of Dr. Susan Semple-Rowland.