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CELL-SELECTIVE EXPRESSION
OF A GLUTAMINE SYNTHETASE FUSION GENE
IN EMBRYONIC CHICK RETINA

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

David Andrew Beard, B.S.

The Ohio State University
1995

Dissertation Committee:
N.J. Uretsky
D.R. Feller
A.H. Burghes

Approved by
A.P. Young
Adviser
Department of Pharmacology
College of Pharmacy
To My Wife, Julie
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VITAE

September 30, 1966 . . . Born - Columbus, Ohio

1988 . . . . . Bachelors of Science in Pharmacy, The Ohio State University

1989 - 1991 . . . . Medical Student
College of Medicine
The Ohio State University

1991 - present . . . . Graduate Teaching Associate
College of Pharmacy
Dept. of Pharmacology
The Ohio State University

FIELDS OF STUDY

Major Fields: Pharmacology
              Medicine
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INTRODUCTION

This thesis addresses specific issues of glutamine synthetase (GS) gene regulation as it relates to cell-specific expression in embryonic chicken retina. While GS is expressed at a basal level in many tissues, expression is highly induced in a cell-specific manner in some tissues (e.g., retina). We have attempted to define the sequences of the GS gene and associated mechanisms that confer GS cell-specific expression in Müller glia of the retina. To ascertain these answers, we have characterized fundamental properties in the utilization of electroporation for transfection of intact retina. An in vitro assay system was also developed to examine cell-specific expression of transfected GS fusion genes in retinal primary cultures. In addition, transgenic mouse models were generated to examine the in vivo behavior of GS fusion transgenes. The goal of this introduction is to familiarize the reader with background information that will allow comprehension of the investigations presented.

Biochemical importance of GS

GS is an important enzyme in the biochemistry of both avian and mammalian species. This enzyme catalyzes a reaction in the only known pathway of glutamine synthesis. The formed product may serve as an intermediate in many reactions involving nitrogen metabolism. Aside from being utilized as a building block for proteins, glutamine also plays a role in various amide and amine transfer reactions (Meister, 1980). These reactions include...
the synthesis of carbamyl phosphate, asparagine, histidine, tryptophan, guanine, cytidine, NAD⁺, and the detoxification of glutamate. Carbamyl phosphate can then be utilized in the synthesis of urea and arginine (Meister, 1984). In prokaryotes, either ammonia or glutamine may be utilized for these reactions. However, high concentrations of ammonia are toxic to eukaryotic nervous tissues. Glutamine thus serves as a non-toxic alternative for the many nitrogen transfer reactions within eukaryotic metabolism. Figure 1 demonstrates the central role of glutamine in nitrogen metabolism. In addition, glutamine has been shown to be a major fuel for the kidney (Pitts, 1975), small intestine (Windmueller and Spaeth, 1980), bone (Biltz et al., 1982), and in HeLa cells (Reitzer et al., 1979). Indirect evidence also exists that indicate it can be consumed as an energy source in the brain (Tildon and Roeder, 1984).

Albeit there exists a large number of enzymatic reactions which may deplete levels of glutamine, most body fluids have a high concentration of this amino acid. In man, the highest concentrations of glutamine occur in the blood, where it accounts for one-fifth of amino acid content, and in the cerebrospinal fluid (CSF), where it composes two-thirds of the amino acid pool (Ferraro and Hare, 1984). Glutamine has also been detected in millimolar concentrations in rat brain, heart, liver, small intestine, and skeletal muscle (Herbert et al., 1966). The high glutamine content in these tissues may in part be due to increased GS activity present in these areas. It is known that GS is present in mammalian retinal, brain, skeletal muscle, liver, kidney, heart, lung, spleen (Cooper, 1988) and adipose tissues (Miller, 1975).
Figure 1: Roles of glutamine in nitrogen metabolism. The bold arrow stresses the importance of GS as the only enzyme known to catalyze the synthesis of glutamine. Glutamine can be broken down by various enzymes to contribute amino groups to diverse products. (Modified from Tate and Meister, 1973).
Glutamate + NH₃

Glutamine synthetase

α-amino acids

NH₂-C-COOH

H

CH₂

CH₂

C

O

NH₂

Proteins

Arginine, urea

Carbamyl phosphate

Pyrimidines

NH₃

Purines

NAD⁺

Histidine

Tryptophan

Asparagine

Glutamate

Figure 1
Distribution and function of GS in relevant tissues

The metabolic significance of GS activity varies in the individual organs in which it is expressed. In the brain and retina, GS acts primarily in the detoxification of ammonia, which if allowed to accumulate is detrimental to neurons. In many tissues the urea cycle functions to remove excess ammonia. However, in the brain, the urea cycle is inoperable (Nicklas, 1988). In addition, GS plays a role in the removal of glutamate, which in high concentrations is also toxic to neurons. GS is also thought to participate in the maintenance of carbon and nitrogen homeostasis. It appears that there is a net export of glutamine out of astrocytes into the blood and CSF and also from astrocytes to neurons (Cooper and Plum, 1987). This export is not surprising when one considers the need to counterbalance the large uptake of ammonia and glutamate. Van Gelder (1983) has suggested that GS, in addition to carbonic anhydrase, may also be important in osmoregulation within the CNS. Still others have found that GS plays a role in the control of pH in the brain and the CSF (Kraig, et al., 1985, Kraig, et al., 1986). Clearly, GS is an integral player in general brain metabolism and function. It has been determined by immunohistochemical means that GS is largely confined to astrocytes in the brain (Martinez-Hernandez, et al., 1977, Norenberg and Martinez-Hernandez, 1979) and Müller glial cells in the retina (Riepe and Norenberg, 1977). However, biochemical evidence exists which indicates the presence of GS within neurons (Weiler, et al., 1979), albeit at lower levels than in glia. It is postulated that the post-synaptic neuron may participate in glutamate uptake followed by synthesis of glutamine by neuronal GS. Overall, it is generally accepted that the glial cell population is the main source of GS in the brain and retina, with a small contribution of GS arising from neurons. A special
anatomical relationship exists between astrocytes and neurons (Bradbury, 1979). At one end, astrocytic end-feet ensheath the capillaries. At the other end, astrocytes cover the neurons. Thus, astrocytes are in a unique position to communicate metabolically with neurons and CSF on one end and the blood compartment on the other.

In skeletal muscle, the specific activity of GS in relatively low compared to that of brain (Iqbal and Ottaway, 1970). However, because of the large mass of muscle present in the body, the contribution of GS in glutamine formation can be quite substantial. It has been suggested that muscle, in fact, is probably the major site of glutamine synthesis (Shróck and Goldstein, 1981). It has been shown that up to 50% of the ammonia in the bloodstream is removed by the skeletal muscle (Lockwood, et al., 1979). Thus a correlation exists between decreased muscle mass and increased levels of ammonia. Skeletal muscle is an important source of amino acids released into the bloodstream. It is estimated that 25-30% of the total amino acid efflux from the myocyte is glutamine which has been formed de novo (Marliss, et al., 1971). In fact, only about 6% of the normal myocyte proteins is glutamine which confirms that most of the exiting glutamine must be newly formed (Kominz, et al., 1954). The carbon for glutamine synthesis may come from glucose or amino acids either in the circulation or from muscle protein breakdown. The nitrogen source may be provided by free ammonia or other amino acids. The glutamine that is released may function in many roles including, as a buffer for excess ammonia, as a substrate in gluconeogenesis, as a source of buffer for urinary acids, and as a fuel for the small intestines (Durschlag and Smith, 1985).

GS specific activity in the liver has been shown to be 3-fold less than that of brain but still 20-fold greater than in muscle (Magnuson and Young, 1988).
This moderate level of activity is not surprising in light of the fact that the liver is a major site for ammonia detoxification. It appears that the urea cycle and GS both play integral roles in preventing hyperammonemia, which if left untreated can lead to confusion and coma. GS is not uniformly expressed in the liver. It functions only in the perivenous cells, whereas the urea cycle operates primarily in the periportal cells (Häussinger, 1983). The hepatic glutamine cycle is also important for the maintenance of pH, as it is thought that the major pathway for bicarbonate ion removal is by hepatic urea synthesis (Atkinson and Camien, 1982, Oliver, et al., 1977).

The specific activity of GS in the kidney shows interspecies differences. In rat, mouse, rabbit, and sheep kidneys (Krebs, 1935, Burch, et al., 1978, Wu, 1963) GS activity is moderate, but in humans and dogs (Lemieux, et al., 1976) GS activity is low or undetectable. This may be due to differences in urinary acidity among the species. In rodents and sheep, the urine is neutral or alkaline in which case GS may function to recover unused ammonium ions. However, in humans and dogs, the urine is normally acidic which causes large amounts of ammonium ions to be consumed for buffering. Thus, in these animals, GS plays a less important role in the recovery of excess ammonium. The expression of GS is not homogenous throughout the kidney and in rats is confined to the proximal tubule of the nephron (Burch, et al., 1978). It is clear that the main function of GS in the kidney is in the maintenance of acid-base homeostasis.

It has also been reported that adipose tissues contain a high level of GS activity (Miller, 1975). The specific activities are comparable to those found in brain and liver. As in other GS-containing tissues, the distribution of GS was found to be cell-specific. In fat cells, the levels of GS are at least two-fold higher
than that of whole adipose tissue. Miller also proposed that the adipocytes may provide for more than 25% of the rapidly metabolized glutamine pool.

*GS gene and protein structure*

In order to determine which sequences may be important in regulation of the GS gene, it is necessary to review basic structural information about the gene. The chicken GS gene structure has been previously determined in our lab (Pu and Young, 1989). It is a single copy gene composed of a 7 exon unit of which exons 2 through 7 contain sequences which appear in the 1119 nucleotide (nt) open reading frame. This transcriptional unit is contained within a 7 kilobase genomic fragment. Pu and Young also cloned a genomic fragment of approximately 7 kilobases of sequence immediately upstream of the transcription start site. It was determined that this fragment contained a functional promoter. These genomic clones served as the basis for the GS constructs utilized throughout this thesis.

The fully functional product of the GS gene is a protein composed of 8 identical polypeptide subunits each weighing approximately 42,000 daltons (Yamamoto, et al., 1987). The octomer is formed by isologous association of two heterologously bonded tetramers (Haschemeyer, 1970). Antibody staining to GS reveals a homogenous cytoplasmic distribution of the enzyme (Linser and Moscona, 1979).

*GS catalytic activity*

GS catalyzes the reversible formation of glutamine from glutamate, ammonia, and ATP (Levintow and Meister, 1954). The synthesis is a condensation reaction in which a divalent cation (Mg$^{2+}$, Mn$^{2+}$, or Co$^{2+}$) is also
required. The reaction equilibrium is attained when about 90% of the glutamate is converted into glutamine (Levintow and Meister, 1954). If hydroxylamine is substituted for ammonia, the reaction proceeds to 99% completion. The resultant γ-glutamyl hydroxamic acid (GGHA) that is formed exhibits a brown color in the presence of acidic ferric chloride. The absorbance of this product at 550 nm has been used as a basis for the quantification of GS activity (Pamiljans, et al., 1962, Rowe, et al., 1970).

GS expression in development

The appearance of GS during development has been studied in both chickens (Moscona, 1972, Moscona and Hubby, 1963, Patejunas and Young, 1987) and rodents (Magnuson and Young, 1988). In chick embryonic retina, GS activity is very low until day 16 (of a 21 day incubation) when it begins to increase sharply. This elevation of GS follows the appearance of systemic corticosteroids from the newly functioning adrenal glands (Moscona, 1972, Piddington, 1970). Over the remaining incubation period and during the first few days after hatching, the GS levels rise to more than 100 fold times the basal level. This induced level of GS is then maintained throughout life (Moscona, 1972). This pattern of increase is also seen in the murine retina, although its time course is somewhat different (Magnuson and Young, 1988). In the mouse, which also has an embryonic period of 21 days, GS does not begin to rise until after birth. This is due to the fact that functional maturation of the retina does not take place in the mouse until after birth (Chader, 1971), whereas functional maturation of the chick eye takes place during embryonic development. The mechanism of this large increase in GS activity has been extensively investigated. It appears that endogenous adrenal corticosteroids may contribute
to the sharp rise in GS in embryonic chick retina (Piddington and Moscona, 1967). However, hormonal induction of GS is not limited to retina of 15 days and older. When exogenous steroids are administered into an egg at embryonic day 8, before the endogenous hormone is present, the GS induction is still appreciable, although at reduced levels. At embryonic days 5 to 7, similar experiments fail to show any hormonal response (Moscona and Moscona, 1979). These results have also been verified in retinal organ cultures, which demonstrate that the retina itself contains the necessary components for hormonal induction of GS (Moscona and Linser, 1983).

An interesting pattern of GS expression is seen in the developing mouse liver. Using GS antiserum, Kuo, et al., 1988, examined the distribution of GS within fetal and postnatal murine livers. Most fetal hepatocytes were found to express GS. This is in agreement with the general theory that a hepatic stem cell exists which gives rise to all functional cells. However, at postnatal ages, GS expression becomes limited to a single-cell layer of hepatocytes surrounding the central veins. The transition to a cell-specific expression pattern begins a few days after birth. The liver assumes the adult pattern of GS expression by postnatal day 12. Kuo, et al. proposes that this cell-specific expression is due to changes in transcriptional regulation. The pericentral hepatocytes show an increased transcription while the remaining adult hepatocytes undergo a decrease in GS transcription. GS expression in the liver is not markedly induced by glucocorticoids in rodents (Magnuson and Young, 1988, De Groot, et al., 1987) or chickens (Patejunas and Young, 1987).
Inducible GS expression in skeletal muscle

In the skeletal muscle, GS does not appear to show a pattern of cell-specific expression. However, glucocorticoids do have effects on the physiology of muscle, perhaps mediated by GS. It is well established that chronic administration of glucocorticoids can cause muscle atrophy (Shoji and Pennington, 1977, Mayer, et al., 1976). Falduto et al. (1989) examined the effects of steroid-mediated atrophy upon the levels of GS activity and GS mRNA. Their findings indicate that chronic dosing of rats with hydrocortisone results in 2.4 and 5.9 fold increases in plantaris muscle GS activity and mRNA, respectively. A priori, increases in GS activity would be expected since 25-30% of amino acid efflux from myocytes is from glutamine during steroid-induced atrophy (Marliss, et al., 1971). However, they also found that if the rats were exercised regularly, the atrophy process was arrested. They proposed that during glucocorticoid-mediated atrophy, the GS gene was transcriptionally upregulated but this response could be attenuated by endurance exercise (Falduto, et al., 1992). The increases in GS seen during atrophy are affected, at least in part, by a post-transcriptional mechanism, as the fold increase in GS activity is not as high as the mRNA increase. It is clearly seen that GS plays a critical role in muscle wasting processes.

Rationale for studying GS in embryonic chickens

The frequent use of embryonic chick retina in experiments presented in this dissertation deserves some general comments. The utilization of chicken eggs offers advantages over the use of other animal models. Full development of a chick embryo requires only 21 days in a humidified incubator at 37 °C. The developmental stages of the chick embryo have been well described by
Hamburger and Hamilton (1951). These descriptors of external features allows one to accurately determine the stage of a particular embryo. This can be critical when examining developmental changes that may occur during a short time period. The experiments presented were performed on retina from embryonic days 6 to 18.

Although avian species have a unique physical appearance after hatching, they undergo a mammalian-like pattern of embryonic development. Perhaps the most unique feature of chick embryos is that the eye is quite large during early stages of development relative to the size of eyes in rodents and other mammals. Without much difficulty, it is possible to dissect the retina from an embryo that is only 5 days old. The retina is easily dissected up to around embryonic day 13, at which time strong attachments begin to form between the retina and the pigmented epithelium. The chick retina closely resembles the mammalian retina in development with its carefully structured laminae of neuron cell bodies. The 5 types of neurons in the retina are ganglion, bipolar, amacrine, horizontal cells, and photoreceptors. The Müller cell is the only type of glia present in the retina. Müller glial fibers span the entire thickness of the retina while the neuronal cell types are organized in three distinct laminae. Figure 2 shows a cross-sectional view of the retina. The major difference between chick and mammalian retina is that the chick retina is avascular while the mammalian retinal layer has its own blood supply.

Normal development of the chick retina proceeds as follows. The early retina is composed of undifferentiated neuroepithelium which primarily undergoes mitosis until embryonic day 8, at which time cell division slows and the retinal cells begin to differentiate into their final phenotype (Moscona and
Figure 2: The adult vertebrate retina. Retinal layers and synaptic relationships are shown between the various neurons and the Müller glia. The major cell types are signified as R, rods; C, cones; H, horizontal cells; B, bipolar cells; I, interplexiform cells; A, amacrine cells; G, ganglion cells; M, Müller glia cells. (Modified from Farber and Adler, 1986).
Figure 2

Choroidal Border

Pigment Epithelium

Photoreceptor Layer

Outer Nuclear Layer

Outer Plexiform Layer

Inner Nuclear Layer

Inner Plexiform Layer

Ganglion Cell Layer

Nerve Fiber Layer

Optic Nerve

Vitreal Border
Moscona, 1979). During this phase, retinal lamination appears as the neurons migrate to their final positions in the retina.

It is also feasible to remove the retina and sustain it in organ culture. GS expression in the cultured intact retina follows a pattern of development similar to that of the retina in ovo. Thus, developmental aspects of GS expression can be easily and accurately studied in vitro. In cultures of dissociated retina, there is no steroidal induction of GS. This implies that the neuronal-glial cell interactions are necessary for proper induction of GS in the retina (Vardimon, et al., 1988, Linser and Moscona, 1979).

Statement of problem

The studies presented in this paper were geared toward defining the control mechanisms of cell-specific expression of GS in the chick retina. This problem was approached by two specific methods. First, a retinal assay system was developed to allow us to localize the expression of transfected plasmids to individual cells. From this system, we have identified a 2.2 Kb fragment of GS promoter that confers cell-selective expression of a reporter gene in the Müller glia of the retina. And secondly, transgenic mice were generated to study the in vivo expression of GS fusion transgenes. As an adjunct to these studies, the technique of electroporation, as it relates to transfection of intact retina, was carefully characterized. The long term goal of this research program is to demonstrate which sequences of the GS promoter region are responsible for Müller glial cell-specific expression in the retina. The identification of such sequences could, theoretically, have clinical implications in the treatment of ocular diseases. As one may be able to target the production of therapeutic proteins to specific cells of the retina.
Chapter I
Electroporation as a Tool for the Transfection
of Embryonic Chick Retina

Introduction

Transfection methodology in cell lines has been instrumental in the study of gene expression. The introduction of specific DNA plasmids into cells has been performed by various techniques which include the utilization of calcium phosphate (Wigler, et al., 1979), DEAE-dextran (McCutchan and Pagano, 1968), lipophilic carriers (Fraley and Papahadjopoulos, 1982), direct microinjection (Capecchi, 1980), viruses (Mulligan, et al., 1979), high-velocity microprojectiles (Fox, et al., 1988), and electric fields (Andreason and Evans, 1989).

Of course, since cell lines consist of only a single cell type, investigations using these lines preclude the study of cell-cell interactions and possible effects upon gene expression. Nervous tissue is primarily composed of two cell types, neurons and glia, whose interactions may affect gene expression. However, studying these complex relationships \textit{in vitro} is not without difficulty. To date, it has not been possible to culture \textit{in vitro} a whole brain or spinal cord and simultaneously introduce exogenous DNA into the cells of the organ. The avascular embryonic chick retina, however, provides a source of complex tissue composed of both neuronal and glial cell types. The retina can be easily removed from the developing chick and cultured as a whole organ (Linser and...
Moscona, 1979). Thus, the natural contacts between neurons and glia are preserved.

For gene expression studies, one would like to introduce reporter constructs into these complex tissues without disrupting cellular contacts. For these purposes, our lab has adopted electroporation for transfection of intact retina. This technique has been widely used in applications ranging from the transfection of plant (Fromm, et al., 1986), yeast (Hashimoto, et al., 1985), and eukaryotic cell lines (Shigekawa and Dower, 1988) to the transformation of bacteria (Dower, et al., 1988). The theory of electroporation is based on the observation that cell membranes partially breakdown when placed in an electric field. These transient "pores" are quickly sealed, but, while open, do allow passage of foreign particles, such as DNA (Potter, 1988), peptides (Raptis, et al., 1995), and antibodies (Lukas, et al., 1994), into the cytoplasm of a cell. In the case of transfected plasmid DNA constructs, transcription may proceed and the protein product may be expressed. Although this technique has been utilized previously in our lab, an in-depth examination of this transfection process for chick retina was lacking. The studies which are presented in this chapter attempt to answer basic questions concerning the use of electroporation in transfection of embryonic chick retina. These topics include the choice of electroporation devices and their optimal settings, the effects of varying culture conditions both before and after transfection, and the quantification of plasmid DNA which enters into the retinal cells.
Materials and Methods

Plasmid DNA constructs

pRSV-CAT (Gorman, et al., 1982), pRSV-βgal (MacGregor, et al., 1987), and pRSV-LUC (De Wet, et al., 1987) were all obtained as gifts. These constructs are all driven by the Rous Sarcoma Virus long-terminal repeat (RSV-LTR). The reporter genes expressed from these plasmids produce chloramphenicol acetyl transferase (CAT), β-galactosidase (β-gal), and firefly luciferase (LUC), respectively. The GS promoter constructs used in this chapter, p7f-CAT and pXP242, were both cloned in this laboratory. The details of their constructions are provided in Chapter 3.

Large-scale preparation of plasmid DNA

The preparation of plasmid DNA on a milligram level was based largely on the alkaline lysis method detailed by Sambrook, et al., 1989. A frozen plasmid-containing bacterial stock was streaked onto a 1.2% LB (Luria-Bertani) agar (for 400 mls: 4.8 grams agar, 4 grams tryptone, 2 grams yeast extract, 2 grams NaCl) petri dish which contained 50 μg/ml ampicillin. The culture was grown overnight at 37 °C. A single colony was picked with a sterile toothpick and added to a 15 ml plastic Corning tube which contained LB medium (for 250 mls: 2.5 grams tryptone, 1.25 grams yeast extract, 1.25 grams NaCl) and 50 μg/ml ampicillin. This tube was grown overnight in a rotating incubator at 37 °C and 250 RPM. After the tube became cloudy with bacterial growth, its contents were added to a 2.8L Fernbach flask which contained 1 liter Terrific Broth with 50 μg/ml ampicillin. The large prep was grown overnight in the rotating incubator at 37 °C at 250 RPM. This solution was then centrifuged in the Sorvall
for 15 minutes at 4000 RPM, 4 °C. The supernatant was poured off and discarded. The pellet was completely resuspended in 40 mls Solution 1 (50 mM glucose, 25 mM Tris-Cl pH 8.0, 10 mM EDTA pH 8.0). The alkaline lysis step was performed by the addition of 80 mls of freshly prepared Solution 2 (0.2 N NaOH, 1% SDS). The contents were mixed by gently inverting the closed bottle several times. The bottle was left unagitated at room temperature for 5-10 minutes preceding the addition of 40 mls of ice-cold Solution 3 (5 M potassium acetate 60 mls, glacial acetic acid 11.5 mls, water 28.5 mls). The bottle was shaken several times and then stored on ice for 10 minutes. The prep was then centrifuged for 20 minutes at 6000 RPM at 4 °C. The supernatant was filtered through 4 layers of cheesecloth into a 250 ml centrifuge bottle. Isopropanol 0.6 volume (96 mls) was added to the supernatant and the solution mixed. The solution was stored undisturbed for 10 minutes at room temperature. The precipitate was collected by centrifugation at 6000 RPM at room temperature for 20 minutes. The supernatant was discarded and the pellet rinsed with 70% ethanol. The ethanol was then allowed to evaporate and the pellet was resuspended in 6 mls of autoclaved water. Cesium chloride (9 grams) was added to the dissolved pellet and stirred until completely dissolved. This solution was placed in a 10 ml Beckman Quickseal centrifuge tube (#1610) with 500 μl of ethidium bromide 10 mg/ml solution. The remainder of the tube was filled with a stock solution of 88 grams CsCl mixed with 80 mls water. The final density of the solution in the centrifuge tube was ~1.55 g/ml. The sample was spun at 50,000 RPM in a 70.1Ti rotor at 20 °C for 20 hours. The tube was gently removed from the rotor and the lower band of DNA collected using a syringe and large bore needle. The DNA was placed into a new centrifuge tube and filled with the CsCl stock solution. The gradient was spun for 20 hours at 50,000
RPM. The DNA band was collected as before and placed into a 15 ml plastic Corning tube. To extract the ethidium bromide, an equal volume of water-saturated butanol was added to the tube and mixed by vortexing. Separation of the phases was done by centrifugation at 1500 RPM for 3 minutes at room temperature. With a pasteur pipette, the organic phase (on top) was removed and the extraction repeated with equal volumes of water-saturated butanol until the pink color of the EtBr disappeared from both the aqueous and organic phases. At this point, the CsCl needed to be removed from the DNA solution. The preparation was loaded into dialysis tubing (Spectra/Por 2, MW 12,000-14,000) and the bag placed in a 1 liter beaker with distilled water. Using a magnetic stir bar and plate, the dialysis solution was gently stirred at 4 °C. Exchanges were made with distilled water at least three times separated by at least 1-2 hours each. The solution was removed from the dialysis bag and placed into a 30 ml glass Corning tube. The DNA was precipitated by the addition of 0.1 volume 5 M NaCl and 2.5 volumes 95% EtOH and mixed by inversion. The tube was stored at -80 °C for 1 hour to assist DNA precipitation. The DNA was pelleted by centrifugation at 9000 RPM for 20 minutes at room temperature. The EtOH was discarded and the pellet briefly rinsed with 70% EtOH before air drying. DNA was resuspended in TE (10 mM Tris-Cl pH 7.4 and 1 mM EDTA pH 8.0). Using the spectrophotometer, the OD at 260 nm was measured of 5 μl sample in a total volume of 1000 μl in a quartz cuvette. An OD of 1 corresponds to 50 μg/ml for double-stranded DNA.

Incubation of chick embryos

Fertile White Leghorn chicken eggs were obtained from The Ohio State University Department of Poultry Science. Eggs were kept at 14 °C until ready
for incubation. The eggs were allowed to warm up at room temperature for 1 hour before placement in an incubator at 38 °C for 6 to 18 days. The chambers were kept humidified with water.

**Removal of retina from embryonic chicks**

Chick embryos were removed from the egg and staged according to Hamburger and Hamilton, 1951. The embryos were killed by complete transectioning of the neck. The eyes were removed and placed into a dissecting dish and immersed in a solution of 1X Hanks (for 1 liter: CaCl$_2$·2H$_2$O 0.185 g, KCl 0.4 g, KH$_2$PO$_4$ 0.06 g, MgCl$_2$·6H$_2$O 0.1 g, MgSO$_4$·7H$_2$O 0.1 g, NaCl 8 g, Na$_2$HPO$_4$·7H$_2$O 0.09 g, glucose 1 g, NaHCO$_3$ 0.35 g). With the aid of a dissecting stereo microscope, the lens of the eye is removed followed by the vitreous humor. The retina can then be visualized and gently removed from the optic cup paying particular attention to separate the retina from the pigmented epithelium. The tissue can then be used for transfection or preparation of primary flat-cell cultures.

**Electroporation with the Biorad Gene Pulser**

The retina was dissected and either precultured for 24 hours in Dulbecco's modified Eagle's medium with high glucose (DMEM-HG) supplemented with 10% (v/v) fetal bovine serum (Irvine Scientific) and streptomycin 50 μg/ml and penicillin 50 units/ml (BRL-GIBCO), or immediately placed in a 0.4 cm Biorad electroporation cuvette containing 1 ml of DMEM-HG (1X PBS is also acceptable, see discussion [10X PBS: NaCl 80 g, KCl 2 g, Na$_2$HPO$_4$·7H$_2$O 27.2 g, KH$_2$PO$_4$ 2.4 g, brought up to 1 liter with water and pH adjusted to 7.4 with concentrated HCl]). The supercoiled plasmid for
transfection was also placed in the cuvette. The desired capacitance and voltage settings were selected on the device (standard 960 μFd, 370 V). The cuvette was placed in the holder and the pulse delivered to the sample. The sample was left undisturbed for 1-2 minutes while other samples were transfected. The entire contents of the cuvette were then poured into a 25 ml organ culture flask containing 7 mls of DMEM-HG supplemented with 10% (v/v) fetal bovine serum (Irvine Scientific) and streptomycin 50 μg/ml and penicillin 50 units/ml (BRL-GIBCO). The flasks were then equilibrated with 5% CO₂/95% air for 30 minutes before sealing. Samples were incubated in a dark shaking incubator at 60 RPM and 38 °C for 1 to 14 days. For the duration of the experiment, the medium was replaced every 2-3 days depending on its color change from pink to yellow.

**Electroporation with the BTX-800 transfection device**

The retina were dissected and precultured for 24 hours in DMEM-HG supplemented with 10% (v/v) fetal bovine serum (Irvine Scientific) and streptomycin 50 μg/ml and penicillin 50 units/ml (BRL-GIBCO). The samples were rinsed twice with 1X PBS to remove medium and the retina added to a sterile, plastic spectrophotometric cuvette (P/N 471, 1 ml, 0.4 cm wide) which contained the plasmid DNA diluted in 1 ml of 1X PBS. The parameters of amplitude, length of pulse, and number of repeats of pulse were selected on the device. The 0.4 cm electrodes were placed into the cuvette and the pulses delivered. The sample was then handled in the same manner as the Biorad procedure (i.e. grown in serum-supplemented growth medium).
Preparation of retinal cellular extracts

The retinal samples were harvested by aspirating most of the medium and pouring the remaining fluid and retinal tissue into a 1.5 ml eppendorf tube. The samples were spun at full speed in an Eppendorf benchtop centrifuge for 5 minutes at which time the supernatant was removed. The samples were then rinsed twice with 1X PBS with gentle vortexing to resuspend the pellets. A 5 minute spin followed each rinse step. After the final PBS rinse, each pellet was resuspended in 300μl 0.25 M Tris-Cl pH 8.0 and sonicated for 8 pulses using a Branson Sonifier 450 at 50% duty cycle, output control at 7, and the timer on hold. Samples were kept on ice before, during, and after sonication. The cellular debris was then spun down by centrifuging for 5 minutes at full-speed at 4 °C. The supernatants were then assayed by the methods described below.

Luciferase assay

This protocol is based on that described by De Wet, et al, 1987. Luminescence measurements were made with a Berthold Lumat luminometer (model 9501). From 5 to 50 μl of retinal extract was added to a 12×75 mm plastic test tube (Sarstedt) which contained 350 μl of substrate 2 (25 mM gly-gly buffer pH 7.8, 5 mM Na₂-ATP pH 6-8, 15 mM MgSO₄). The tube was inserted into the luminometer and the injection and measurement period started. The injection consisted of 100 μl of substrate 1 [1 mM luciferin (Jersey Labs) in water] and the emitted light was monitored for 10 seconds. Raw data from the luminometer was reported in relative-light units (RLUs). These values were normalized to either extract protein concentrations or to raw data from internal controls (i.e. activities arising from cotransfection with pRSV-βgal or pRSV-CAT). The use of internal controls is necessary to distinguish differences in the level of transcription from
differences in the efficiency of transfection when studying the effect of promoters on gene expression.

**Chloramphenicol Acetyl Transferase (CAT) assay**

Extracts to be tested for CAT activity were heated in a water bath at 65 °C for 10 minutes followed by centrifugation at full-speed for 10 minutes. Samples of the supernatant to be tested were placed in a new eppendorf tube and the volumes were brought up to 300 µl with 0.25 M Tris-Cl pH 8.0. Fifty µl of a master mix [5 µCi/ml 14C-chloramphenicol (NEN-Dupont), 1 mg/ml butyryl-CoA (Sigma), 0.2 M Tris-Cl pH 8.0] was added to each sample and mixed. The samples were incubated in a water bath at 37 °C for 1 to 24 hours. Incubations were stopped when 10 to 30% of the chloramphenicol substrate had been converted into the acetylated product. The reactions were terminated by the addition of 700 µl of a 2:1 TMPD/xylene solution. Samples were thoroughly vortexed and spun for 3 minutes at full-speed at room temperature. Scintillation counting was performed by removing 500 µl of the top phase and adding it to a scintillation vial containing 5 mls of Scintiverse. Samples were counted using a Packard scintillation counter. Raw data was reported in counts per minute (CPM). These values were normalized to either extract protein concentrations or to raw data from internal controls (i.e. activities arising from cotransfection with pRSV-βgal or pRSV-LUC).

**β-galactosidase (β-gal) assay**

For each sample to be tested, the following was added to a 1.5 ml centrifuge tube: 3 µl of 100X Mg solution (0.1 M MgCl₂, 4.5 M β-mercaptoethanol), 66 µl of 1X ONPG (4 mg/ml α-nitrophenyl-β-D-
galactopyranoside in 0.1 M sodium phosphate pH 7.5), 30-231 \mu l retinal cell extract, and 0-201 \mu l 0.1 M sodium phosphate pH 7.5 (retinal cell extract and sodium phosphate buffer should total 231 \mu l). Samples were incubated in a water bath at 37 °C for 30-240 minutes. Reactions were incubated until a faint yellow color developed at which time 500 \mu l 1 M Na\textsubscript{2}CO\textsubscript{3} was mixed in each sample. The reaction mixture was poured into a 1 ml spectrophotometer cuvette and the optical density was read at 420 nm. The linear range for this reaction is 0.2-0.8 OD. The raw data was converted into specific activity (S.A.) by the formula reported by Norton and Coffin (1985). \beta-gal S.A. equals \frac{380 \times \text{OD}_{420}}{\text{reaction time (min)}} / \text{mg protein in sample}. One unit is equivalent to 1 nmol of ONPG hydrolyzed per minute per mg protein. These values were normalized to either extract protein concentrations or to raw data from internal controls (i.e. activities arising from cotransfection with pRSV-CAT or pRSV-LUC).

**Protein assay by Bradford method**

The protein concentrations of the retinal extracts were determined by the method of Bradford, 1976. One ml aliquots of protein assay reagent (Biorad) at room temperature were added to 1 ml spectrophotometer cuvettes. An aliquot of retinal extract (usually 5 \mu l) was added to the cuvette and mixed by inversion. The optical densities of the samples were read at 595 nm in a Beckman spectrophotometer. Bovine serum albumin (BSA) standards from 0.1 mg/ml to 5 mg/ml were also added (5 \mu l) to create a standard curve. The curve was fitted by a linear regression method using the computer-based program Kaleidagraph. The resulting equation was used to convert the retinal extract ODs to concentrations of protein in mg/ml.
Harvest of total DNA from retinal cultures

This procedure is based on that described by Davis, et al.(1986). Retinal cultures were rinsed in an eppendorf tube 7 times with 1X PBS to ensure that no remaining untransfected plasmid DNA would contaminate the prep. The tissue was transferred to a 10 ml polypropylene tube containing 5 ml 1X RSB buffer (10 mM Tris-Cl pH 7.4, 10 mM NaCl, 25 mM EDTA pH 8.0). One-tenth volume of 10% SDS was added followed by enough powdered fungal proteinase K (GIBCO BRL) for a final concentration of 1 mg/ml. The mixture was incubated in a 37 °C water bath for 1-2 hours or until the tissue was completely dissolved. An additional 1 mg/ml proteinase K was added if necessary. After the incubation, 1/10th volume 5 M NaCl was added and the entire solution extracted with an equal volume of 1:1 phenol / chloroform mixture. Following gentle mixing and centrifugation at 1500 RPM for 3 minutes in the IEC benchtop centrifuge, the aqueous phase was transferred to a clean polypropylene tube and extracted with 2 volumes of diethyl ether. The ether phase (on top) was removed by aspiration and the remaining aqueous phase (on bottom) was precipitated with 2.5 volumes of ice-cold 95% EtOH. The precipitated DNA strands were removed from the solution with a glass rod and inserted into a 15 ml plastic Corning tube with 5 ml 0.1X SSC buffer (for 20X SSC stock: NaCl 175.3 g, trisodium citrate 88.2 g, brought up to 1 liter with water and pH adjusted to 7.0 with 5 N NaOH). The solution was placed on a Nutator at 4 °C overnight to allow for complete dissolution of the DNA. RNA was removed by the addition of 10 μl of 10 mg/ml DNase-free RNase A (dissolve RNase A in 10 mM Tris-Cl pH 7.5 and 15 mM NaCl, boil for 15 minutes and cool slowly, store at -20 °C) and incubation for 30 minutes in a 37 °C water bath. Following the enzyme reaction, 1/10th volume 5 M NaCl was added and the entire solution was
extracted with 2 volumes 1:1 phenol / chloroform. The samples were centrifuged for 5 minutes at 1500 RPM in IEC for phase separation. The aqueous phase (on top) was removed with a large bore pipette and transferred to a new tube. The DNA was EtOH precipitated as before using 2.5 volumes ice-cold 95% EtOH. The DNA strands were removed with a glass rod and dissolved overnight in 5 mls TE buffer on the Nutator at 4 °C. The DNA concentration was determined either by spectrophotometric methods or by the DABA assay (see next section).

**Determination of DNA concentration by DABA method**

This procedure is based on the method described by Schy and Plewa, 1989. Herring sperm DNA standards were prepared in concentrations of 25 ng/μl to 1000 ng/μl. Five μl of sample DNA, standard DNA, or water was added to separate 10x75 mm glass culture tubes. In a room with minimal light, 15 μl of 400 mg/ml diaminobenzoic acid (DABA) (prepared in water with light exposure minimized) was added to each tube. Parafilm was used to seal the tubes and incubation was carried out in a 60 °C heat block for 45 minutes in the dark. Following the incubation, the tubes were removed from the heat and allowed to cool for 5 minutes. Then, 2.6 mls 0.6 M perchloric acid was added to each tube and the contents mixed thoroughly by inversion. The fluorescence of the samples was determined by using a fluorimeter (Turner model 111) with filters to allow excitation at 404 nm and emission at 520 nm. The standard DNA samples were used to construct a standard curve from which the sample DNA concentrations could be calculated. This method allows for the detection of as little as 100 ng of DNA and is not interfered with by contaminating RNA.
Southern blotting of DNA preps

After quantitation of the total retinal DNA samples, 10 µg of DNA was cut overnight at 37 °C by restriction digest (7.5 units enzyme/µg DNA) in a volume of 100-200 µl. After cutting was complete, DNA was precipitated by the addition of 1/10th volume 5 M NaCl and 2.1 volumes 95% EtOH. The DNA pellets were resuspended in 20 µl water. DNA loading buffer (for 6X stock: 0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll 400) was added to a final concentration of 1X and the samples were loaded onto a 0.7% agarose gel with appropriate size markers. The gel was electrophoresed overnight at 25-35 V. After taking a picture of the gel on the UV lightbox, the gel was denatured by gentle agitation at room temperature for 45 minutes in several volumes of a solution of 1.5 M NaCl and 0.5 N NaOH. The gel was briefly rinsed in deionized water before neutralization in several volumes of a solution of 1 M Tris Cl pH 7.4 and 1.5 M NaCl. This step was performed for 30 minutes before changing to fresh neutralization solution and continuing to agitate for an additional 15 minutes. The DNA was transferred from the gel to a Duralon-UV nylon membrane with the assistance of a Posiblot (Stratagene) apparatus. The transfer was performed in the presence of 10X SSC solution for 2 hours at a pressure of 75 mmHg. Following transfer, the lanes of the gel were marked on the membrane and excess fluid was removed by placing the membrane on a paper towel. While still damp, the membrane was placed in a Stratalinker (Stratagene) and the DNA cross-linked to the membrane by UV irradiation. The blot was then placed in a Seal-a meal bag with prehybridization solution (50% formamide, 5X SSC, 5X Denhardt's [For 100X stock: polyvinylpyrrolidone 10 g, BSA 10 g, Ficoll 400 10 g, brought up to 500 mls with water], 0.1 mg/ml denatured herring sperm DNA) and incubated on a Nutator at 42 °C for 30
minutes. Following prehybridization, the solution was replaced with a hybridization mixture (50% formamide, 5X SSC, 2X Denhardt's, 0.1 mg/ml denatured herring sperm DNA, 0.1% SDS) and the denatured probe was added to the bag. Hybridization was carried out on a Nutator at 42 °C for 18 hours. To remove excess probe, the blot was washed in a solution of 2X SSC, 0.1% SDS for 20 minutes at 65 °C. The blots were further rinsed twice in 0.1X SSC, 0.1% SDS for 25 minutes each at 65 °C. The blots were then exposed either in a Phosphorimager cassette or a standard autoradiography cassette with Kodak film.

**Synthesis of $^{32}$P labeled probe**

$^{32}$P labeled probes were synthesized by the random primer method. First, linearized DNA fragments to be used for probe synthesis were generated by cutting with appropriate enzymes followed by gel electrophoreses in 0.7% low-melting point agarose. The fragment of interest was band isolated from the gel in the smallest possible volume (50-100 µl) and placed in an eppendorf tube with enough TE to bring up to 500 µl. The sample was heated at 65 °C for 10 minutes to completely melt the agarose. The sample was then extracted with an equal volume of phenol followed by extraction with 1:1 phenol/chloroform followed by a final extraction with chloroform. DNA in the aqueous phase was precipitated by the addition of 1/10th volume 5 M NaCl and 2.1 volumes 95% EtOH. The sample was kept at -80 °C to aid precipitation. The DNA was pelleted by centrifugation at 4 °C, full-speed, for 30 minutes. The DNA pellet was dissolved in a small volume of TE (25-50 µl). The concentration of the sample was determined by the DABA method described elsewhere. Fifty ng of the linearized DNA was placed in an eppendorf tube with 1 µl random hexamer
oligonucleotide (90 OD$_{260}$ units/ml) and brought up to a final volume of 13 μl with water. The solution was boiled for 5 minutes to denature and then cooled on ice. The reaction mixture was added to the tube as follows: 2 μl 10X Pol I buffer (500 mM Tris-Cl pH 7.2, 100 mM MgCl$_2$, 1mM DTT, 500 μg/ml BSA), 4 μl nucleotide mix (3.3 mM each with respect to dATP, dGTP, and dTTP), 2 μl α-³²P dCTP (10 μCi/μl, NEN-Dupont), and 0.5 μl Klenow enzyme (6 units/μl, BRL). The sample was then stored in a lead container at room temperature for 3-16 hours. Free nucleotides were removed by using a NucTrap probe purification column (Stratagene). A 3 μl sample was taken for scintillation counting. Specific activities of probes were always greater than 1x10⁸ cpm/μg.

Results

Optimization of electroporation parameters

In order to optimize transfection of intact embryonic chick retina, parameters affecting luciferase-gene activity following electroporation by either the Biorad Gene Pulser or the BTX-800 transfection apparatuses were examined. These electroporation devices operate on the same basic principle that cells placed in an electric field develop micropores in their membranes. Foreign substances such as DNA or proteins may gain entry into the cytoplasm during this process. However, these devices differ in the manner in which the electric field is produced. The Biorad apparatus utilizes an exponential decay waveform, which can be generated by complete discharge of a capacitor. In this case, the voltage rises very rapidly to a peak amplitude and then declines as an exponential function of the resistance and the capacitance. With the BTX apparatus, a square waveform is generated by rapidly increasing the voltage to
the desired amplitude, holding that voltage for a specified time, and rapidly reducing the voltage to zero. This is accomplished using a partial-discharge capacitor. There is some disagreement in the literature concerning which device results in higher electroporation efficiencies. The argument arises when one considers whether the length of time the electric field is applied (i.e. pulse width) affects overall transfection. The BTX device allows for complete control of pulse width. However, in the Biorad system, the pulse width is primarily a function of resistance in the sample. It is on this basis that both methodologies were examined in the context of chick retinal transfection.

Electroporation of E10 retina was examined by monitoring transfected luciferase gene (pRSV-LUC) activities while altering both the capacitance and voltage settings of the Biorad unit (Figure 3). pRSV-LUC gene expression is reported as relative light units per milligram of protein (RLU/mg protein). When voltages were held constant, a general trend emerged indicating that 960 μFd is the optimal capacitance setting for enzyme activity. This value is the highest capacitance allowable with this device. From this figure, we also noted that maximal enzyme activities were associated with the higher voltages tested, 400 volts and 450 volts. Further characterization of the optimum voltage is shown in figure 4. Part A of this figure is derived from the data in Figure 3 and shows the effect of varying voltage when the capacitance is kept constant at 960 μFd. Part B of Figure 4 takes a closer look at voltages ranging from 370V to 410V in 10 volt increments. From these data we conclude that a voltage of 370 volts (equivalent to 0.925 kV/cm) and a capacitance of 960 μFd gives maximal luciferase activity using the Biorad Gene Pulser.

The trials of the BTX-800 electroporation system are depicted in Figure 5 Part A shows the effect on luciferase activity when the retina is subjected to 1 to
Figure 3: Effects of varying capacitance and voltage of the Biorad Gene Pulser on transfected luciferase activity. E10 chick retina were dissected and grown for 24 hours in DMEM-HG with 10% FBS and streptomycin/penicillin. Following the preculture period, retina were electroporated with pRSV-LUC 20 μg/ml and cultured an additional 22 hours before harvest and assay. Each bar represents the average of two samples.
Figure 4: Optimization of electroporation voltage using a Biorad Gene Pulser. E10 chick retina were dissected and grown for 24 hours in DMEM-HG with 10% FBS and streptomycin/penicillin. Following the preculture period, retina were electroporated with pRSV-LUC 20 µg/ml and cultured an additional 22 hours before harvest and assay. (A) Examination of voltages from 100 to 450 volts at a capacitance of 960 µFd. Each bar represents the mean of 2 samples. (B) Examination of voltages from 370 to 410 volts at a capacitance of 960 µFd. Error bars represent standard deviations from 3 samples at each condition.
Figure 4
Figure 5: Utilization of the BTX-800 electroporation system in transfection of embryonic chick retina. E10 chick retina were dissected and grown for 24 hours in DMEM-HG with 10% FBS and streptomycin/penicillin. Following the preculture period, retina were electroporated with pRSV-LUC 20 μg/ml and cultured an additional 22 hours before harvest and assay. (A) The number of consecutive 99 μsec square wave pulses is varied from 1 to 50. Each bar represents the mean of 2 samples except as designated by a "***" which implies one sample only. (B) The number of consecutive 99 μsec square wave pulses is varied from 10 to 30. Error bars represent standard deviations from 3 samples at each condition.
50 consecutive 99 µsec pulses in an electric field of 1.4 kV/cm. Fifteen pulses appears to give maximal expression in this experiment. A range of 10 to 30 consecutive 99 µsec pulses was examined in part B, which shows a peak in reporter activity at 17 pulses. However, all enzyme activities in this range were within 3 fold of each other. Thus, the optimization of this system was not dramatic. Further characterizations were not performed since there were no significant increases in transfected enzyme activities as compared to the Biorad system.

Due to its ease of use, the Biorad Gene Pulser at settings of 370 volts and a capacitance of 960 µFd was adopted into the standard electroporation procedure and was utilized for all subsequent experiments.

Retinal variables which affect electroporation

Experiments were undertaken to determine if preculture of the retina had any effect on the subsequent expression of transfected plasmids. Figure 6 shows that when retina are electroporated following a 24 hour preculture period, luciferase activities are 5-fold lower than when the retina are immediately electroporated after dissection. Thus, the adoption of no preculture period was incorporated into the standard transfection protocol.

Figure 7 demonstrates the effect of the embryonic age of the retina upon electroporation with pRSV-LUC. Luciferase gene expression is shown to decline as the embryonic age of the retina increases. That is, the highest luciferase expression is seen at embryonic day 6 and declines, as measured at three day intervals, until embryonic day 18, at which time the level of expression has decreased approximately 20 fold from expression at embryonic day 6. This relationship of declining expression with age provides a possible explanation
Figure 6: Effects of a 24 hour preculture period on transfected luciferase activity. One group of E10 chick retina was dissected and grown for 24 hours in DMEM-HG with 10% FBS and streptomycin/penicillin before electroporation. The other group of retina was electroporated immediately following dissection. Electroporation was performed using the Biorad Gene Pulser with pRSV-LUC 20 μg/ml at 960 μFd and 370 volts. Retina were cultured for 22 hours after transfection before harvest and assay. Error bars represent standard deviations from 4 samples at each condition.
Figure 7: Effects of embryonic age of chick retina on transfected luciferase activity. Retina of varying embryonic ages were dissected and immediately electroporated with pRSV-LUC 20 μg/ml. A Biorad Gene Pulser was used at 960 μFd and 370 volts. Retina were then cultured for 48 hours in DMEM-HG with 10% FBS and streptomycin/penicillin before harvest and assay. Error bars represent standard deviations from 3 samples at each condition.
for the difference of expression seen in the preculture experiments. In this case, retina were dissected at E10, but were actually E11 retina when transfected. Thus based on figure 7, one would expect to find lower expression in the precultured retina compared to the immediately transfected E10 retina.

**Long-term culture of electroporated retina**

Transient assays have been traditionally performed 24-72 hours following transfection. However, we have examined the effects of culturing electroporated retina up to 14 days before assay as shown in figures 8 and 9. Two reporter genes were tested in this system to discern any possible differences in the patterns of activity of the reporter proteins. First, pRSV-LUC was transfected into both E6 and E10 retina and cultured from 1 to 14 days. Figure 8 demonstrates a decline in luciferase expression over a two-week culture period independent of the age of the retina at transfection. Similar experiments were performed (figure 9) with transfected pRSV-CAT to determine whether this pattern of declining activities was independent of the reporter gene. In part A of this figure, we see that in E6 retina, there is a reduction in CAT activity over the two-week culture period. However, as seen in part B, CAT activity increases over the study period in E10 retina. This unexpected result was confirmed in three different experiments.

**Fate of transfected plasmid DNA**

To address these paradoxical findings in retina transfected with pRSV-CAT, we quantified the disappearance of transfected plasmids over a two-week culture period. This was accomplished by Southern blotting of total DNA prepared from these cultures. These results could then be compared with their
Figure 8: Effects of long-term culture of electroporated retina on luciferase expression. Retina of either E6 or E10 chick embryos were dissected and immediately electroporated with pRSV-LUC 20 μg/ml using a Biorad Gene Pulser at 960 μFd and 370 volts. Retina were then cultured in DMEM-HG with 10% FBS and streptomycin/penicillin for 1 to 14 days before harvest and assay. Error bars represent standard deviations from 3 samples at each condition. (A) Embryonic day 6 retina. (B) Embryonic day 10 retina.
Figure 8
Figure 9: Effects of long-term culture of electroporated retina on chloramphenicol acetyl transferase expression. Retina of either E6 or E10 chick embryos were dissected and immediately electroporated with pRSV-CAT 60 μg/ml using a Biorad Gene Pulser at 960 μFd and 370 volts. Retina were then cultured in DMEM-HG with 10% FBS and streptomycin/penicillin for 1 to 14 days before harvest and assay. Error bars represent standard deviations from 3 samples at each condition. (A) Embryonic day 6 retina. (B) Embryonic day 10 retina.
Figure 9

A

CAT / mg protein (10^-5)

Incubation (days)

1 3 4 6 8 11 14

B

CAT / mg protein (10^-5)

Incubation (days)

1 2 4 6 8 10 12 14
respective enzyme expression data. As shown in figure 10, amounts of both
transfected plasmids, pRSV-LUC and pXP242, decline with time in
electroporated E6 retina. pXP242 is a luciferase expression vector driven by
~4.2 Kb of chicken GS promoter (see Chapter 3). By transfecting with this
plasmid, a single probe to a portion of GS promoter could detect both the
endogenous gene and the transfected plasmid. It is the endogenous copy of the
GS promoter which provides an internal control for the blot. One can compare
the intensities of the endogenous GS bands in individual lanes to ensure that
equivalent amounts of genomic DNA were analyzed. The decrease in amount
of pRSV-LUC agrees with the declining enzyme activity detected in analogous
cultures.

Similar experiments were performed with the CAT constructs, pRSV-CAT
and p7f-CAT. p7f-CAT is a CAT-expressing homolog of pXP242 and thus allows
for the detection of an internal control. Figure 11 shows the fates of CAT
constructs in E6 and E10 retina. In part A, the amount of p7f-CAT is shown to
decrease with time. Parts B and C illustrate that pRSV-CAT also decreases with
time in both E6 and E10 retina. Thus, even though in E10 retina CAT activity
increases over a two-week period, the amount of actual plasmid within the
transfected retina declines.

Estimations of the amounts of transfected plasmids were made using two
methods. First, the intensities of the transfected plasmid bands were compared
to the bands of standard amounts of the DNA used for transfection. For the
purpose of these estimations, a genome equivalent was defined as the amount
of transfected plasmid that gives an equivalent signal intensity as a single copy
gene within the genome. A sample calculation demonstrating the relationship
between the amount of marker and the genome equivalency is shown below.
Figure 10: Southern blotting of luciferase-construct electroporated retina. Embryonic retina were electroporated with 20 μg/ml of either pRSV-LUC or pXP242. The samples were organ cultured for 2 to 14 days at which time total DNA was isolated. Each lane represents 10 μg of total DNA digested overnight with EcoRI. The mock (M) lanes represent retina which were identically treated but were not electroporated. Marker lanes consist of 10 μg carrier DNA doped with picogram quantities of plasmid before digestion. (A) E6 retina transfected with pXP242. (B) E6 retina transfected with pRSV-LUC. (A) was probed with a 2.8 Kb BamHI fragment from the promoter region of the chicken GS gene. (B) was probed with a 656 bp Eco RI fragment from the luciferase cDNA.
Figure 10
Figure 11: Southern blotting of CAT-construct electroporated retina. Embryonic retina were electroporated with 20 μg/ml of either pRSV-CAT or p7f-CAT. Samples were organ cultured for 2 to 14 days at which time total DNA was isolated. Each lane represents 10 μg of total DNA digested overnight with EcoRI. The mock (M) lanes represent retina which were identically treated but were not electroporated. Marker lanes consist of 10 μg carrier DNA doped with picogram quantities of plasmid before digestion. (A) E6 retina transfected with p7f-CAT, (B) E6 retina transfected with pRSV-CAT, (C) E10 retina transfected with pRSV-CAT. (A) was probed with a 2.8 Kb BamHI fragment from the promoter region of the chicken GS gene. (B) and (C) were probed with a 228 bp Eco RI fragment from the CAT gene.
Figure 11
size of marker DNA (p7f-CAT) = 6.5\times 10^3 \ \text{bp}

size of chicken genome = \sim 2\times 10^9 \ \text{bp}

amount of genomic DNA loaded per lane = 10 \ \mu g

\begin{align*}
6.5\times 10^3 \ \text{bp} / 2\times 10^9 \ \text{bp} &= x / 10 \ \mu g \\
x &= 3.3\times 10^{-5} \ \mu g (33 \ \text{pg})
\end{align*}

Thus, one genome equivalent of p7f-CAT

is equal to 33 pg in a 10 \ \mu g sample.

The second method involves simply estimating the ratio between the intensity of the transfected plasmid band and that of endogenous GS to determine the genome equivalency. It is clear that this procedure is only useful when an internal control is present.

In the p7f-CAT transfection, calculations reveal that 2 days after transfection there were as many as 50 genome equivalents of the plasmid. The amount of p7f-CAT declined to \sim 8 and \sim 0.5 equivalents by days 7 and 14, respectively. Similar estimations in the pXP242 experiment reveal genome equivalents of \sim 1.4, \sim 0.7, and \sim 0.14 respectively at days 2, 4, and 14. There were no significant differences in genome equivalency estimations by either method.
The aforementioned studies were initiated to carefully characterize the optimal conditions for electroporation of intact embryonic chick retina and to determine the fate of transfected plasmids. We were also interested in the average quantity of plasmid transfected into the cells and the subsequent expression of these plasmids on a gross enzyme level.

Comparison of the Biorad Gene Pulser and the BTX-800

The Biorad Gene Pulser and the BTX-800 represent two types of electroporation devices. While both operate on the premise that an electric field can induce pore formation in cell membranes, the manner in which each apparatus generates these fields is fundamentally different. The Biorad device generates an exponential decay waveform due to the utilization of a complete discharge capacitor. In the BTX-800 however, a square waveform is produced using a partial-discharge capacitor. The functional difference between these waveforms is that the length of the pulse (i.e. pulse width) can be strictly controlled in a square waveform, whereas the pulse width of an exponential decay waveform is primarily dependent upon resistance of the sample. A literature review reveals inconsistencies regarding which type of device is superior for transfection. We therefore examined both devices in the context of retinal electroporation.

The optimization of the Biorad apparatus in chick retinal transfections was performed by varying the physical parameters of the device. The major variable parameters are the capacitance, the resistance, and the voltage applied to the sample.
The capacitance variable indicates the amount of charge that the capacitor will store at a given voltage. Using a higher capacitance increases the time that the voltage will be applied to the sample. The length of time the voltage is applied is reflected by its time constant. It was demonstrated that at any given voltage, increasing the capacitance resulted in a higher level of pRSV-LUC expression. We postulate that this increased enzyme activity reflects a greater uptake of plasmid by the retinal cells. Based on this experiment, a capacitance value of 960 μFd, the maximum allowable with this device, was chosen as a standard setting for future electroporation trials.

The resistances of the samples and the electroporation medium were not explicitly examined. In early experiments, phosphate-buffered-saline (1X PBS) was used as the electroporation solution. However, this necessitated a wash step preceding the subsequent growth in the culture medium. The culture medium, DMEM-HG, without serum was also tested as an electroporation solution. This allowed for the direct addition of the contents of the electroporation cuvette to the final culture medium following transfection. There were no obvious differences detected between 1X PBS and DMEM-HG in either the time constants or enzyme activities (data not shown). DMEM-HG without serum was utilized as the electroporation solution in all subsequent experiments.

Perhaps, the most important variable in successful electroporation is the voltage applied to the sample. It is known that in order to maximize transfection of a given tissue or cell line, one must increase the voltage to a point where the creation of transient pores in the cell membrane is maximal but which permits the cells to recover and express the plasmid. It is this balance of cell-uptake of DNA versus cell-death which gives optimal expression. The data in figure 4
indicate that the maximal expression of pRSV-LUC occurs at 370 volts (0.925 kV/cm) in the Biorad system. Other experiments to define optimum voltage suggest that a range exists between 350V and 400V where expression levels are quite similar (data not shown). When the voltage was increased to 450 volts, the maximal allowable setting, enzyme activities were shown to decrease, presumably from increased cell death associated with higher voltages.

The BTX-800, as mentioned earlier, generates a square electrical waveform which means that a given voltage can be held for a finite amount of time. It has been suggested that high transfection efficiencies can be attained by carefully titrating this pulse width (Hoffman, 1988). In our trials of this device, a voltage of 1.4 kV/cm was used. This value is higher than the maximal attainable voltage of the Biorad unit, 1.125 kV/cm. The generated pulse lengths may range from 1 to 99 µsec and may be repeated in rapid succession. The sum total of which behaves essentially as a single pulse. The trials shown in figure 1-3 show optimum enzyme activity at 17 pulses of 99 µsec each for a total time of 1.7 msec. However, when one compares the luciferase activities per milligram of protein generated between the two electroporation devices, it is seen that in both cases the luciferase activities are on the order of $10^5$ RLU/mg protein. Thus there does not appear to be any great advantage of choosing the BTX-800 over the Biorad device for this application. Since the Biorad Gene Pulser is technically easier to use, all subsequent electroporation experiments were performed with this system.

Non-electrical variations in electroporation procedures

The protocol previously used in our lab incorporated a 24 hour preculture period preceding electroporation. This practice was examined in figure 6 which
shows that the deletion of the preculture period has a profound effect on transfected enzyme activity by increasing luciferase levels 5-fold compared to the precultured samples. A possible explanation for this phenomenon arises when one considers the effect of embryonic age upon expression (figure 7). In these experiments, increasing age of the retina correlated with lower enzyme activities. Thus, an E11 retina (dissected at E10 and precultured for 24 hours) would be expected to show lower activities than that of an immediately-transfected E10 retina. The standard electroporation protocol was amended to immediately electroporate the intact retina after dissection.

As mentioned above, the embryonic age of the retina was shown to have an effect on transient gene expression. We postulate that the decline in enzyme activities seen from E6 to E18 (figure 7) may, in part, be linked to the decline of the mitotic process. At earlier developmental stages of the chick embryo, the retina is strongly engaged in mitosis. By embryonic day 8, the retina begins to differentiate and mitosis slows. At E12, most mitotic divisions are complete and differentiation proceeds. It is generally accepted that transfection of cells in the logarithmic growth phase results in increased expression of a vector. This observation appears to be independent of the method of transfection employed and has been noted in techniques utilizing, calcium phosphate (Okayama and Chen, 1991), DEAE-dextran (Lake and Owen, 1991), and electrical poration (Spencer, 1991). In fact, successful retroviral-mediated gene transfer requires that the cells be actively dividing (Morgenstern and Land, 1991). In this regard, these findings in chick retina are neither novel nor surprising. Another explanation for these data involves cellular phenotypes and their possible effects on transfection efficiency. There may exist an inherent difference in the
ability to transfect non-differentiated retinal cells (E6) versus the differentiated neuronal or glial cell types (≥E12).

**Long-term culture of electroporated retina**

Culture of transfected cell lines is usually only carried out for a reasonable amount of time to allow for adequate expression of the plasmid. This length of time varies by the types of cells used, but is often 24-72 hours. However, the organ cultured retina continues to follow its developmental program (Moscona and Linser, 1983). Thus, perhaps it would be possible to study expression of a transfected gene in the context of normal development. We monitored the expression of transfected plasmids in retinal cultures over a two-week period following electroporation. These studies reveal that the stage of the retina at transfection can profoundly alter reporter gene expression in these long-term transient assays. In retinal transfection experiments using pRSV-LUC, the reporter gene activities were shown to decline over the 14 day period. This finding was independent of the age of the retina at transfection. In similar experiments using pRSV-CAT, opposing trends in enzyme activities are observed in E6 and E10 retina. In E6 transfections, CAT activity decreases with time in culture. However, in transfected E10 retina, an accumulation of CAT activity with time was observed. Thus, when using this particular plasmid, the age of the retina at transfection appears to have a critical effect on CAT expression in long-term cultures. Experiments examining the disappearance of transfected plasmids (figures 10 and 11) have shown that, independent of the retinal age at transfection, these same constructs are degraded with time. To explain our enzyme data, we postulate that there is a fundamental difference in the degradation of CAT mRNA in E6 retina relative to E10. The cellular milieu of
an E6 retina may favor CAT mRNA instability. While in E10 retina, CAT mRNA may be stabilized by unknown factors. It should be noted that in electroporated HeLa cells, CAT activity has been observed to remain at high levels for up to 40 hr after transfection, whereas luciferase activity declines after an initial peak (Maxwell and Maxwell, 1988). These investigators postulate that this observed difference in reporter activities is presumably explained by differential stability of the respective reporter mRNAs and/or enzymes. Their results suggest that much of the CAT activity seen at later times may have been expressed as a result of transcription within the first 12 hours following electroporation. Additional contributions to our results may have come from an enhancement in translation of CAT mRNA in the older retina and/or an attenuation of CAT degradative processes.

From these experiments, we show that the choice of a reporter gene may have important ramifications if one wishes to examine the expression of a plasmid over a long culture period. If the experimenter is only assaying time points which are relatively short (i.e. 24-72 hours) then these differences in reporter gene activities may be less important.

**Plasmid identification by Southern blotting**

Aside from the expression of a particular construct, we were interested in the fate of the transfected plasmid on a quantitative level. Southern blot experiments examined both luciferase and CAT reporter constructs. Culturing was performed identically to the long-term transient enzyme assay experiments followed by isolation of total DNA (i.e. genomic DNA and transfected DNA) at various time points. When these Southern blot results (figures 10 and 11) are coupled with their respective enzyme activity data, we observe a general trend
for the amount of transfected plasmid to decrease as does the enzyme activity over the two-week study period. This was true for E6 retina transfected with pRSV-LUC or pRSV-CAT. However, we were surprised to find at E10, even as CAT enzyme activities increased, the quantitative amount of pRSV-CAT decreased with time. Possible explanations for this finding have been previously discussed above.

In similar long-term culture experiments, both pGS-LUC and pGS-CAT constructs were quantitatively observed to decrease with time in E6 retina. Thus, disappearance of transfected plasmids in retina appears to be independent of either promoter or reporter gene effects. Enzyme assays of these GS constructs were not determined due to the relatively low transcriptional activity of the GS promoter with comparison to the RSV promoter.

The number of copies of transfected plasmid within an "average" cell were estimated by comparison of the intensities of the transfected plasmid bands with those of known standards (see Results). By visual estimation, the average number of copies of plasmid per cell varied from one to fifty copies two days after transfection declining to 0.14 to 0.5 copies after two weeks in culture. One must keep in mind that these estimations do not imply there are a certain number of copies of plasmids within each cell but that the "average" cell contains this estimated amount. The literature suggests that the efficiency in electroporation of cell lines is on the order of $10^{-1}$ to $10^{-4}$ cells and thus the majority of cells are not actually transfected (Zheng and Chang, 1991). Based on retinal primary culture experiments (see Chapter 2), we estimate that electroporation of intact retina results in a transfection frequency of 1 in 10,000 cells. This transfection ratio coupled with the "average" cell estimates reveals
that successfully transfected cells may contain on the order of $10^3$-$10^5$ plasmid copies.

These studies of electroporation techniques of intact embryonic chick retina help define its potential uses and limitations. One major flaw of these studies is that the exact cell types transfected are not identified. This is important since the retina is composed of different neuronal and glial cell types. In addition, when studying putative cell-specific promoter constructs, it is imperative to know the transfected cell type responsible for reporter expression. These problems are addressed in the following chapter.
Chapter II
Müller Glia Cell-selective Expression of a GS Fusion Gene in Electroporated Retina

Introduction

In the previous chapter, we have examined the process of electroporation as it applies to the transfection of intact chick retina. These experiments measured the total reporter enzyme activities of the transfected samples. However, since the chick retina is composed of a heterogeneous population of neurons and glia, it is possible that overall reporter gene expression may be affected by the types of cells transfected. Using traditional enzyme assay methods, the structural integrity of the tissue is often completely disrupted. Therefore it is not possible to determine the relative contributions of reporter expression by two or more populations of cells. This shortcoming is a central problem in our study of putative Müller cell-specific GS promoters. Thus far, our enzyme data with GS-fusions lacks critical evidence that proves the activities are primarily a result of Müller glial expression rather than aberrant neuronal expression.

This chapter describes the development of a histochemical procedure by which promoter selectivity in the retinal populations can be studied. This method answers two important questions. First, do neurons and glia each have the ability to be transfected and express reporter genes following electroporation? And
second, can Müller glia be targeted for expression by transfection with GS-reporter fusion genes?

To answer these questions, both cell-type specific and non-specific promoters were employed. The GS promoter fused to the lacZ gene was used in this system to test for Müller glial cell-specific expression. Two strong viral promoters, the Rous sarcoma virus long terminal repeat (RSV-LTR) and the SV40 major late promoter, were used to test for non-cell-specific expression.

Materials and Methods

Plasmid constructs

pRSV-ßgal (MacGregor, et al., 1987), pCH110 (Hall, et al., 1983), and pRSV-hGR (Giguere, et al., 1986) were all obtained as gifts. The first two plasmids are composed of the lacZ gene under transcriptional control of the RSV-LTR and the SV40 major late promoter, respectively. pRSV-hGR encodes for the human glucocorticoid receptor (GR) under transcriptional control of the RSV-LTR. A GS promoter construct, pNASSß-2192+51, was made by placing the lacZ gene of pNASSß (Clontech) under control of a region of the GS promoter spanning -2192 nt to +51 nt. Details of this construction may be found in Chapter 3.

Establishment of primary retinal cultures

E12 chick retina were electroporated as described in Chapter 1 using 300 µg/ml of the lacZ reporter construct (pRSV-ßgal, pCH110, or pNASSß-2192+51) and where indicated 40 µg/ml of the GR-expressing plasmid, pRSV-hGR. Following 16-18 hours of organ culture, retina were rinsed 3 times with 1.5 mls of...
Hank's solution and then incubated for 30 minutes at 37 °C in 1 ml of 0.125% (w/v) trypsin, in Hank's solution (Lemmon, 1986). One-tenth volume of fetal bovine serum and DNAse I (final concentration 20 μg/ml) were added to terminate the trypsin activity and the retina were then triturated 30-40 times with a pasteur pipette to obtain a single cell suspension. The cells were counted using a haemocytometer and plated on Permanox 2-well tissue culture chamber slides at a density of 1.5 x 10^6 cells/cm^2. Cultures were grown in DMEM-HG with 10% (v/v) fetal bovine serum which had been charcoal stripped and dialyzed to remove endogenous steroids. Fifty units/ml penicillin and 50 μg/ml streptomycin were also added to the growth medium. Dexamethasone was added to selected cultures to a final concentration of 10^-7 M.

**Immunofluorescent staining**

Immunofluorescent staining was based on procedures modified from Harlow and Lane, 1988. Following 1-6 hours of culture, slides were rinsed twice with 1X PBS, briefly air-dried, and fixed for 15 minutes with 2% (w/v) paraformaldehyde in a 0.1 M sodium phosphate buffer pH 7.5. The samples were then blocked by a 15 minute incubation with 5% (w/v) bovine serum albumin, 5% (v/v) lamb serum, and 0.2% (v/v) Triton X-100. Primary antibody incubations were carried out for 60 minutes in a humidified chamber at 37 °C followed by three 5 minute rinses with 1X PBS. Secondary antibody incubations were performed under the same conditions except the incubation period was limited to 30 minutes. Slides were rinsed three times as before. Coverslips were applied using Aquamount containing 2.5% (w/v) DABCO (Sigma). Slides were visualized using a Zeiss Axiophot microscope equipped with epifluorescence optics using a
150 W xenon lamp with excitation at 450-490 nm and emission at 515-565 nm for fluorescein, or excitation at 530-585 nm and emission ≥ 615 nm for Texas Red.

Antibodies were diluted in 1X PBS before use. The following primary and secondary antibodies were used at the dilutions indicated: rabbit anti-*E.coli* β-galactosidase 1:1000 (Cappel); mouse monoclonal antibody 5E10 directed against a chicken filamin 1:50 (Lehmann, 1986); mouse monoclonal antibodies directed against sheep GS 1:500 (Chemicon); goat anti-rabbit Texas Red-conjugated antibodies 1:500 (Jackson Labs); and goat anti-mouse fluorescein-conjugated antibodies 1:100 (Cappel).

**Scoring procedure of β-gal positive cells**

After the culture slides were prepared for analysis by double immunofluorescence, β-galactosidase positive cells were identified by their Texas Red fluorescence. The cells were then scored as either a neuron or a Müller glial cell based on morphology of the Texas Red fluorescence. This was confirmed by visualization of the fluorescein-conjugated secondary antibody to a Müller cell antigen. Only cells which could be scored unambiguously were included in the data. In all experiments, it was possible to assign more than 90% of the β-gal positive cells to either the neuronal or glial cell groups. The culture slides were scanned for β-gal positive cells beginning in an upper corner of the square and proceeding horizontally to the opposite edge. The slide was then moved vertically by a distance that just exceeded one field of view, followed by horizontal scanning. This process was continued for the entire slide. The use of this scanning method ensured that no cells were scored twice. The summary data shown in Figure 14 are all means ± standard deviation based on the results obtained after 4 or 5 separate transfections.
Results

Qualitative identification of β-gal expressing cell types

The main purpose of these studies was to ascertain whether 2.2 Kb of GS promoter could confer Müller cell specific expression of the lacZ reporter gene. Depicted in figure 12 is the experimental procedure used to determine which retinal cell types express transfected genes. Intact E12 retina were electroporated and cultured overnight before trypsinization to a single-cell suspension. This mixture was plated onto tissue culture chamber slides for 1 to 6 hours. The slides were then stained with antibodies to β-gal and to a Müller cell specific antigen, either 5E10 or GS. The number of cells expressing β-gal could then be assigned based on morphology as either a neuron or a glial cell.

This method takes advantage of the distinct differences in cell morphology between neurons and Müller glia in retinal primary cultures. When CNS tissues are dispersed and cultured, the cultures rapidly develop a layer of flat cells upon which the neuronal cells grow (Meller, 1979). Previous immunohistochemical studies using antibodies directed against glial fibrillary acidic protein (GFAP) (Li and Sheffield, 1984) or glial filamin (5E10) (Lemmon, 1986) have shown that the flat cells seen in primary culture are indeed derived from the Müller glia of the intact retina. This observation was confirmed in our hands by staining retinal primary cultures with both antibodies to 5E10 and GS. This is shown in Figure 13 where phase contrast (a-c) and immunofluorescent (d-h) micrographs were taken of primary cultures that had been labeled for immunofluorescent analysis. Particular attention should be made to the comparison between 13d-e (5E10) with 13f (GS). Both of the staining patterns observed show a wide flattened
Electroporation of E12 chicken retina with a β-galactosidase-encoding fusion gene

organ culture for 16-18 hr

Prepare single cell suspension and plate onto tissue culture slide

primary culture for 3 hr

Immunofluorescent staining with antibodies directed against β-galactosidase and a glial cell specific marker (filamin or GS)

Score β-galactosidase-positive cells as neuronal or glial based on morphology and the presence or absence of the glial marker

Figure 12: Assay protocol for cell-specific gene expression in electroporated retina. This flow chart outlines the experimental procedure followed to examine which types of retinal cells, neurons or glia, express transfected β-gal plasmid constructs under the controls of various promoters.
Figure 13: Immunofluorescent analysis. E12 retina were electroporated and organ cultured overnight and primary cultures prepared and analyzed by phase contrast microscopy (a-c) and immunofluorescence with antibodies directed against filamin (d and e), GS (f), or β-galactosidase (g and h). (a, d, and g), (b, e, and h), and (c and f) each represent a single field of different cultures. The fields shown in (a, d, and g) and (b, e, and h) were derived from retina electroporated with pRSV-βgal or pNASSB-2192+51, respectively. The field shown in (c and f) was derived from retina treated overnight and in primary culture with 10^{-7} M dexamethasone.
appearance. When comparing these micrographs to their respective phase contrast photos, it is easily seen that 5E10 and GS antibodies only label the flat cells and that the neuronal cells which appear as round under phase contrast are not labeled. These data help validate the differential classification of neurons and Müller glia based on morphology. The flat cells can be identified within 1 hour of culture which demonstrates their rapid attachment to the slides.

The retinal primary cultures used to demonstrate GS staining were grown in the presence of 10^{-7} M dexamethasone. This was necessary to raise the level of GS in the cell to a detectable threshold. Previous studies have also shown that glucocorticoid treatment is required for the immunologic detection GS in Müller cells of the intact embryonic retina (Linser and Moscona, 1979). As a result in nearly all other experiments, 5E10 was utilized to identify Müller glia since the presence of the 5E10 antigen is not dependent upon steroid treatment.

Figure 13 also contains examples of β-galactosidase expressing neurons and Müller glia. Figure 13a,d, and g show phase contrast, filamin immunofluorescence, and β-gal immunofluorescence, respectively, of a field obtained after transfection with pRSV-βgal. The single β-gal positive cell in figure 13g is round and filamin-negative and thus is classified as a neuron. Figure 13b,e,and h show phase contrast, filamin immunofluorescence, and β-gal immunofluorescence, respectively, of a field obtained after transfection with pNASSβ-2192+51. The single β-gal positive cell in figure 13h is flat and filamin-positive and is thus scored as a Müller cell.

**Quantitative assay of β-gal expressing cell types using viral promoters**

This procedure was used to quantify the distributions of neurons and Müller glia which were identified as β-gal expressing cells following
electroporation with pRSV-βgal. The primary cultures were assayed at several times between 1 and 6 hours after plating. These results are summarized in Table 1. Three conclusions can be made concerning these data. First, the total number of β-gal positive cells attached to the slide before assay is shown to increase from 1 to 3 hours at which time a plateau is reached. Further culture time does not result in the identification of more β-gal positive cells. Second, the time in primary culture prior to assay does not affect the percentage of β-gal positive cells identified as Müller glia. Based on these two points, further experiments were performed with a three hour culture period. The third observation is that following electroporation with pRSV-βgal, there are approximately twice as many neurons as Müller glia which express β-gal.

Figure 14 shows the results of additional transfections with pRSV-βgal and pCH110, both of which are lacZ reporter constructs driven by strong viral promoters. An approximate 2:1 ratio of neurons to Müller glia which were β-gal positive was obtained after four separate transfections (65% neurons, 35% Müller glia) with pRSV-βgal. The addition of dexamethasone 10^{-7} M to the cultures had no affect on the neuron to glia ratio (66% neurons, 34% Müller glia). As an additional positive control, pCH110 was transfected into retina and assay of these primary cultures resulted in a similar distribution of β-gal expressing neurons and glia (67% neurons, 33% Müller glia). Thus, both of these viral promoters, which are known to be highly expressed in many eukaryotic cell types, direct expression of the lacZ gene to approximately twice as many neurons as Müller glia in this retinal transfection system.
TABLE 1: The time in primary culture does not affect the distribution of β-galactosidase in neurons and Müller cells in E12 retina electroporated with pRSV-βgal.  

<table>
<thead>
<tr>
<th>Time in primary culture</th>
<th>Number of β-galactosidase positive cells</th>
<th>Percentage of β-galactosidase expressing cells that are Müller glia</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 hr</td>
<td>88</td>
<td>35.7</td>
</tr>
<tr>
<td>2 hr</td>
<td>118</td>
<td>37.4</td>
</tr>
<tr>
<td>3 hr</td>
<td>196</td>
<td>36.0</td>
</tr>
<tr>
<td>4 hr</td>
<td>204</td>
<td>41.1</td>
</tr>
<tr>
<td>6 hr</td>
<td>223</td>
<td>31.9</td>
</tr>
</tbody>
</table>

Mean ± SD 36.4 ± 3.3

*Retina were electroporated in the presence of 300 μg/ml pRSV-βgal and cultured overnight, followed by preparation of primary cultures for the times indicated prior to fixing and histochemical analysis.*
Figure 14: Distribution of β-gal positive neurons vs. β-gal positive Müller glia following electroporation of E12 retina. E12 retina were electroporated with 300 μg/ml of the β-gal construct (pRSV-βgal, pCH110, or pNASSB-2192+51) along with 40 μg/ml pRSV-hGR where indicated. The retina were cultured overnight in the presence or absence of dexamethasone 10⁻⁷ M. The retina were then stained with antibodies and scored as described in Materials and Methods. Data shown are the mean values ± standard deviation obtained after 4 or 5 separate transfections. Actual data for this figure may be found in the Appendix.
Figure 14: Distribution of β-gal positive neurons vs. β-gal positive Müller glia following electroporation of E12 retina.
Quantitative assay of β-gal expressing cell types using a GS promoter

Also shown in figure 14 are the results of retinal transfections using pNASSB-2192+51 which is composed of the β-gal reporter gene under control of sequences -2192 nt to +51 nt of the GS promoter. In this case, we see a dramatically altered ratio of β-gal positive neurons to glia when compared to the 2:1 ratio obtained using the viral promoters. The 2.2 Kb of GS promoter directs β-gal expression to 2-3 times more Müller glia than neurons. Transfection with this construct followed by organ culture in the absence of dexamethasone resulted in a ratio of 3:1 Müller glia to neurons expressing β-gal (77% Müller cells, 23% neurons).

The use of α-amino adipic acid to selectively kill Müller glial cells of the retina has been shown to abolish the hormonal induction of pΔG46TCO, a highly glucocorticoid-responsive CAT expression vector (Grossman, et al., 1994). Western blots of glia-depleted retinal cultures reveal that the levels of GR are one-fifth the normal level of whole retina. This suggests that most of the GR in the retina is contained in the Müller glial compartment. This implies that the localization and functioning of the GR may contribute to Müller cell specific expression of GS. To this end, we tested the hypothesis that dexamethasone treatment may enhance the selectivity for β-gal expression in the Müller glia. In Figure 14, we see that the addition of a glucocorticoid treatment had no effect on the distribution of β-gal positive cells which were identified as glia vs. neurons (73% Müller cells, 27% neurons).

Grossman, et al. also demonstrated that when glia-depleted retina were cotransfected with a rat GR-expressing construct, induction of pΔG46TCO could be returned to a level similar to that of whole retina. This suggests that the mechanism for the low levels of GS expression in neurons lies with their inherent
deficiency of adequate amounts of GR. We examined the effects of exogenous GR in our system by cotransfection of pRSV-hGR along with pNASSβ-2192+51 into E12 retina. As shown in figure 14, slight increases in the proportion of β-gal positive neurons are observed after cotransfection. However, the differences are small and are glucocorticoid-independent, thus the significance of these data is not clear.

**Gross enzyme activities of pNASSβ-2192+51 transfection**

We have shown that neither dexamethasone treatment nor the addition of exogenous GR alters the distributions of β-gal positive cells in retina transfected with pNASSβ-2192+51. However, it is possible that overall expression levels may be altered without disturbing the neuron to glia ratio. To address this potential, we performed experiments identically as before except that following the overnight organ cultures, the retina were assayed for β-gal activity as described in Chapter 1. Figure 15 shows the relative activities of β-gal under control of the RSV promoter and the GS-2192+51 promoter fragment. Each construct was tested in the presence and absence of glucocorticoid treatment. Transfection with pRSV-βgal resulted in β-gal activities 20-fold higher than those produced by pNASSβ-2192+51. These relative promoter strengths agree with previous data obtained using luciferase as the reporter gene. The presence of dexamethasone had no effect on the expression of either construct alone. Cotransfection of pNASSβ-2192+51 with pRSV-hGR was also examined. This resulted in a 2-fold induction of β-gal activity in the presence of dexamethasone. These data suggest that expression of pNASSβ-2192+51 is only slightly inducible, even in the presence of exogenous GR and dexamethasone. It should be pointed out that these data are somewhat suspect due to the relatively low sensitivity of the β-gal assay. When
Figure 15: β-galactosidase enzyme activity assay in E12 electroporated retina. E12 retina were electroporated with 300 μg/ml of the β-gal construct (pRSV-βgal or pNASSβ-2192+51) and 40 μg/ml pRSV-hGR where indicated. The retina were cultured overnight in the presence or absence of dexamethasone 10⁻⁷ M. The samples were then harvested and assayed for β-galactosidase enzyme activity as described in Chapter 1. Data shown are the mean values ± standard deviation representing 3 samples of each condition.
similar experiments were carried out with analogous luciferase constructs, steroid treatment was shown to result in a 2-4 fold induction. Cotransfection with pRSV-hGR in these experiments produced a 5-fold dexamethasone induction.

Discussion

This chapter describes an immunological technique that allows one to quantitate the distribution of an expressed reporter gene among neurons and Müller glia of the electroporated retina. This method combines the unique advantages of a transient assay system with the study of cell-specific expression in a complex tissue. In corroboration with assays that measure gross levels of expression in whole tissues, this procedure provides a powerful means to study the mechanisms regulating GS gene expression during development in the embryonic chick retina.

A more direct approach would have provided for the electroporation of intact retina followed by the identification of reporter positive cells without disruption of the tissue. However, the use of primary cultures has two major advantages. First, relatively few cells of the retina express plasmid constructs following electroporation. This made the identification of β-gal positive cells from frozen sections of intact retina quite laborious and for the large part futile. Thus, by dispersion of the cells, we have an increased chance of detecting β-gal expressing cells as the slide cultures represent an "average" field of cells. Second, primary cultures allow for the exploitation of the characteristic differences between the morphologies of the neuronal and glial cell populations.

We have estimated the extent to which systematic errors may affect the ratio of β-gal positive neurons and Müller glia. The experimental design ensured
that the retina spent most of the time as an intact organ culture as opposed to the dispersed primary cultures. Thus, the β-gal expression should result primarily from the time spent intact and not be due to an artifact of primary culture. As shown in Table 1, the time spent in primary culture (from 1 to 6 hours) did not affect the 2:1 ratio of β-gal expressing neurons to Müller glia. However based upon estimates made using phase contrast and immunofluorescent techniques, the actual ratio of neurons to Müller glia in culture is approximately 5:1. This reflects a 2.5-fold (5:1/2:1) selectivity for Müller glia expression which may be due to a variety of possible mechanisms. Perhaps the Müller glia of the intact retina are more easily transfected compared to neurons, or the glia may have a survival advantage in the primary cultures. Enhanced expression of the viral promoters in Müller cells must also be considered a possibility. As a result, the 2.5-fold selectivity for Müller glia represents the upper limit on the systematic error of this experimental design.

Electroporation with pNASSβ-2192+51 resulted in a β-gal positive cell ratio of 0.3:1 neurons to Müller glia. When compared with the 5:1 ratio of neurons to glia in the primary culture, this represents a >15-fold enhancement of Müller cell selectivity. Assuming a maximal systematic error of 2.5-fold, the β-gal positive Müller cell enrichment is still at least 6-fold.

As previously mentioned, studies with the glial toxin α-amino adipic acid suggest that glucocorticoid receptors are present in Müller cells but not neurons of the retina (Grossman, et al., 1994). It is therefore surprising that treatment with dexamethasone did not have any effect on the β-gal expressing cell ratio. In experiments with the same GS promoter fragment driving luciferase expression, we see a 2-4-fold induction in enzyme activity induced by glucocorticoid treatment. This slight increase in gross enzyme activity may not significantly
change the distribution of β-gal cells which display a threshold level of β-galactosidase necessary for immunologic detection.

The works of Grossman, et al., 1994, would have predicted that the Müller cell selectivity displayed by the GS-fusion gene would be reduced by the cotransfection of GR. This is based on their findings that the glucocorticoid-inducible construct pΔG46TCO could be steroid-induced in glia-depleted retina only if exogenous GR was supplied. Since this result was not observed in our system, we can speculate that either the expression of pRSV-hGR is impaired in neurons or that the receptor produced is not functional. However, it is unlikely that expression of the GR construct is impaired as the RSV promoter has been shown to direct expression of β-gal to twice as many neurons as Müller glia. In fact, the observed ratio of β-gal positive cells in the presence of exogenous GR and dexamethasone did not come close to this 2:1 neuron to glia ratio but was only slightly less selective for Müller cell expression than in the absence of exogenous receptor (see Figure 14). One other explanation for this phenomenon should be strongly considered. It is entirely possible that the induction of GS expression is partially based upon factors separate from steroid-induced mechanisms. Patejunas and Young, 1990, described an increase in chick retinal GS activity even in the absence of steroids. They argue that although glucocorticoid hormones may potentiate the transcription of GS, the constitutive increase in GS seen during development may be mediated by a steroid-independent mechanism.

This analytical method for cell-specific expression has demonstrated that sequences from -2192 nt to +51 nt of the GS promoter confer expression of a reporter gene primarily to Müller glia. Since this effect is not dependent upon glucocorticoids, we propose that steroid-independent mechanisms may
contribute to the pattern of GS expression seen during retinal development. Further studies utilizing this technique should allow for the identification of a cis-acting element which directs GS transcription in Müller glia of the retina.
Chapter III
Cloning and Testing of GS-promoter Fusion Constructs

Introduction
This section focuses on the cloning of GS promoter-driven fusion genes. Many of the GS-promoter constructs previously studied in our lab were cloned using CAT expression vectors. (Pu and Young, 1990, Zhang and Young, 1991). For the current experiments, we chose to utilize the luciferase and β-gal reporter genes as each of their products offers unique advantages.

The assay of luciferase involves the measurement of photons produced by the luciferase-luciferin reaction in the presence of ATP and Mg$^{2+}$ (DeWet, et al., 1987). The analytical device used in this procedure, a luminometer, contains photomultipliers which allow for the quantitation of extraordinarily small amounts of light and thus detect minute amounts of luciferase (Gould and Subramani, 1988). It has been estimated that depending on the instrumentation employed, the luciferase assay may be 30 to 1000 fold more sensitive than assaying CAT expression (DeWet, et al., 1987). Therefore, the major impetus for using luciferase is derived from the ability to detect small quantities of the protein which can be invaluable in the study of weak promoters such as GS.

Although the level of sensitivity offered by β-gal is not as great as that of luciferase, β-gal can be employed in simple histological staining techniques. Aside from immunodetection by specific antibodies to β-gal, a histochemical
reaction exists which allows for the intracellular localization of the β-gal protein. In this reaction, β-gal catalyzes the conversion of 5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside (X-gal) to a blue precipitate which deposits in the cell (MacGregor, et al., 1987). Therefore, the use of a β-gal expressing fusion gene may facilitate the study of GS-promoter expression in individual cells.

The remainder of this chapter is devoted to expression studies of these constructs in embryonic chick retina at various stages of development. Specific GS-constructs which show normal patterns of expression in this system may be useful in the generation of transgenic mouse lines to provide for the study of GS regulation in vivo.

**Materials and Methods**

**Preparation of M13 GS construct**

A double-stranded replicative form of bacteriophage vector containing the GS promoter, dIIIIB21-IVf (Pu, 1991), was received in a lyophilized form. The pellet was resuspended in a small volume of water and used to transform (see methods below) competent DH5αF' E. coli. The mixture was plated on a 0.7% LB agar plate with no selective additive. The sample was grown overnight at 37 °C until clear plaques formed. A toothpick was touched to individual plaques and then used to inoculate 2.5 ml cultures of LB which contained 100 µl of an overnight culture of DH5αF'. These cultures were grown for 16 hours at 37 °C and worked up as minipreps (see below) to extract the plasmid DNA.
Preparation of competent DH5αF'

A single colony of DH5αF' was picked from a fresh plate and grown overnight at 37 °C in 5 mls of LB. Five-hundred μl of this stock was added to 250 ml flasks with 50 mls of LB. These were grown at 37 °C until the OD₆₀₀ was 0.2 to 0.3. The culture was transferred to a 50 ml plastic Corning tube and spun at full speed for 10 minutes in the IEC centrifuge. The supernatant was poured off and the cells resuspended in the remaining LB. Ten mls of ice-cold trituration buffer (100 mM CaCl₂, 70 mM MgCl₂, and 40 mM sodium acetate pH 5.5) was slowly added to the cells over a period of 5 minutes. The sample was spun for 10 minutes as before and the supernatant discarded. The cells were resuspended in remaining trituration buffer. The cells were brought up to a volume of 1 ml and glycerol added to a final concentration of 15%. Aliquots of 50 μl were stored at -80 °C until use.

Transformation of competent DH5αF' cells

The ligation mixture or DNA prep was added to 50 μl of competent DH5αF' cells and placed on ice for 15 minutes. The mixture was heat shocked by placing the sample in a 37 °C water bath for 5 minutes. The cells were then returned to the ice for an additional 10 minutes. One ml of LB was added to the sample before incubating for 1 hour at 37 °C in the shaker at 140 RPM. The cells were pelleted by a 10 second spin in the microfuge. The supernatant was poured off and the cells gently resuspended in residual LB. In the case of M13 vectors, the suspension was mixed with 2.5 mls of 0.7% LB agar (liquid) containing 50 μg/ml ampicillin, 640 μM IPTG, and 0.25 mg/ml X-gal and then plated onto 1.2% LB agar plates containing 50 μg/ml ampicillin. For plasmids which give bacterial colonies, the mixture was plated directly onto a 1.2% LB
agar plate with 3 µl X-gal 40 mg/ml for each 100 µl of suspension. X-gal was only added for those constructs which give blue selection (i.e. pBluescript SK+).

**Miniprep methods of obtaining plasmid DNA**

Two methods were used to obtain plasmid DNA from small cultures of transformed bacteria. The first method is a scaled down version of the alkaline lysis procedure presented in Chapter 1. A 1.5 ml centrifuge tube was filled with overnight bacterial culture and spun at full speed for 30 seconds. The medium was removed by aspiration and the pellet resuspended in 100 µl of ice-cold Solution 1. Two-hundred µl of freshly prepared Solution 2 was added followed by inversion of the tube 5 times. The sample was then placed on ice for 5 minutes. To the tube, 150 µl of ice-cold Solution 3 was added and mixed by gentle vortexing for 10 seconds. The sample was returned to the ice for 5 minutes before centrifuging for 5 minutes at full speed at 4 °C. The supernatant was transferred to a new tube which contained 400 µl of a 1:1 phenol/chloroform solution. The sample was mixed by vortexing and then spun for 2-3 minutes. The aqueous phase was transferred to a new tube and the DNA precipitated by the addition of 0.1 volume 5 M NaCl and 2.1 volumes of ice-cold 95% EtOH. The sample was spun for 5 minutes at full speed at 4 °C and the supernatant discarded. The pellet was rinsed briefly with 70% EtOH and then dried in the Speedvac. DNA pellets were resuspended in a small volume (25-100 µl) of water or TE.

The second miniprep procedure is based on the boiling of the DNA. This boiling method gives DNA which is not as clean as the above method but is well suited for restriction digest and most cloning applications. The overnight culture was added to a 1.5 ml eppendorf and the cells were pelleted by a 10 second spin at full speed. The supernatant was removed and the pellet was
resuspended in 350 μl of boiling prep buffer (8% sucrose, 0.5% Triton X-100, 50 mM EDTA pH 8.0, 10 mM Tris-Cl pH 8.0). Twenty-five μl of freshly prepared lysozyme (a pinch in 10 mM Tris-Cl pH 8.0) was added and the tube gently inverted 5 times. The sample was placed in a boiling water bath for 40-50 seconds. The tube was then spun for 8 minutes at full speed. The pellet of cellular debris was removed with a toothpick and discarded. To precipitate the DNA, 40 μl of 3 M sodium acetate and 500 μl isopropanol were added. After thorough mixing, the samples were spun for 3 minutes at full speed. The supernatant was removed and the remaining DNA pellet was rinsed with 70% EtOH. The supernatant was again removed and the dried pellet was resuspended in 25-100 μl of either water or TE.

Ligation of DNA fragments

Both vector and insert were gel isolated and mixed in a ratio of 3 moles of insert to 1 mole of vector. The total ligation mixture contained both DNA fragments in a total volume of 10 μl with 1X T4 DNA ligase buffer (GIBCO) and 1 unit of T4 DNA ligase (GIBCO). The ligation reaction was incubated overnight at 16 °C before transforming competent DH5αF' cells.

Verification of insert junctions by sequencing

Sequencing reactions were carried out by first denaturing a miniprep DNA pellet. This was done by resuspending the pellet in 16 μl of water and 4 μl of 2 M NaOH, 2 mM EDTA pH 8.0. The sample was left at room temperature for 5 minutes before the addition of 6 μl of 3 M sodium acetate and 150 μl of 100% EtOH. The sample was placed at -80 °C for 15 minutes to assist DNA precipitation. DNA was pelleted by a 30 minute centrifugation step at 4 °C. The
pellet was rinsed once with 70% EtOH before drying briefly in the Speedvac. The pellet was then resuspended in 6 μl of water and 2 μl of 5X Sequenase reaction buffer (USB) and 2 μl of primer (~10 pmoles) were added. The sample was heated in a 65 °C water bath for 3 minutes and then cooled at room temperature to allow primer annealing. Separate 0.6 ml centrifuge tubes containing 2.5 μl of ddNTP termination mix (Sequenase 2.0 kit) (i.e. each ddNTP mix in its own tube) were prepared and kept on ice. A master reaction mix was also prepared for which each reaction planned contained 0.45 μl 0.2 M DTT, 0.36 μl dGTP labeling mix (Sequenase 2.0 kit), 1.58 μl enzyme dilution buffer (Sequenase 2.0 kit), 1.91 μl water, 0.5 μl α-35S dATP and 0.2 μl Sequenase (USB) for a total of 5 μl per reaction. Five μl of master mix was added to each of 4 tubes of primer annealed template (at ~40 °C) and kept at room temperature for 5 minutes. Of this solution, 3.4 μl was added to each of four ddNTP tubes which had been immediately prewarmed to 37 °C for 1-2 minutes. This mixture was incubated at 37 °C for 5 minutes before the addition of 4 μl of stop solution (Sequenase 2.0 kit). The samples were stored at -80 °C until ready for loading onto the gel.

Sequencing plates were prepared by scrubbing with a bleach-containing soft abrasive followed by a 70% EtOH rinse. The plates were then coated with 100 μl dimethyldichlorosilane (SIGMA) in 10 mls chloroform. The plates were dried and appropriate spacers were placed before taping and clamping. The poured gel consisted of 6.5% "insta-gel" (210 g urea, 81.25 mls 40% acrylamide, 50 mls 10X TBE [ 10X TBE: 121.1 g trizma base, 55 g boric acid, 7.4 g EDTA brought up to 1 liter], and water to bring up to 500 mls). Before pouring, ammonium persulfate was added to the insta-gel to a final concentration of 0.1%. This solution was degassed by applying a vacuum for 5 minutes. For
After the gel was polymerized, the sequencing apparatus was assembled and the gel run for 1 hour at 65 watts. The lanes were then loaded with the sequencing reaction samples which had been heated to 80 °C for 2 minutes. The loaded gel was run until the bromophenol blue band had migrated to the bottom. The gel was transferred to Whatman paper and covered by Saran wrap and dried in the gel dryer. The gel was then placed in an autoradiography cassette with Kodak film at -80 °C for 24 hours before development.

Results

The figures presented in this chapter describe the pathways followed to assemble various GS-fusion gene constructs. The 4.2 Kb GS promoter was derived from an M13 clone, dIIIB21-lVf, which originated from the screening of a genomic library (Pu and Young, 1989). This promoter fragment spans from -4200 nt to +51 nt with respect to the transcriptional start site. The full-length promoter was cloned into promoterless luciferase-encoding vectors, pXP1 and pXP2 (Nordeen, 1988). The promoter was inserted in both the forward, pXP242 (Figure 16), and reverse, pXP142 (Figure 17) orientations. The reverse orientation construct serves as a negative control in the retinal transfections. The GS promoter and the luciferase-encoding region were removed as a cassette from pXP242 and cloned into pBluescript SK+. The resulting plasmid, pGSLUC-4200+51 (Figure 16), facilitates the easy removal of any contaminating bacterial sequences, which is essential for DNA intended for the generation of transgenic mice. The full-length promoter was also incorporated directly into pBluescript SK+ in two orientations, pSK242 and pSK142 (Figure
isolate vector with Sal I and Kpn I sticky ends

ligate and transform

isolate 4.2 Kb Sal I to Kpn I fragment

ligate and transform

isolate 7.5 Kb fragment with Sal I/partial Eco RI

isolate vector with Sal I and Eco RI sticky ends

ligate and transform

Figure 16: Cloning of pXP242 and pGSLUC-4200+51
Figure 17: Cloning of pXP142, pSK142, and pSK242
Figure 18: Cloning of pGSLUC-2192+51 and pGSLUC-2192+34
Figure 19: Cloning of pGSLUC-2120+34
Figure 20: Cloning of pNASSβ-4200+51 and pNASSβ-2192+51
Figure 21: Cloning of pG46LUC

pG46TCO (4.6 Kb)

isolate 236bp Hind III to Bgl II fragment

pXP2 (6.1 Kb)

isolate vector with Hind III and Bgl II sticky ends

ligate and transform

pG46LUC (6.3 Kb)
for further cloning manipulations. A β-gal expressing construct was also made in which the 4.2 Kb promoter region was also placed upstream of the lacZ gene (pNASSβ, Clontech) resulting in pNASSβ-4200+51 shown in Figure 20. pXP242, pGSLUC-4200+51, and pNASSβ-4200+51 were sequenced across their vector-insert junctions to ensure that the ends of the inserts were not deleted or altered.

Previous data from our lab indicate that smaller length GS promoter fragments result in better expression of the CAT reporter gene than the -4200 nt to +51 nt region. Thus, shorter length GS promoter constructs were placed upstream of the luciferase and β-gal reporters. Zhang and Young, 1991, demonstrated a functional glucocorticoid response element (GRE) at position -2120 nt which is responsible for the hormonal inductiveness of GS. Therefore, promoter constructs originating from shorter than -2120 nt were not made. pGSLUC-2192+51 (Figure 18) and pNASSβ-2192+51 (Figure 20) represent the luciferase and β-gal reporter constructs respectively with the GS promoter truncated slightly upstream of the GRE. The vector-insert junctions were confirmed by sequencing for pGSLUC-2192+51.

Our lab has also shown that the 3' end of the full-length GS promoter may influence expression. It has been demonstrated that sequences from +34 to +51 diminish reporter gene expression. This is postulated to result from folding of the mRNA in the +51 construct into a hairpin loop which is less readily translated than the +34 construct. Thus, GS-luciferase fusions were made to examine this possible effect. Figures 18 and 19 demonstrate the assembly of pGSLUC-2192+34 and pGSLUC-2120+34. The latter construct has also been shortened at the 5' end of the promoter to just include the GRE. The vector-insert junctions of pGSLUC-2192+34 were confirmed by sequencing.
Table 2: Testing of GS promoter-luciferase fusion genes. The fold inductions of construct luciferase expression caused by dexamethasone are represented in this table. GS-promoter constructs 120 μg/ml and appropriate cotransfectants, pRSV-HGR 40 μg/ml and/or pRSV-Cat-α 10 μg/ml, were electroporated into chick retina of the designated embryonic day. All cultures were grown in DMEM-HG with 10% FBS which was free of endogenous glucocorticoids. Glucocorticoid induction was provided by the selective addition of dexamethasone 10⁻⁷ M to the growth medium. Cultures were grown for 24 hours before assaying for luciferase activity. All experiments were cotransfected with pRSV-βgal 40 μg/ml for normalization of the luciferase data except where noted.
## Table 2

**Fold Inductions by Dexamethasone**

<table>
<thead>
<tr>
<th></th>
<th>pXP242</th>
<th>GSLUC4200+51</th>
<th>GSLUC2192+51</th>
<th>GSLUC2192+51</th>
<th>GSLUC2192+34</th>
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<tr>
<td></td>
<td>+GR</td>
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<td>+GR</td>
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<td>+GR</td>
</tr>
<tr>
<td><strong>E5.75</strong></td>
<td></td>
<td>4.7</td>
<td>4.4</td>
<td>2.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>E6</strong></td>
<td>2.2</td>
<td>1.6</td>
<td>4.4</td>
<td>2.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>E7</strong></td>
<td>1.9</td>
<td></td>
<td>3.0</td>
<td>4.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>E11</strong></td>
<td>0.8*</td>
<td></td>
<td>4.8*</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

<table>
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<tr>
<th></th>
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<th>GSLUC2120+34</th>
<th>GSLUC2120+34</th>
<th>GSLUC2120+34</th>
<th>G46LUC</th>
<th>G46LUC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+GR</td>
<td>+PKAα</td>
<td>+GR/+PKAα</td>
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<td>1.1</td>
<td>3.9</td>
<td>2.0</td>
<td>10.9</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td><strong>E10</strong></td>
<td>2.4</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

*normalized to protein concentration*
Figure 21 displays the construction of pG46LUC which is the luciferase-encoding analog of pG46TCO (Sakai, et al., 1988). The parent construct, pG46TCO, contains a 46 nt synthetic GRE with a 166 nt region of the thymidine kinase promoter upstream of the CAT gene. The promoter region along with the enhancer region was shuttled into a luciferase-expressing vector to serve as a positive control.

The testing of these constructs in embryonic chick retina is shown in Table 2. These constructs were analyzed with the intention of identifying a strongly glucocorticoid-inducible GS-fusion gene which could be used in the production of a transgenic mouse line. Only generalized comments will be presented here as the Discussion section more closely examines these data. All GS-promoter fusions were demonstrated to have a 2-4 fold dexamethasone induction with or without the cotransfection of a glucocorticoid receptor (GR)-expressing plasmid (pRSV-hGR, Giguere, et al., 1986). The cotransfection of a protein kinase A (PKA) expressing vector (pRSV-Cat-α, Maurer, 1989) alone also resulted in a low level of induction. However, if PKA and GR were both introduced, the GS-construct was induced 10-fold. The positive control, pG46LUC, was demonstrated to be 100-fold inducible in the presence of GR and dexamethasone.

**Discussion**

This section deals only with the retinal transfection data presented in Table 2. The original purpose of examining these constructs was to screen for a highly inducible candidate which could be used to generate a transgenic mouse line. Thus, exhaustive studies were not performed on these plasmids as the goal was only to confirm their glucocorticoid responsiveness. Previous
studies with GS promoter fusions show a 4-5 fold glucocorticoid-mediated induction in retina of embryonic day 8 or older (Zhang and Young, 1993a). These experiments failed to show an induction in E7 retina. Although GR is present in early stage retina (Koehler and Moscona, 1975), it appears to remain non-functional until E8 at which time it may confer glucocorticoid inducibility. However, Zhang also demonstrated that if a GR-expressing plasmid is cotransfected into retina at E5.5, GS promoter activity is induced ~10 fold. The data shown in Table 2 reveal that the GS constructs were 2-4 fold inducible, both in E6 and E10 retina. The unexpected activation in the E6 setting reveals that there may be additional influences which contribute to hormonal responsiveness. The addition of exogenous GR did not increase expression in either early or mid-stage retina. Since exogenous GR failed to enhance inducibility, the positive control plasmid, pG46LUC, was tested. This construct resulted in a 100-fold induction with the cotransfection of GR which demonstrated that the exogenously supplied GR was functional.

In later studies Zhang, et al., 1993b, demonstrated the ability to render the early stage retina glucocorticoid inducible through the cotransfection of cAMP-dependent protein kinase (PKA). This is postulated to result from the phosphorylation and subsequent activation of endogenous GR. In the present studies, this PKA treatment resulted in only a 2-fold induction. However, when both GR and PKA were cotransfected, GS promoter activity increased ~10 fold. This shows an additive effect between the GR and PKA treatments.

These data, although somewhat variable, appear to confirm basic premises about the hormonal responsiveness of the GS-luciferase fusions. While a strongly steroid-inducible construct was being sought, these results show no advantage of any particular GS-luciferase fusion construct.
Chapter IV
Generation and Analysis of Transgenic Mice which Express GS-promoter Fusion Genes

Introduction

In Chapter 2, we examined the Müller cell-selective expression of GS fusion genes in the context of an in vitro retinal transfection system. Knowledge gained from these experiments include the fact that sequences from -2192 nt to +51 nt of the GS promoter are sufficient to direct appropriate expression of a reporter gene primarily in Müller glia. We considered whether a similar fusion gene when introduced into the genome of a mouse would display appropriate expression in the retina as well as in other tissues where GS is normally found.

In this section, we attempt to generate such transgenic mouse lines using GS promoters of various lengths in order to display faithful cell-specific expression of a reporter gene. Again, one must consider which reporter gene will give the most useful information from these experiments. Due to the extreme sensitivity of the luciferase assay, using a GS-luciferase transgene construct may be quite valuable in monitoring the levels of transgene expression in tissues which normally display low GS activities. Such tissues, in order of decreasing GS activities, include liver, kidney, and skeletal muscle (Magnuson and Young, 1988). The retina and brain have been shown to demonstrate moderate to high GS activities and thus an appropriately expressed GS-
luciferase transgene could be easily monitored. Thus luciferase offers an excellent opportunity to address transgene expression in varying physiological contexts. The major drawback to using luciferase is that it does not easily allow for monitoring of expression within individual cells. Of course, indirect methods such as antibody detection and in situ hybridization could be applied to try to elucidate the patterns of cell-specific expression.

If one is concerned only with the proper distribution of expression of a transgene, then perhaps the use of the β-gal reporter gene is more appropriate. Although the sensitivity of the β-gal enzyme assay is much less than that of luciferase, β-gal offers a direct approach to examining exactly which cells express the transgene. The reaction of the substrate, X-gal, with the intracellular β-gal enzyme results in the precipitation of a blue product which can easily be detected with light microscopy. Thus, to date, many transgenic models have incorporated β-gal transgenes into the analysis of in vivo cell-specific expression.

We have generated transgenic mouse lines which utilize either luciferase or β-gal as the reporter gene under control of a chick GS promoter fragment which is 2.2 Kb or 4.2 Kb in length. The analysis of GS-transgene expression in these lines will be discussed.

**Materials and Methods**

**Preparation of transgene DNA**

Both luciferase and β-gal reporter genes were incorporated into the transgene constructs. In the luciferase construct, the promoter sequence of GS from -2192 nt to +51 nt was placed upstream of the luciferase cDNA (see
pGSLUC-2192+51 in Chapter 3). A 5.5 Kb Sac II fragment was isolated from pGSLUC-2192+51 for the microinjections. The β-gal transgene was driven by GS promoter sequences from ~-4200 nt to +51 nt. The specific fragment for microinjection was an 8.1 Kb Sal I piece isolated from pNASSβ-4200+51 (see Chapter 3 for details).

After digestion of the plasmid DNA, the specific fragments of interest were gel isolated in a 0.7% agarose gel. The bands were cut out and the DNA electroeluted in dialysis bags with 1X TAE buffer. The DNA in solution from these bags was concentrated using an Elutip-d column (Schleicher & Schuell). The DNA fragment was gel purified a second time followed by electroelution and concentration using an Elutip-d. The DNA solution was then extracted once each with phenol, 1:1 phenol/chloroform, and chloroform before being ethanol precipitated 3 times. The resulting DNA pellet was resuspended in a suitable solution for microinjection containing 5 mM Tris-Cl pH 7.4, 0.1 mM EDTA pH 8.0.

**Identification of transgenic mice**

Transgenic mice were identified either by Southern blotting, PCR, or luciferase assays of tail tissue.

**Southern blots**

For the Southern blots, genomic DNA was derived from tail clips using a modified SDS/proteinase K procedure. Briefly, ~0.7 cm of tail was clipped from mice at least one week old. The sample was placed in an eppendorf tube with 500 μl of tail digestion buffer (50 mM Tris-Cl pH 8.0, 100 mM EDTA pH 8.0, 0.5% SDS) and 50 μl of 10 mg/ml proteinase K. The samples were incubated overnight at 55 °C on a rotating device. The digestions were then serially
extracted with equal volumes each of phenol, 1:1 phenol/chloroform, and chloroform. The DNA was precipitated by the addition of 50 μl 5 M NaCl and 1000 μl 95% EtOH. The DNA was pelleted by centrifugation followed by a brief 70% EtOH rinse. Pellets were dried briefly in the Speedvac. Samples were resuspended in 100 μl TE overnight on a shaker. Contaminating RNA was degraded by the addition of 5 μl of 10 mg/ml RNase A and incubation at 37 °C for 30 minutes. The DNA was ethanol precipitated and resuspended in 50 μl TE for use.

Ten micrograms of DNA was analyzed in each lane. The random primer probe used to identify GS2.2LUC transgenic mice was a 656 bp Eco RI fragment from luciferase cDNA. The probe used to detect the GS4.2β-gal transgene was a 2.2 Kb Sac I fragment isolated from the pNASSB vector. Details of the Southern blotting procedure are provided in Chapter 1.

Polymerase chain reaction (PCR)

This procedure was utilized only to identify GS4.2βgal transgenic mice. Genomic DNA was isolated from ear or tail clips with the following protocol. Each clip was received in an eppendorf tube to which 100 μl of 1X PCR/detergent buffer (50 mM KCl, 10 mM Tris-Cl pH 8.3, 2.5 mM MgCl₂, 0.45% NP-40, 0.45% Tween-20) was added along with 10 μl of 10 mg/ml proteinase K. Samples were incubated overnight at 55 °C on a rotator. Proteinase K was denatured by boiling samples for 10 minutes. Two μl of samples were added to PCR tubes with 18 μl PCR reaction mix (200 μM dNTPs, 1 μM each primer, 1 unit Taq Polymerase per 20 μl reaction, diluted in 1X PCR buffer without detergents). The primers for β-gal transgene detection were as follows: LacZ 5' - GGC AGG GTG AAA CGC AGG TC; LacZ 3' - CAT TTT CAA TCC GCA CCT
Reaction mixtures were overlaid with 40 μl mineral oil. The thermocycler conditions were as follows: Hold at 94 °C for 5 minutes to denature followed by 30 cycles of 94 °C for 30 sec, 60 °C for 20 sec, and 72 °C for 1 minute. DNA loading dye was added to the reactions before loading onto a 2% agarose gel. The LacZ product could be identified as a band of size 237 base pairs.

**Luciferase assay**

All solutions and methods for this assay are from the Promega Luciferase Assay System. Approximately 0.2 cm of tail clip was crushed and digested for 10 minutes in 50 μl of lysis buffer and pelleted by centrifugation. Twenty μl of this extract was assayed with the addition of 100 μl luciferase assay reagent mix. Samples were counted in a luminometer for a 10 second period.

**Transcardial perfusion**

Mice were deeply anesthetized with ~400 mg/kg avertin. The beating heart was exposed by an anterior approach through the rib cage. A 27g needle was placed in the left ventricle to supply a perfusion solution of 2% buffered paraformaldehyde. The right atrium was nicked to relieve venous pressure. The mice were perfused with ~15-20 mls of paraformaldehyde. Tissues were dissected and post-fixed for 1 hour in fresh perfusion solution. Tissues designated for cryostat sectioning were immersed in a solution of 22% sucrose in 1X PBS.

**Preparation of cryostat tissue sections**

Tissues which had been cryoprotected in a 22% sucrose solution were embedded and frozen in O.C.T. Sections of 20-30 microns were cut in a cryostat
at -20 °C. These tissue sections were placed on gelatin-coated microscope slides for staining procedures.

**X-gal staining**

Whole tissues or slide-attached sections were incubated at 37 °C in X-gal staining solution (Oberdick, et al., 1990, [1 mg/ml X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl₂, 0.02% (w/v) NP-40, and 0.01% (w/v) sodium deoxycholate]) until a blue color developed (1-18 hours). Slides and tissues were rinsed in 1X PBS before microscopic examination.

**Cresyl violet counterstaining**

Cresyl violet was applied to counterstain previously stained slide sections. Hydrated slides were dipped in cresyl violet solution (cresyl violet 0.5 gm, 10% acetic acid 0.3 ml, distilled water to 100 mls) for 30-40 seconds followed by two brief distilled water exchanges. Slides were destained in an acetic formalin solution (37% formaldehyde 4 mls, glacial acetic acid 0.2 ml, distilled water to 100 mls) for 60 seconds. Slides were rinsed 3 times in distilled water followed by an ethanol dehydration series.

**Surgical sectioning of sciatic nerve**

The sciatic nerve of one leg was transected as follows. The animal was given an anesthetic dose of avertin (125 mg/kg) i.p. A small incision was made in the hip region on the ventral surface. Blunt dissection of the adjacent muscles allowed for exposure of the sciatic nerve. The nerve was either transected or left undamaged (sham-operated). The wounds were closed with surgical wound
clips and the animal returned to its cage for a 10 day-recovery period. Mice were monitored for any signs of infection or complications. At the end of the recovery period, the mice were killed by cervical dislocation and the soleus muscle harvested for weighing and luciferase assay.

**Assay of GS activity**

This assay is based upon a reaction catalyzed by GS which converts glutamine to Y-glutamyl hydroxamic acid (GGHA) in the presence of hydroxylamine. The GGHA product turns brown in the presence of acidic FeCl₃ which allows for colorimetric detection.

Tissue pellets were sonicated in 500 μl sonication buffer (68.1 mg imidazole in 100 mls water, pH adjusted to 6.6 with HCl) followed by centrifugation to pellet cellular debris. Reactions were performed in 12x75 mm glass culture tubes. To each tube, 500 μl of reaction mix A (240 mM glutamine, 20 mM Na₂AsO₄·7H₂O, 0.2 mM ATP, 2 mM MnCl₂·4H₂O, 93 mM imidazole, pH adjusted to 6.6 with HCl) and 150 μl reaction mix B (200 mM hydroxylamine-HCl, pH adjusted to 6.6 with NaOH) were added before the addition of 350 μl cell extract. Samples were incubated at 37 °C for 1 hour. One ml of stop solution (8.75% (w/v) TCA, 0.875 M HCl, 5.24% FeCl₃·6H₂O) was added to each tube after the incubation. Samples were centrifuged for 5 minutes in the IEC centrifuge before OD₅₀₀ readings were measured using a spectrophotometer. Standard reactions spiked with known amounts of GGHA were used to construct a standard curve. The method of Bradford (described in Chapter 1) was used to determine protein concentrations. Glutamyl transferase specific activities were calculated and expressed as μmoles Y-glutamyl hydroxamic acid formed at 37 °C per hour per mg protein.
Generation and Identification of GS2.2-LUC Transgenic Mice

At the Ohio Edison Institute of Biotechnology at Ohio University, Dave Wight, coordinator of transgenic services, performed microinjections of the GS2.2-LUC DNA fragment into fertilized mouse embryos. This 5.5 Kb linear construct, derived from pGSLUC-2192+34 (Chapter 3), consisted of GS promoter sequences from -2192 nt to +34 nt placed upstream of the luciferase gene. These embryos were implanted in pseudopregnant females and their offspring assayed for transgene integration. Using PCR, 5 male founders were identified to contain the transgene. These F₀ males were mated to non-transgenic females. The pregnant females were then transferred to the Sisson Hall vivarium at The Ohio State University. Following birth, the F₁ offspring of each litter was analyzed by Southern blots of genomic DNA derived from tail clips. The results of this screening are depicted in Table 3. Only two founders who were PCR positive passed the transgene to some fraction of their offspring (Founder #13 [F₀ 13] 8/16, Founder #35 [F₀ 35] 8/18). We can assume that these founders contained the transgene in all of their cells. Otherwise, in the case of a chimeric founder, the fraction of offspring receiving the transgene would have been less than 50%. From the Southern blot data (not shown), we estimated that the F₀ 13 line contained ~50 copies of the transgene per genome while the F₀ 35 line showed an integration of ~3 transgene copies per genome. The three founders that did not give rise to any transgenic offspring could have either been chimeras or initially misidentified as transgenic.

It is interesting to note that the two positive founders were also identified by other means. While at OU following initial identification by PCR, the tail clips
TABLE 3: Screening of F₁ offspring from transgenic founders

<table>
<thead>
<tr>
<th>male founder</th>
<th>number of transgenic pups a</th>
<th>total number of pups born</th>
</tr>
</thead>
<tbody>
<tr>
<td>F₀₃</td>
<td>0/3</td>
<td></td>
</tr>
<tr>
<td>F₀₈</td>
<td>0/21</td>
<td></td>
</tr>
<tr>
<td>F₀₁₃</td>
<td>8/16</td>
<td></td>
</tr>
<tr>
<td>F₀₃₅</td>
<td>8/18</td>
<td></td>
</tr>
<tr>
<td>F₀₄₀</td>
<td>0/10</td>
<td></td>
</tr>
</tbody>
</table>

*aGenomic DNA samples (10 μg each) from tail clips were analyzed by Southern blot to identify F₁ offspring which carried the GS2.2LUC transgene. The probe consisted of a 656 bp Eco RI fragment of luciferase cDNA.*
were also tested for luciferase activity using the Promega Luciferase Assay System. $F_0_{13}$ and $F_0_{35}$ tail tissues were the only samples tested which resulted in luciferase activities significantly above background, >250 fold and 30 fold, respectively (data not shown). Each transgenic offspring from these two founders also demonstrated similar luciferase activities from tail clips. Therefore, this direct assay of luciferase expression could be used to greatly simplify the identification of transgenic animals. This method alone was performed in screening all subsequent litters.

**Analysis of GS2.2LUC transgene expression**

Initial characterizations of these GS2.2LUC transgenic lines were performed on mice 20 days after birth (P20). $F_1$ offspring from each of the two founders, $F_0_{13}$ and $F_0_{35}$, were dissected and selected tissues assayed for luciferase enzyme activity using the luciferase procedure described in Chapter 1. Tissues examined include retina, whole eye, liver, brain, skeletal muscle, ear skin, and tail. As shown in figure 22a, the only luciferase activity which could be detected was found in the muscle and eye in the $F_0_{13}$ line. These data are reported in raw form to point out the exceedingly low amounts of luciferase in these tissues. These values are only 3-4 fold above background while typical luciferase data range from 20-2000 fold above background. In order to increase the sensitivity of the luciferase assay, the protocol was changed to that of the Promega Luciferase Assay System. This procedure incorporates the use of coenzyme A (CoA) which when reacted with luciferase and luciferin results in the product, luciferyl-CoA, which emits light at a nearly constant level for measurements of up to several minutes. This is in contrast to the protocol previously described in Chapter 1 which displays a characteristic initial peak in
### A

Luciferase activity (RLU-background)

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<tr>
<th>Tissue</th>
<th>F013</th>
<th>F035</th>
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<tbody>
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<td>retina</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>brain</td>
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<td>N.D.</td>
</tr>
<tr>
<td>whole eye</td>
<td>400</td>
<td>N.D.</td>
</tr>
<tr>
<td>skeletal muscle</td>
<td>400</td>
<td>N.D.</td>
</tr>
<tr>
<td>kidney</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

### B

Luciferase activity

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<th>Tissue</th>
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<th>F035</th>
</tr>
</thead>
<tbody>
<tr>
<td>tail</td>
<td>6x10⁵ RLU/mg</td>
<td>6x10³ RLU/mg</td>
</tr>
<tr>
<td>skin/core ratio</td>
<td>~3</td>
<td>~10</td>
</tr>
</tbody>
</table>

Figure 22: Basal expression of GS2.2LUC in transgenic mice. Selected tissues of untreated transgenic mice from lines F013 and F035 were collected and assayed for luciferase expression. (A) shows raw luciferase assay data for various tissues in each line measured by the protocol described in Chapter 1. (B) shows luciferase activities (RLU/mg protein) of tail samples and also the ratio of luciferase activity arising from the outer skin compared to the inner core of the tail. These samples were measured using the Promega Luciferase Assay System.

N.D. = not detectable
light production followed by a rapid exponential decay of luminescence. Thus by utilization of the CoA product, the sensitivity of the enzyme assay is increased. When tail tissues were examined using this protocol (Figure 22b), luciferase levels were readily detectable with activities of $6 \times 10^5$ RLU/mg protein (raw RLU~100,000) and $6 \times 10^3$ RLU/mg protein (raw RLU~1000) for lines F₀13 and F₀35, respectively. Also shown in this figure are the skin to core ratios of luciferase expression which were determined in order to ascertain which tissues of the tail (outer skin vs. the inner core of cartilage, bone, and muscle) most contributed to the luciferase activities. It was shown that from 75 to 90% of the luciferase activities arose from the skin compartment. The Promega protocol, when applied to the other tissue samples, resulted in higher RLUs in skeletal muscle only. The brain, liver, kidney, and retina continued to show a lack of luciferase activity in spite of the increased sensitivity of the Promega assay. Detection of luciferase expression was also examined by immunofluorescent methods. Staining with antibodies directed against luciferase failed to detect expression within any tissues.

Theoretically, glucocorticoids would be expected to induce luciferase expression from the GS2.2LUC transgene. However, experiments utilizing a one-time only injection of dexamethasone, cortisone, or hydrocortisone failed to show any reproducible luciferase inductions when assayed 12-18 hours following treatment (data not shown). Although in separate experiments the transgene was shown to be inducible in the brain and muscle of F₀35 mice, these data could not be repeated in follow-up studies. Of all other tissues assayed in these experiments, only the tail consistently showed luciferase levels above background but these activities were independent of glucocorticoid treatment.
Experiments were performed to confirm the effect of glucocorticoids on endogenous GS induction in skeletal muscle. Falduto, et al., 1989, showed that treatment with glucocorticoids leads to atrophy of skeletal muscle with an increase in GS activity. The study depicted in figure 23 shows the inducibilities of endogenous GS expression in hindlimb skeletal muscles following vehicle-injection, cortisone-injection, or no injection in non-transgenic mice. Our data are reassuring in the fact that GS activity is induced 2-fold following a single glucocorticoid treatment.

Induction of the transgene was also studied in organ culture. Figure 24 shows experiments in which the hindlimb muscle group was dissected from transgenic mice and organ cultured in media with or without 1 μM dexamethasone for 24 hours. As shown, the F₀₁₃ line resulted in a reduction of transgene expression by dexamethasone whereas the F₀₃₅ line muscle samples showed a 2.1 fold induction of luciferase activity in the presence of steroid. This small induction agrees with the 2-fold induction of GS detailed in figure 24.

As a final test of transgene induction in skeletal muscle, we performed surgeries on F₀₃₅ mice in which the sciatic nerve of one leg was completely severed while the other sciatic nerve was surgically exposed but not damaged. Following a 10 day recovery period, the soleus muscles of each leg were removed and weighed before assaying for luciferase activity. As shown in Table 4, the denervated soleus muscles were 40% smaller by weight than their innervated counterparts. If the transgene were expressing properly, we would have predicted that while the soleus underwent a process of atrophy, expression of the transgene would increase similar to the response of GS expression seen in atrophied plantaris muscle of the rat (Falduto, et al., 1989).
Figure 23: Effect of glucocorticoid treatment on endogenous GS activity in skeletal muscle. Non-transgenic mice were injected with cortisone 100 mg/kg, an equal volume of vehicle, or were not injected. Eighteen hours after treatment, the mice were sacrificed and the hindlimb muscle groups removed for analysis of GS activity.
Figure 24: Effect of dexamethasone on transgene expression in hindlimb organ cultures. The hindlimb muscle groups of transgenic mice were organ cultured in the presence or absence of 1 μM dexamethasone for 24 hours. The samples were then assayed for luciferase activity using the Promega Luciferase Assay System.
<table>
<thead>
<tr>
<th></th>
<th>soleus weight (mg)</th>
<th>RLU/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>sciatic nerve severed</td>
<td>4.37 mg ± 0.95</td>
<td>N.D.</td>
</tr>
<tr>
<td>sham operated</td>
<td>7.23 mg ± 3.65</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

Transgenic mice from F₀35 had the sciatic nerve of one leg surgically cut while the sciatic nerve of the other leg was exposed but not damaged. Following a 10-day recovery period, the soleus muscles were removed and weighed. The samples were then assayed for GS2.2LUC transgene expression using the Promega Luciferase Assay System.

N.D. = not detectable
However, the luciferase activities were not above background which indicates that if an induction of GS2.2LUC did occur, its magnitude was not large enough to be detected. It is likely that the small size of the soleus muscle in these mice does not permit detection of luciferase activity since the basal levels of activity are themselves difficult to detect in assays of entire hindlimb muscles.

**Development of emulsion technique for histochemical detection of GS2.2LUC transgene expression**

Currently, the most commonly employed technique available for detection of luciferase protein *in situ* is based upon luciferase-specific antibodies. Of course *in situ* hybridization (ISH) could be applied to detect luciferase mRNA but this does not indicate the status of functional enzyme. However in plants (Millar, et al., 1995), bacteria (Langridge, et al., 1994), and some cell lines (Inouye, et al., 1992), *in situ* luciferase-mediated luminescence has recently been examined using low-light imaging systems (i.e. charge-coupled device (CCD) cameras). These imaging methods are currently being adapted for the study of luciferase expression in animal tissues.

The use of nuclear emulsions in ISH to detect radiolabeled probes led to the following hypothesis. Perhaps the light produced by the luciferase reaction could also be detected with these emulsions. In conjunction with Dr. Richard Burry of The Ohio State University, we designed and initiated the following experiments.

In initial trials on actual tail samples from F_{0}13 transgenic mice, both nuclear emulsions tested, Kodak and Ilford brands, failed to produce any silver granule deposition above background. The experiments were performed utilizing two procedures with slides which contained attached tail cross-sections. In the first method, the chemical mixture needed for reaction (i.e.
substrate and co-factors) was placed directly on the tail sections and the slide then coated with molten emulsion. The second procedure involved mixing the substrate and co-factors directly into the emulsion and then dipping the slides to allow the reaction to proceed. Neither of these techniques resulted in any areas of silver granule formation within a specific area of the tail section.

To examine emulsion chemistry at a basic level, we coated microscope slides with both Kodak and Ilford nuclear emulsions. Drops of an ongoing luciferase-luciferin light-producing reaction were placed onto the coated slides for various times. The slides were then developed and fixed according to normal ISH protocols. The slides were then examined by light and darkfield microscopy to detect the presence of silver granules which had been generated by the light-producing reaction. The areas of granule formation were easily identified, however, the density of the granules was relatively low. This result is not surprising in light of the fact that the nuclear-type emulsions are composed of silver halide crystals which are natively most sensitive to blue light (~400 nm). Without the aid of sensitizing chemicals, these emulsions would not be expected to be sensitive to the yellow-green light (~560 nm) produced by the luciferase reaction.

With this knowledge, we investigated the use of photographic emulsions in this procedure. Photographic emulsions are also based on silver halide crystals but have sensitizing dyes added. We purchased a standard base emulsion, Ag-Plus (Rockland), which had been specifically sensitized to light of 560 nm wavelength. In experiments similar to above, light-producing droplets resulted in a much denser field of developed silver granules than the nuclear-type emulsions. However, the areas surrounding the strong development displayed an unacceptably high level of granule deposition. This background is
likely due to the nature of the photographic emulsion which is not produced with the concern for minimizing background to the lowest possible level since the unaided eye cannot discern such small imperfections. From the above data it is clear that the ideal emulsion would have no background (i.e. nuclear-type) but still be sensitive to light in the 560 nm range.

The next logical step was to try the addition of a sensitizing dye to a nuclear-type emulsion. The dye, 3,3' diethylthiacarbocyanine, was added to the nuclear emulsion at varying final concentrations. Slides were then coated and a droplet of reacting luciferin-luciferase placed as before. These slides showed no increased density of silver granule deposition compared to the original emulsion without dye. This indicates that the emulsion was not adequately sensitized to the wavelength ~560 nm. This is most likely due to the inaccessibility of the silver halide crystals since they are already emulsified. In order to ensure dye contact with the silver halide crystals, it should be incorporated into the synthetic process of emulsions before the crystals are suspended in gelatin.

**Generation and Identification of GS4.2βgal Transgenic Mice**

At the transgenic facilities of the Ohio State University Biotechnology Center, Jan Parker-Thornburg performed microinjections of the GS4.2βgal fragment into fertilized mouse embryos. This 8.1 Kb linear construct, derived from pNASSβ-4200+51 (Chapter 3), consisted of GS promoter sequences from ~4200 nt to +51 nt placed upstream of the β-gal gene. These embryos were implanted in pseudopregnant females and their offspring analyzed for transgene integration. Using a PCR-based approach, we were able to detect 2 founders which contained the GS4.2βgal transgene, Fₐ2 (female) and F₀24
Each of these mice were mated to non-transgenic counterparts. The $F_1$ transgenic offspring were identified by PCR which showed 7 of 11 pups were transgenic from $F_02$ but only 1 of 33 pups were transgenic from $F_024$. Thus it appeared that $F_02$ was a pure transgenic while $F_024$ was a chimeric mouse. The single positive $F_1$ transgenic from $F_024$ was mated to provide a 50% transgenic ratio as it was necessarily a pure transgenic. Southern blot data of genomic DNA (data not shown), revealed that the $F_02$ line contained $\sim$5 transgene copies per genome while the $F_024$ line contained $\sim$ 50 transgene copies per genome.

**Analysis of GS4.2βgal transgene expression**

Transgenic mice were analyzed by the X-gal histochemical technique. In short, the mice underwent transcardial perfusion with a buffered 2% paraformaldehyde solution. Selected tissues were collected for X-gal staining. These tissues included brain, eye, liver, kidney, skeletal muscle, and tail. Tissues were divided into two halves for the purpose of whole mount staining and staining of cross-sections mounted on slides. Overnight X-gal incubations were performed for staining of whole tissues and slide sections. The whole mount tissues were examined *in toto* before sectioning of the stained specimen. Selected X-gal stained slide sections were counterstained with cresyl violet to determine the cell-specific distribution of β-gal expression. Prepared slides were examined by light microscopy.

Whole mount X-gal staining of the brains of mice from the $F_02$ line revealed that particular regions developed an intense blue color, signifying β-gal expression. By correlation with a rat brain anatomy reference, the regions of transgene expression were determined to include a number of brain structures
as listed in Table 5. The hippocampal region exhibited particularly intense staining but many other areas showed moderate to light staining by X-gal. Examples of X-gal stained whole mounts and slide sections are shown in figures 25 and 26. As compared to the cross-sections cut from whole mount tissue which had been stained with X-gal, there were no differences in areas of β-gal expression when cross-sections were made first and then followed by X-gal staining. Counterstaining with cresyl violet revealed that transgene expression was limited to the neuronal cell population. No glial cell types could be found which expressed the transgene. In an effort to induce transgene expression, cortisone (or vehicle) was administered followed by analysis of the mice 18 hours after injection. This experiment did not result in any difference of transgene expression compared to the initial studies which had been performed on untreated mice. Similar areas of the brains from both glucocorticoid and vehicle-injected mice showed X-gal staining. There was no significant change in intensities of reaction between the two groups.

A similar pattern of analysis was used to characterize transgene expression in the F_{0}24 line mice. There were no tissues from mice of this line that could be shown to contain β-gal activity by X-gal staining. This line was discarded due to its lack of GS4.2βgal transgene expression.
TABLE 5: Areas of the transgenic brain which stain for β-galactosidase

<table>
<thead>
<tr>
<th>Brain regions which show X-gal staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>dentate gyrus</td>
</tr>
<tr>
<td>CA1, CA2, and CA3 of hippocampus</td>
</tr>
<tr>
<td>central gray</td>
</tr>
<tr>
<td>subiculum</td>
</tr>
<tr>
<td>superior colliculus</td>
</tr>
<tr>
<td>inferior colliculus</td>
</tr>
<tr>
<td>medial cerebellar nuclei</td>
</tr>
<tr>
<td>pontine nuclei</td>
</tr>
<tr>
<td>various reticular nuclei</td>
</tr>
<tr>
<td>purkinje cell (occasional)</td>
</tr>
<tr>
<td>olfactory nuclei</td>
</tr>
<tr>
<td>thalamus</td>
</tr>
<tr>
<td>hypothalamus</td>
</tr>
<tr>
<td>deep cortical neurons</td>
</tr>
</tbody>
</table>

$F_0^2$ transgenic mice were perfused with 2% buffered paraformaldehyde. The brains were removed and incubated in X-gal staining solution for 18 hours as a whole mount or as cross-sections on slides. Cresyl violet counterstaining was employed to help discern neuroanatomical regions.
Figure 25: Gross β-gal expression pattern in GS4.2βgal transgenic mice. An F$_1$ mouse from F$_0$2 was transcardially perfused with 2% paraformaldehyde. The brain was removed and stained in an X-gal solution for 18 hours at 37 °C. (A) lateral aspect of brain. (B) medial aspect of brain.
Figure 26: Cellular patterns of β-gal expression in GS4.2βgal transgenic mice. An F₁ mouse from F₀2 was transcardially perfused with 2% paraformaldehyde. The brain was removed and stained in an X-gal solution for 18 hours at 37 °C. Frozen sections were counterstained with cresyl violet. (A) dentate gyrus, 100X. (B) thalamic nuclei, 100X. (C) subiculum, 40X.
Discussion

The overall results of the GS2.2LUC and GS4.2βgal transgenic mice are disappointing. In neither case did we find transgene expression or activation in a pattern similar to that of endogenous GS. There are many hypotheses to help explain these findings.

The most obvious explanation for these data is that perhaps the promoter sequences used in these transgene constructs, 2.2 Kb and 4.2 Kb in length, are not sufficient to direct reporter expression in a GS-like pattern. However, the cell-specific expression studies presented in Chapter 2 revealed that 2.2 Kb of GS promoter could confer Müller cell selective expression in the chick retina. Whether or not this same promoter sequence directs GS expression in other tissues is unknown. Thus, at least we would hope to see faithful expression of the GS transgenes in the retina. Since this was not the case, we must consider other alternatives.

The number of transgenic founders generated in these experiments was very low as each set of microinjections resulted in only 2 founders for each construct. A more ideal situation would involve analyzing 5-7 transgenic lines for each construct as each line would likely have a different transgene integration site. Thus, an average effect could be derived which lends credibility to the argument that the detected expression of the transgene would be due to the construct itself and not greatly influenced by surrounding DNA sequences. It appears that in the GS4.2βgal line, which showed expression of the transgene in many neurons of the brain, the transgene may have been affected by nearby regions of DNA. For example, it is possible that at this particular site of integration, the transgene was near an enhancer region and that expression of
the construct was not driven by the GS promoter sequence but rather by the transcriptional activity caused by the enhancer. This would result in the aberrant expression of the transgene in a pattern similar to that of the endogenous gene which is normally affected by the enhancer. In cases where the transgene was not at all expressed at detectable levels, the site of integration in the chromosome may have been such that the transgene sequences were not in a position to be actively transcribed (i.e. positioned in a stretch of sequence which is bound tightly to a histone protein).

Another obvious explanation for these data involves the placing of a chicken GS promoter into the genome of a mouse. It is possible that mice lack some factor necessary for transcription of the chick GS DNA. However, we propose that this possibility is quite small due to our experience with in vitro studies. Zhang and Young, 1991, has demonstrated that chicken GS-promoter driven CAT constructs are glucocorticoid-inducible in rat L6 myoblast cell cultures. Thus, we feel that the underlying reason for lack of expression in the transgenic mice is probably not based upon interspecies differences in transcriptional machinery.

One must also consider the choice of reporter gene used for construction of the transgene. From a current literature search, it is apparent that the β-gal gene is quite frequently employed in transgenic mouse studies. However, reported trials which utilize luciferase as the reporter are 10-fold less frequent. We do not know if this difference is due to the relatively new use of luciferase reporters or due to the fact that β-gal is more amenable to histochemical detection and localization.

An important question which will be answered in time is whether faithful expression of luciferase reporters in mice is adversely affected in any particular
tissues. From the GS2.2LUC transgenic lines, we show that luciferase transgene expression is highest in the tail. *A priori*, one would not expect to see this phenomenon as GS is not known to be highly expressed in the tail or skin (data not shown). But the fact that both of the GS2.2LUC lines show tail expression of luciferase causes us to consider the possibility that the luciferase-encoding sequences are responsible. Tail expression of a luciferase construct has also been detected by others (H. Zhang, personal communication) in which the promoter-construct was successfully expressed in targeted lymphocytic cells.

This discussion would not be complete without a brief review of the known variations of transgene constructs which have been shown to direct cell-specific expression. In many instances, investigators have successfully directed cell-specific expression of a transgene using only the 5' flanking sequences of the gene under study. For example, 6.5 Kb of ZP3 gene 5'-flanking sequence is sufficient to direct correct expression of a reporter gene in mouse oocytes (Lira, et al., 1990). This strategy was followed in our attempts to study GS cell-specific expression. However, there are cases in which the 5'-flanking sequences do not confer cell-specific expression. Nestin gene regulation provides an example of this phenomenon. Zimmerman, et al., 1994, showed that 5.8 Kb of rat nestin upstream sequences did not direct cell-specific expression in transgenic mice. In fact, faithful expression of a lacZ reporter construct was directed by various introns of the nestin gene placed 3' of the reporter. Even when the upstream sequences of the gene were substituted with a basal promoter (Herpes Simplex Virus thymidine kinase), the introns conferred cell-specific expression. Others have found that faithful transgene expression may require both 5' flanking sequences and intronic sequences (Belecky-Adams, et al., 1993). It is evident
from these cases that regulatory sequences may be located anywhere within a
gene. Given these references, it is not surprising that genomic DNA constructs
are often more appropriately expressed than cDNAs in transgenic animals
(Wight and Wagner, 1994).

While the results of our GS-reporter transgenic lines are dissatisfying, we
hope to identify exact elements or sequences which are responsible for
directing GS expression in Müller glia. This will be performed by utilizing the
techniques developed in Chapter 2. The elucidation of such an element could
lead to transgene constructs which are driven by element-multimers. This may
result in higher transgene expression which would assist in detection of the
reporter protein. In addition, the incorporation of GS introns into a transgene
construct may contribute to cell-specific expression.
APPENDIX

Data Relevant to Chapter II (Figure 14)

<table>
<thead>
<tr>
<th>Construct</th>
<th>Dex</th>
<th>expt #</th>
<th>β-gal positive</th>
<th>neuronal (round)</th>
<th>glial (flat)</th>
<th>% glial</th>
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<tbody>
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a) approx 5-10% of βgal positive cells could not be clearly classified as neurons or glia
Data Relevant to Chapter II (Figure 14)

(continued)

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a) approx 5-10% of βgal positive cells could not be clearly classified as neurons or glia
LIST OF REFERENCES


