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Regulation of chicken retinal glutamine synthetase during development

Patejunas, Gerald, Ph.D.
University of Illinois at Chicago, 1989
REGULATION OF CHICKEN RETINAL
GLUTAMINE SYNTHETASE DURING DEVELOPMENT

BY
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B.S., University of Illinois at Chicago, 1984

THESIS
Submitted as partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Chemistry
in the Graduate College of the
University of Illinois at Chicago, 1989

Chicago, Illinois
I hereby recommend that the thesis prepared under my supervision by

Gerald Patejunas

entitled: Regulation Of Chicken Retinal Glutamine Synthetase

During Development

be accepted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

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The Graduate College
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GP
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NOMENCLATURE

When referring to the age of an embryo, the letter "E" precedes the age in days, for example, "E12" would refer to the twelfth day of embryogenesis. A number preceded by the letter "H" indicates the age in days after hatching, for example, "H6" would refer to a six-day-old chick.

Other abbreviations are as follows:

ATP  adenosine triphosphate
CDNA complementary deoxyribonucleic acid
cpm  counts per minute
EDTA ethylenediaminetetraacetic acid
GS  glutamine synthetase
HRE hormone response element
MOPS morpholinopropanesulfonic acid
OD  optical density
SDS sodium dodecylsulfate
SSC standard saline citrate
TRIS tris (hydroxymethyl) aminomethane
UTP uridine triphosphate
Glutamine synthetase (GS) catalyzes the formation of glutamine from glutamate and ammonia, a reaction that is used in recycling the neurotransmitter glutamate. In the chicken retina, levels of this enzyme rise more than one hundred-fold late in embryogenesis. Prior to this rise, GS is expressed at low levels but is inducible to the extent of about 20-fold by glucocorticoids. Since an increase in the production of glucocorticoids by the adrenals coincides with the elevation of GS expression, it has been suggested that glucocorticoids are responsible for the developmental rise. If true, this would represent a rather unusual mode of glucocorticoid action, since these hormones typically serve to reversibly modulate gene expression rather than induce life-long changes.

To study the mechanism of GS induction and the role that glucocorticoids play in its developmental rise, I cloned a cDNA encoding this enzyme. A survey of mRNA expression in several tissues throughout development indicates GS mRNA levels correspond well with GS activity. I characterized the hormonal induction by using intact retinas in organ culture. Prior to the eighth day of embryogenesis (E8), GS mRNA expression is low and not inducible by steroids, despite the presence of glucocorticoid receptors. From E8 to embryonic day 15 (E15), mRNA levels rise very slowly and the tissue
acquires inducibility. After E15, GS message levels increase more than one hundred-fold and plateau shortly after hatching, which occurs on the 21st day of development. In contrast, GS transcription increases less than 10-fold during this time, which suggests that post-transcriptional events are involved in this developmental rise. Investigation of these events is hampered by the lack of viability of hatching retinas in culture.

Using organ cultures of embryonic retinas, I found that glucocorticoids induce a rapid, transcriptionally-based, 20-fold rise in GS mRNA expression. This induction is not dependent upon protein synthesis and is readily reversible upon removal of steroid. These findings are consistent with other steroid-inducible genes. In addition, I have found that retinal GS mRNA levels begin to exhibit an increase in culture in the absence of steroids which is similar to the rise that occurs in ovo. From these data I conclude that glucocorticoids are not responsible for triggering the developmental rise in retinal GS expression.
INTRODUCTION

A. Background

During embryogenesis, an animal develops from a single totipotent cell into a complex organism with a set of well-organized, differentiated tissues and cell types. This process involves the coordinated temporal and spatial regulation of the expression of thousands of genes. The mechanisms by which this regulation is achieved are not well understood.

To begin to study a problem of such complexity, it is necessary to restrict oneself to simple model systems. The chicken glutamine synthetase (GS) gene has been put forward as an interesting model (26). The product of this gene catalyzes the formation of glutamine from glutamate and ammonia. In the developing chicken retina, levels of GS rise some 200-fold late in embryogenesis (45). In addition, glucocorticoids induce expression of the enzyme early in development (30). Thus, chick retina GS is a potentially useful system for studying both developmental and glucocorticoid regulation of gene expression.

Previous reports in the literature have suggested that glucocorticoids are the causative agent behind the large, lifelong increase in GS activity that begins late in devel-
opment (36, 53, 27). If true, this would represent a rather
unusual mode of glucocorticoid action, since steroids most
commonly act as reversible modulators of gene expression
(60). The objective of my thesis project has been to explore
the mechanisms of GS regulation and to investigate the role
that glucocorticoids play in the developmental rise in the
expression of this gene.

B. Biology of the Retina

A chicken egg typically takes 20 to 21 days to hatch. The
retina is derived from the neural tube and begins to
form around embryonic day 3 (E3). Nerves from the retina
reach the optic lobes of the brain by E5 (43), which is also
when the retina is first able to be surgically removed. By
E12, cell division ceases, although differentiation and
specialization of the cells continues (27). At E17, the
first electrical activity from the retina is detectable, and
a day later the embryo responds to light (13). The chick
hatches with its eyes open and is able to engage in visually
mediated behavior (such as pecking at food) immediately.

The avian retina is a multilayered, avascular tissue.
There are two types of cells in the retina: neurons and
glia. Aside from the photoreceptors, there are four
varieties of neurons: the ganglia, which transmit output
from the retina to the brain; the bipolar cells, which
connect the photoreceptors to the ganglia; and the horizon-
tal cells and amacrine cells, which apparently modulate the
transmission of information from receptor to ganglion (9) (see Figure 1).

About 90% of the cells of the retina are neurons and the other 10% are glia. There is only a single type of glial cell in the avian retina and it is called the Muller cell. These are the only cells which span the entire thickness of the retina and they make contact with every type of neuron. Indeed, the glia send out arborizations which occupy nearly all the space in the retina which is not occupied by neurons, thus creating a compact mass of tissue (43) (see Figure 1). They serve as a scaffold for the retina, and like most glial cells, they provide various metabolic services to the neurons, such as storage of energy in the form of glycogen and uptake of excess neurotransmitters (3). Muller cells are the only cells of the chicken retina which contain GS (21).

One of the important neurotransmitters in the retina is the amino acid glutamate. Muller cells have a high affinity transport system with which they absorb the glutamate that is released when a glutamatergic neuron fires. Once inside the Muller cells, the glutamate can be converted to glutamine by GS. Glutamine diffuses out of the glia and is taken up by the neurons where glutaminase converts it back to glutamate. When present in excess, both substrates of GS, glutamate and ammonia, are neurotoxic. Thus GS plays a vital role in the maintenance of the retina (3).
Figure 1. Schematic representation of the structure of the retina. Five types of cells are highlighted: P=Photoreceptor cell, M=Muller glia cell, B=Bipolar cell, H=Horizontal cell, A=Amacrine cell, and G=Ganglion cell. Arrow indicates direction from which light enters the eye. Re-drawn from ref. 9.
C. Steroid Hormone-Mediated Regulation of Gene Expression

The effects of steroid hormones on gene expression have been studied extensively (for reviews see refs. 2 and 60). The initial sequence of events involved in steroid-mediated gene activation has been elucidated. First, the hormone diffuses into the cell and binds to a receptor. The hormone-receptor complex binds to a specific sequence in or near the target gene. This sequence is known as the hormone response element (HRE) and consensus sequences for several different steroid receptors have been identified (2).

An HRE acts as an enhancer (60), i.e., it is a DNA sequence which increases the frequency of transcription initiation at a nearby promoter in a more or less distance- and orientation-independent manner. The exact mechanism by which an enhancer accomplishes this is not well understood. Current theories propose that an enhancer serves as a binding site for a protein which may then confer some sort of conformational change on the local DNA structure or perhaps recruit transcription factors for the nearby promoter (2).

Although there is some evidence that the glucocorticoid receptor may be associated with the HRE in the absence of hormone (59), the steroid molecule is necessary for enhancer function. Upon removal of steroids, transcription levels that had been affected by the hormones generally revert to their basal level of activity (60). The ease with which steroid effects are reversed is illustrated by the fact that
steroids frequently play a role in conditions which temporarily alter the expression of certain genes, such as the stress response (11).

It should be noted, however, that not all steroid effects are completely reversible. There are several examples of apparent alterations in chromatin structure, as manifested by nuclease hypersensitive sites, which persist after removal of hormone. A particularly interesting example of this phenomenon is the estradiol induction of chicken liver vitellogenin (5). Upon initial exposure to estradiol, vitellogenin is induced with a lag phase of 4 to 6 hours. This induction is reversible. But upon subsequent administration of hormone, no lag is observed and the extent of induction is greater. This behavior is believed to be associated with the persistence of two hypersensitive sites near the gene which appear after the initial exposure to estradiol.

In addition, there is one example of a steroid-mediated transcriptional induction which is maintained even after removal of the inducer. In the estradiol induction of retinol binding protein in Xenopus laevis liver, a single injection of hormone yields a ten-fold increase in transcription which continues for at least four months (24). It is postulated that persistent binding of the hormone-receptor complex to the HRE plays a role in the continuation
of induction, since treatment with anti-estrogens abolishes the elevated levels of transcription.

D. Previous Studies of Chicken Retinal Glutamine Synthetase

In all higher eukaryotes examined thus far, GS has eight identical subunits each with a molecular weight of about 42,000 to 49,000 (17, 48). The amino acid composition and general properties of the enzyme are similar in the eu­karyotes, but bacterial GS has a dodecameric structure and its activity is subject to controls not found in the eu­karyotic protein, such as de-activation by adenylylation (54). Based on the cDNA sequence, the chicken retinal GS subunit has 372 amino acids with a predicted molecular weight of 41,996 daltons (Pu and Young, in press).

The principal reaction catalyzed by GS is the formation of glutamine from glutamate and ammonia. The enzyme can also exchange the amide nitrogen of glutamine with hydroxylamine to form gamma-glutamylhydroxamic acid, as shown in Figure 2. This reaction is called the glutamyltransferase reaction and it serves as the basis of a simple colorimetric assay for GS (discussed in greater detail in Chapter II, section C).

In the 1950's, Rudnick at Columbia University used the glutamyltransferase assay to survey expression of GS in embryonic chick tissues (44, 45). She found that expression is low throughout development in most non-neural tissues except the liver where it was moderately high. In retina, levels were low but slowly rose through E17, when they in-
Figure 2. The major reactions catalyzed by GS. Top: Glutamine synthesis. Bottom: Glutamyltransferase.
creased sharply. There was a rise in brain which was similar but less dramatic.

Moscona at the University of Chicago expanded upon this work by culturing embryonic retinas and finding that GS levels rise rapidly when cultured in medium containing serum (26). Specifically, GS levels in retinas from about E8 through E15 exhibit a 10- to 30-fold increase after 24 hours of culturing, and younger retinas show a lag phase before the rise begins. Working on the assumption that some aspect of the culturing conditions was bringing about the premature rise, Reif-Lehrer determined that the serum which supplemented the culture medium contained some dialyzable component which was responsible for the induction (39). Analysis of the serum suggested that steroid hormones were involved (30). Further study indicated that 11-beta-hydroxycorticosteroids, such as the glucocorticoids, induced GS activity in the retina (31).

Several researchers contributed to the growing body of knowledge concerning the steroid-mediated induction of GS. Experiments with cytosine arabinoside, an inhibitor of DNA synthesis, showed that cell proliferation was not required for the induction (32). No alteration in the physical properties of the enzyme could be detected in steroid treated tissue relative to untreated tissue (18). Quantitation of the enzyme by immunoprecipitation established that the rise in GS activity could be accounted for by accumulation of GS.

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protein (1). Cycloheximide, which inhibits protein synthesis, blocked the increase in GS activity, suggesting that the steroids were increasing the synthesis of the protein (18). Levels of GS mRNA were quantitated in a crude fashion by isolating polyadenylated RNA from retinas treated with and without glucocorticoids. The RNA was translated in vitro and GS protein was immunoprecipitated. This experiment showed that GS mRNA accumulated in response to hormonal challenge (49).

E. Objectives

Several investigators noted that the rise in GS expression that occurs around E16 coincides with an increase in the production of glucocorticoid hormones by the adrenal glands (36, 27). This led to the hypothesis that glucocorticoids served as the "developmental trigger" (53) for increasing GS expression. Yamamoto has put forth a speculative mechanism by which glucocorticoids might cause such an irreversible change in gene expression (60). He suggests that the binding of the hormone-receptor complex may provide an entry site for factors which subsequently bind irreversibly to the promotor of the target gene, thus bringing about a persistent change in the transcriptional activity of the gene. At present, there are no confirmed examples of this type of gene activation by glucocorticoids.

As I discussed earlier in section C, steroids are generally thought of as reversible modulators of transcrip-
tion rather than effectors of long lasting alterations in gene expression. Consequently, if glucocorticoids are responsible for the switchover from low- to high-level expression of GS, this would represent a significant departure from the usual mode of steroid action. Previous measurements involving the chick retinal GS system were made at the level of enzyme activity. To more fully understand how the expression of this enzyme is regulated at the molecular genetic level, it is necessary to make direct measurements of changes in mRNA and in transcription rates. In the chapters that follow, I will present data which extends the previous work on GS and which more clearly defines the role of glucocorticoid hormones in the regulation of this gene.
Chapter II

MATERIALS AND METHODS

A. Animals

Eggs from White Leghorn chickens were used in these experiments. This is the same breed that has been used by most of the other investigators cited in this thesis, although some of the early work was done on Rhode Island Reds. Eggs were incubated at 37 to 38 degrees and embryos were staged according to Hamburger and Hamilton (13). Hatchlings and late stage embryos were anesthetized with ether before sacrifice. Experiments involving the injection of drugs into eggs were performed as follows. The eggs were inverted (prolate end up), and a hole just large enough to admit a 22 gauge needle was made. The needle of the syringe carrying the liquid to be injected was inserted into the hole and the egg was turned over (oblate end up) before injection. Afterwards, the hole was plugged with sterile vacuum grease.

B. Organ Culture

Retinas from embryos earlier than E15 were dissected and rinsed in Tyrode's solution (a balanced saline solution with glucose). Retinas from older embryos and from hatchlings are held in place by processes sent out by the pigment epithelium layer. Growth of these processes is stimulated by light
(43). To facilitate removal of these tissues, calcium- and magnesium-free Tyrode's solution was used and the dissections were carried out in subdued light.

The dissected tissue was cultured in Dulbecco's Modified Eagle's medium with 4.5 grams of glucose per liter. The medium was supplemented with 10% fetal bovine serum, and with penicillin and streptomycin. To remove any endogenous steroids, the serum was dialyzed against 0.15 M NaCl overnight followed by treatment with 10 mg/ml activated charcoal before sterile filtration. For some of the experiments, particularly the ones involving long term cultures or frequent medium changes, 25 mM hydroxyethylpiperazine-ethanesulfonic acid at pH 7.2 was added to the medium as a buffer.

The culturing took place in sealed 25 ml flasks which were flushed with 5% CO₂. In general, one retina was cultured per flask in 3 ml of medium, but retinas from the younger, smaller embryos (E6-E8) could be cultured 2 or 3 to a flask. Five to seven ml of medium per flask were used when the culturing lasted more than a few hours, with daily medium changes. Three retinas were usually used for each data point.

C. Assay of Glutamyltransferase Activity

Glutamyltransferase assays were performed as described (20).Briefly, tissues to be assayed are disrupted by freezing/thawing followed by sonication. A portion of the
sonicate is added to a pre-warmed mixture of glutamine, hydroxylamine, ATP, and divalent cations. After incubation for up to an hour at 37 degrees, a solution of FeCl₃ in dilute hydrochloric and trichloroacetic acids is added. The FeCl₃ forms a brown chelate with the hydroxamic acid that is formed during the incubation. After removing debris by centrifugation, the optical density (OD) of the solution is measured and compared to standards made with gamma-glutamylhydroxamic acid. The amount of protein in the original sonicate is determined by Bradford's method (4) and the glutaminyltransferase specific activity is expressed as micromoles of gamma-glutamylhydroxamate formed per hour per mg of protein at 37 degrees. Further details concerning this assay can be found in the Appendix.

D. Viability Testing by Uridine Labeling

Retinas were cultured with 1 microcurie/ml of 51 curie/mmol ³H-uridine for up to three hours. Afterwards, they were washed with Tyrode's solution then disrupted and precipitated three times in cold 0.5 M perchloric acid. Labeled uridine incorporated into RNA was liberated by NaOH hydrolysis and the remaining cellular debris was precipitated with perchloric acid. The supernatant from this step was analyzed by scintillation counting. DNA in the pelleted material was hydrolyzed by two treatments with perchloric acid at 80 degrees. The remaining debris was precipitated on ice and the supernatant was analyzed spectrophotometrically.
at 268 and 284 nM to determine deoxyribonucleoside content (61). This procedure is discussed in greater detail in the Appendix.

E. Nucleic Acid Preparation

Total RNA isolation was accomplished essentially as described in reference 7. Briefly, tissues were rinsed in Tyrode's solution and were homogenized in a 4 M guanidine isothiocyanate solution. The homogenate was then layered over a 5.7 M CsCl cushion and spun overnight at 50000 x g. The pelleted RNA was resuspended in 10 mM Tris + 1 mM EDTA + 0.1% SDS (TE+SDS), ethanol precipitated, and stored at -20 degrees. RNA was quantitated by taking the optical density (OD) at 260 nM. The OD \(_{280}\) was also taken as an internal control for the purity of the RNA. A ratio of OD \(_{260}\) to OD \(_{280}\) of about 2 was considered acceptable. Polyadenylated RNA was isolated by twice passing total RNA over a column of oligo dT cellulose (23).

F. Blotting Procedures

Northern blotting was performed essentially as described in reference 23. Ethanol precipitated RNA's were resuspended in TE+SDS and quantitated as described above. Four to ten micrograms were then denatured in MOPS buffer (20 mM morpholinopropanesulfonic acid, 5 mM sodium acetate, 0.5 mM EDTA), 2.5 M formaldehyde, and 50% formamide at 60 degrees. RNA was loaded onto a 1% agarose gel in MOPS with 1 M formaldehyde in both the gel and the running buffer.
phoresis was generally carried out overnight at 25 volts. Afterwards, the gels were stained with 500 ng/ml acridine orange, photographed, and the RNA transferred to a nylon membrane by capillary action overnight using 20x SSC (1x SSC is 0.15 M NaCl + 0.015 M sodium citrate). The RNA was fixed to the membrane by baking for an hour at 80 degrees in vacuo.

Dot blots were generated by quantitating RNA as described above, then denaturing in 6x SSC plus 2.5 M formaldehyde at 60 to 65 degrees. RNA's were brought up to 15x SSC and were serially diluted. The dilution mixtures contained enough yeast tRNA so that each dot contained a total of 10 micrograms of RNA. Samples were applied to nylon membranes using a dot blotting manifold.

Phosphorus-32-labeled nucleic acid probes were prepared either by oligolabeling (10) or by making complementary RNA using a plasmid containing bacteriophage promotors (pBSM13+ system from Stratagene). For DNA probes, prehybridization and hybridization were performed at 42 degrees using 50% formamide, 5x SSC, 2x Denhardt's solution (23), 0.1% SDS, 100 microgram/ml boiled salmon sperm DNA, and 0.1% sodium pyrophosphate. For RNA probes, the hybridization temperature was raised to 65 degrees and the formamide concentration was lowered to 40%.

Blots were washed in 0.2x SSC plus 0.1% SDS at 50 degrees. Blots hybridized with RNA probes were treated with 1
microgram/ml RNaseA. Quantitation of dot blots was accomplished by autoradiography followed by densitometry. In general, two to four dilutions in each lane were of a suitable intensity for densitometric analysis. The intensities were normalized and averaged, and a standard deviation was calculated where possible. Several of the dot blots were washed and re-probed with labeled oligo dT to check to see that equal quantities of polyadenylated RNA were loaded in each lane.

G. Transcription Measurements

Transcription rates were measured by nuclear run-on (8). Nuclei were isolated by lysing the tissue in a hypotonic buffer followed by dounce homogenization with a non-ionic detergent (Nonidet P-40). Nuclei were removed from the homogenate by spinning at 500 x g and purified over a sucrose gradient. All manipulations were performed at 4 degrees.

After resuspension and quantitation by hemacytometry, the nuclei were incubated at 27 degrees with ribonucleotides, including $^{32}\text{P}$-labeled UTP, so that elongation of nascent RNA chains could occur. After 35 minutes, RNA was isolated by the guanidine isothiocyanate method described above. Any remaining traces of unincorporated radiolabel were removed by two rounds of ethanol precipitation. Further information on these procedures can be found in the Appendix.

Filters for quantitation of the run-on material were
made by denaturing intact plasmids bearing the cloned genes of interest using 0.1 M NaOH followed by neutralization and addition of SSC to 15x. The DNA was then applied to nylon membranes using a dot blot manifold. These filters were then hybridized using the conditions described above for DNA probes, but washed using conditions described for RNA probes. The filters were exposed to X-ray film and quantitated by densitometry.
Chapter III

ISOLATION AND INITIAL CHARACTERIZATION OF A GLUTAMINE SYNTHETASE CLONE

A. Construction of the Library

Previous studies on chick retinal GS expression were limited to measurements of GS enzyme; either directly through immunochemical means or by assaying for enzyme activity. Because I wanted to make direct measurements of mRNA accumulation and transcription rates, it was necessary to clone the GS gene. Since GS levels rise 200-fold during development (45), it seemed reasonable to suppose that GS mRNA was abundant in the retina after hatching. Hence I used polyadenylated RNA from hatchling chick retinas as starting material for the construction of a cDNA library.

The following strategy was used to enrich the starting material for sequences encoding GS. I fractionated the RNA by electrophoresis on a methylmercury agarose gel (5 mM methylmercury in a 1.2% low melting point agarose gel, see ref. 23) and cut out 2 mm slices each containing different sizes of RNA. After eluting the RNA, I translated a portion of each fraction in vitro in the presence of $^{35}$S-methionine and immunoprecipitated with anti-GS antibodies (a gift from P. Linser). The fraction having the most GS mRNA (see Figure 3) was reverse transcribed using oligo dT as the primer.

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Figure 3. Enrichment of mRNA for GS sequences. Immunoprecipitations were performed on proteins translated in vitro using the following templates: (1) polyadenylated hatchling retina RNA, (2) no template, (3-6) fractions 6 through 9 of RNA excised from a gel as described in the text. Mobilities of molecular weight markers are shown on the right.
Complementary DNA (cDNA) was made according to standard methods (12) and linkers for the restriction enzyme EcoRI were ligated onto the ends. The cDNA was cloned into the phage lambda gt10 (15). Approximately 40,000 recombinants were obtained.

B. Identification of a Glutamine Synthetase Clone

I used a differential screening strategy to locate possible GS clones (55). This method allows one to identify a clone on the basis of its greater abundance in one of two otherwise similar populations of nucleic acid sequences. I prepared radiolabeled cDNA from El2 and H2 retinas and screened about 1000 recombinant plaques using replica blots. Three recombinants which gave a more intense signal with the H2 probe than with the El2 probe were isolated for further analysis. Two of the three cross-hybridized and on Northern blots detected a 3 kilobase mRNA which exhibited an increase in abundance during development and in response to glucocorticoids. The 900 base pair insert from the larger of these two clones was subcloned into a plasmid and named pCRGSI.

To demonstrate that pCRGSI encoded chick retinal GS, I performed an experiment aimed at determining whether or not pCRGSI bears sequence homology to genuine GS mRNA. Using anti-GS antibodies, I immunoprecipitated proteins that were in vitro translated from mRNA's selected by homology to pCRGSI (34). I made anti-sense RNA from pCRGSI using T7 RNA
polymerase, denatured it, and bound it to a small piece of nylon membrane which I then incubated with polyadenylated RNA from hatchling chick retinas. In tandem, a membrane carrying RNA synthesized from the vector was similarly incubated as a control. The membranes were washed to remove non-specifically bound RNA and were then boiled to remove material that hybridized to the plasmid-derived sequences.

The eluted material was translated in the presence of $^{35}$S-methionine using a rabbit reticulocyte lysate. The translation products were immunoprecipitated using antibodies against chick retina GS and electrophoresed on an SDS-acrylamide gel. A 42 kilodalton band was evident in the lane with the material derived from the pCRGSI hybridization, but not with the vector hybridization (see Figure 4).

Subsequently, the cDNA library and a chick genomic library were screened by Haifeng Pu using pCRGSI as a probe, and several more clones were obtained, as shown in Figure 5. Sequence analysis (Pu and Young, in press) shows extensive homology between these GS clones and GS clones from other eukaryotes that were later isolated in our laboratory (47) and in others (14). There appears to be only one GS gene detected by our cDNA clones. Evidence for this fact was obtained by Southern blotting chick genomic DNA digested with three different restriction endonucleases and probed with pCRGSI (data shown in ref. 34). Only one band is apparent in the HindIII and EcoRI digests, while two bands...
Figure 4. Immunoprecipitation of chick retinal GS. Proteins were derived from in vitro translation reactions driven by: (1) polyadenylated hatching retina RNA, (2) no template, (3) RNA which hybridizes to pCRGSI, (4) RNA which hybridizes to pBSM13. Mobilities of molecular weight markers are shown on the right.
Figure 5. Structure of GS cDNA clones. The structure of five different cDNA clones is shown along with a composite map of the entire cDNA.
are present in the BamHI digest. Since pCRGSI contains no HindII or EcoRI sites and only a single BamHI site, these data suggest that pCRGSI detects the product of one gene.

C. Expression of Glutamine Synthetase Messenger Ribonucleic Acid during Development

Using pCRGSII and pCRGSIV (see Figure 5) as probes in Northern blots, I surveyed the expression of GS mRNA in retina, brain, and liver during development. In the retina, there is an approximately 130-fold increase in GS mRNA beginning around E16 which is of the same order of magnitude as the developmental increases in glutamyltransferase activities (see Figure 6). The brain shows a smaller increase but with approximately the same time course. In contrast, the levels in liver are high throughout development and do not show a trend toward increasing (data shown in ref. 34). These data are in agreement with previous studies of glutamyltransferase activity (52, 44).

D. Conclusions

I have isolated a cDNA clone homologous to chick retinal GS mRNA. The clone detects a 3 kilobase message in retina, brain, and liver whose abundance parallels that of glutamyltransferase activity in the corresponding tissue. Message levels increase during development in the neural tissues, but are high and steady in hepatic tissue. Despite these variations in expression among different tissues, GS mRNA is the product of a single gene; which suggests that there are
Figure 6. Developmental increase in GS mRNA. Left: Northern blot with each lane having 10 micrograms of retinal RNA from embryos of the indicated ages. Positions of ribosomal RNA's are indicated. Right: A dot hybridization of E12 and H6 retinal RNA; the amount of RNA in micrograms is shown adjacent to each dot.
tissue specific factors involved in the regulation of GS expression.
Chapter IV

MECHANISM OF THE GLUCOCORTICOID INDUCTION OF GLUTAMINE SYNTHETASE

A. Glucocorticoid Inducibility of Glutamine Synthetase

Initial experiments exploring the inducibility of GS mRNA by glucocorticoids were accomplished by injecting eggs at E10 with 100 microliters of a 10 mg/ml solution of hydrocortisone-21-phosphate in saline. Retina and brain tissue harvested four days later showed severalfold increases in glutamyltransferase activity as well as GS mRNA levels (data shown in ref. 34). The liver showed no changes in either parameter, although in later experiments involving the injection of steroids into hatchling chicks, GS mRNA levels in liver exhibited a 3-fold increase (see Figure 7).

The responses in these experiments tended to be somewhat variable, probably due to differences in the efficiency of the absorption of the steroid by the embryo. For the retina, it is possible to perform a more thorough analysis by maintaining the tissue in organ culture. When hormone is added to cultured E12 retinas, there is a 15- to 30-fold increase in GS mRNA (see Figure 8). Using this organ culture system, I was able to perform a series of experiments to characterize the glucocorticoid induction of this gene.

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Figure 7. Glucocorticoid induction of hatchling liver GS. Glutamine synthetase mRNA levels were quantitated using liver RNA from six 2 week old chicks. Three chicks (DEXAMETHASONE) received a subcutaneous injection of 0.05 mg of dexamethasone per gram of body weight. The steroid was at a concentration of 10 mg/ml in a 1% solution of carboxymethyl-cellulose in 0.9% saline. The other chicks (CONTROL) received only the vehicle. Samples of RNA were isolated 24 hours later.
Figure 8. Glucocorticoid-mediated increase in GS mRNA. Embryonic day 12 retinas were cultured overnight and then treated with (+DEX) or without (−DEX) 50 nM dexamethasone for 3 hours. Positions of ribosomal RNA's are indicated. Large arrow indicates position of major 3 kilobase band. On this long exposure, a minor 7 kilobase band (possibly the primary transcript) can be seen (indicated by small arrow).
B. **Dose/Response Curves**

Since the effects of steroid hormones are generally mediated by receptors, one would expect that the magnitude of induction as a function of hormone concentration should mirror the extent of receptor occupancy. To test this, I cultured E12 retinas for 3 hours in the presence of varying concentrations of steroid hormones. I isolated total RNA and quantitated GS message by dot hybridization as described in Chapter II. As shown in Figure 9, the concentration of hydrocortisone giving half maximal induction is approximately 3-4 nM. This coincides with the dissociation constant for the chick retinal glucocorticoid receptor, as determined by Scatchard analysis (19). Although I obtained similar results using dexamethasone (a synthetic glucocorticoid), progesterone, which is not an 11-beta-hydroxycorticosteroid, was a very poor inducer of GS mRNA (data shown in reference 35).

C. **Effects of Protein Synthesis Inhibitors on the Induction**

Another characteristic of the glucocorticoid induction that I investigated is its dependency on protein synthesis. Hormonal inductions that are prevented by inhibitors of translation are called secondary inductions. Presumably, they arise when the hormone affects the accumulation of a protein involved in the production of the target RNA. An induction in which the hormone directly affects the ex-
Figure 9. Dose/response curves for steroids. Top: Dot hybridizations using RNA's from E12 retinas cultured for 3 hours with the indicated hormones. The concentrations in nanomolarity are: (1) zero, (2) one tenth, (3) one, (4) five, (5) ten, (6) one hundred, (7) one thousand. In the "E12" lane, the retinas were not cultured. The amount of RNA in micrograms is indicated next to each row. Bottom: Dose/response curves derived from the corresponding blots.
pression of the target gene and is not influenced by protein synthesis inhibitors is called a primary induction.

To test whether the GS induction is primary or secondary, I incubated E12 retinas for 3 hours with or without 2 micrograms/ml cycloheximide, which prevents chain elongation during translation (58). Thirty minutes into the culturing period, 50 nM dexamethasone was added to half of the cultures. Ribonucleic acid derived from these cultures was analyzed by dot blotting and the results are summarized in Figure 10. Although the cycloheximide inhibited incorporation of labeled methionine into TCA-precipitable protein by 93%, the steroid induction of GS was relatively unaffected. Similar results were obtained at E10. Puromycin, which causes premature chain termination during translation, also gave similar results at E12 (Figure 10).

D. The Role of Transcription in the Induction

In primary inductions, glucocorticoids typically act by increasing the frequency of transcriptional initiation of the target gene (60). To see whether this is the case with GS, I performed nuclear run-on measurements as described in Chapter II, section G. I isolated E12 retinal nuclei under low temperature conditions which prevented transcription, then incubated the nuclei with radiolabeled ribonucleotides under conditions which allow transcription to continue, but do not allow further initiation of transcription to occur. Thus the amount of radioactivity incorporated into RNA
Figure 10. Effect of protein synthesis inhibitors on GS induction. Embryonic day 12 retinas were cultured for 3 hours with (+) or without (-) 2 microgram/ml cycloheximide (CYCLO) or puromycin (PURO). After the first half hour, 50 nM dexamethasone (+DEX) was added to half the cultures, while the other half received an equal volume of water (-DEX). Note that puromycin depressed the basal expression of GS; the ratio of +DEX to -DEX in the presence of inhibitor is the same in the cycloheximide and puromycin experiments.
homologous to the GS gene is proportional to the number of RNA polymerase complexes that were on the gene at the time that the nuclei were isolated.

I performed several tests to determine whether the methodology I employed gave results consistent with those predicted by theory. For example, one would expect that the incorporation of label would initially be proportional to the time of incubation, but since there is no re-initiation, the incorporation should eventually reach a plateau. The plot of incorporation versus incubation time in Figure 11 illustrates that this is the case. To quantitate the amount of transcription from a particular gene, the run-on material is allowed to hybridize to an excess of filter-bound DNA which is homologous to the gene in question. If the DNA on the filter is truly in excess, then the amount of hybridization to the filter should be proportional to the amount of labeled material used in the hybridization. Figure 11 shows that this proportionality exists.

Figure 12 shows the results of a typical nuclear run-on experiment. Labeled run-on RNA from E12 retinas cultured with and without dexamethasone was hybridized to filters containing spots of denatured plasmids. There is little or no hybridization to the spots containing pBSM13+, which has no eukaryotic sequences. The pCR132 spot, which has a chick retinal cDNA clone whose mRNA levels are not glucocorticoid regulated, exhibits the same signal intensity in the control
Figure 11. Test of run-on parameters. Top: Embryonic day 12 nuclei were incubated with labeled UTP and the amount of incorporation into RNA as a function of incubation time is shown. Bottom: Run-on material from E12 retinas was hybridized to filters containing pCRG4SIV. The amount of hybridization as determined by densitometry of autoradiograms is plotted against the amount of run-on material used.
Figure 12. Effect of glucocorticoids on GS transcription. Top: Sample run-on measurement. Embryonic day 12 retinas were cultured for 3 hours with (+DEX) or without (-DEX) 50 nM dexamethasone. Run-on material from these tissues was incubated with filters bearing the indicated plasmids. Bottom: Averages and standard deviations from two sets of three experiments each; in one set, the tissue was exposed to steroid for 3 hours or less, in the other, steroid treatment lasted overnight.
as in the steroid-treated retinas. The same is true for the spots containing 900 base pairs of the coding region of a mouse beta-actin gene which cross-hybridizes to chicken actin (a gift from D. Essig). But the signals given by GS cDNA clones are severalfold stronger with the run-on RNA from steroid-treated retinas relative to the control signals.

The average of three separate experiments shows that the extent of transcriptional increase in E12 retinas treated for 3 hours or less with dexamethasone is about 11-fold when pCRGSII (the more 3' clone) is used in the analysis and about 6.8-fold when pCRGSIV (the more 5' clone) is used. The difference in the extent of induction between the two clones is due to a lower signal in the uninduced tissue as detected by pCRGSII. The reason for this lower signal is unclear, but the overall conclusion of the experiment is that treatment of E12 retinas with glucocorticoids causes a significant increase in the frequency of transcription of GS.

When the tissue is treated overnight with glucocorticoids, the extent of induction drops to about 4.8-fold with pCRGSII and about 4.0-fold with pCRGSIV. A similar diminution in transcriptional induction upon extended exposure to glucocorticoids has also been observed in the rat alpha-1-acid glycoprotein gene (6). In HTC cells, alpha-1-acid glycoprotein signals in run-on assays increase about 50-fold within 4 hours after dexamethasone treatment, but return to
basal levels after an additional 20 hours. The reason for these phenomena is uncertain, but a possible explanation may lie in down-regulation of glucocorticoid receptor levels. It has been shown that in HTC cells, levels of mRNA encoding the receptor drop by 50% to 95% after 24 hours of exposure to dexamethasone (33). It is not known whether such down-regulation of receptor occurs in the chick retina.

E. Reversibility of the Glucocorticoid Induction

In most steroid inductions, the effects of the hormone are reversed when the inducer is removed (60). The question of whether or not the GS induction is reversible was of particular interest because if the induction were irreversible, this would lend credence to the notion that glucocorticoids act as a developmental trigger to turn on GS expression, as some authors have suggested (27, 36, 53). Three separate investigators have performed experiments involving the addition and removal of glucocorticoids to retinal organ cultures (42, 51, 28). In measuring GS activity levels, they found no decrease in expression 24 hours after removal of steroid. Unfortunately, since the GS enzyme has an estimated half-life in excess of 20 hours (29), these experiments are not accurate indicators of the reversibility of the induction.

To examine this issue more closely, I first established a protocol for removing steroids from the retina based on previous reports in the literature (42, 51). I incubated El2
retinas in 50 nM $^{3}$H-dexamethasone for 1 hour and found that changing the medium four times at twenty minute intervals removed 96% of the radioactivity from the tissue (see Figure 13). Using this procedure, I washed E12 retinas that had been cultured with 50 nM dexamethasone for 3 hours, and harvested retinas 0, 1, 2, 4, and 16 hours later. RNA's from these tissues were dot blotted and the results are plotted in Figure 14. These data clearly indicate that the effect of the hormone on GS mRNA is reversible. To show that long-term exposure to steroids does not bring about an irreversible effect, I performed a similar experiment on retinas that had been cultured with hormone for 3 days instead of 3 hours (data shown in Figure 15).

Reversibility can also be demonstrated at the level of transcription, as would be expected. In the experiment shown in Figure 16, I cultured E12 retinas with or without steroid for 1 hour, then washed and continued culturing two sets of cultures either using steroid-free medium or medium that contained steroid. After 3 hours, I isolated nuclei for run-on measurements. The data show that transcription drops when glucocorticoids are removed.

The experiments involving changes in the steady-state levels of mRNA can also provide an estimate of the half-life of GS mRNA. By plotting the logarithm of the fraction of RNA remaining after the steroid wash-out as a function of time, a straight line is obtained whose slope is related to the
Figure 13. Method for removing steroids from the retina. After culturing EI2 retinas with tritiated dexamethasone, the medium was removed and replaced four times at twenty minute intervals. Left: The amount of radioactivity in the medium during each wash is plotted, with the number of cpm in the first wash set equal to 1. Right: One set of retinas was labeled and washed as described above and the other was maintained with label. The next morning they were rinsed, homogenized, and counted. The number of cpm in the unwashed tissue is set equal to 1.

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Figure 14. Reversibility of induction after short-term exposure to glucocorticoids. Embryonic day 12 retinas were cultured with dexamethasone for 3 hours, washed, and RNA was isolated at the times indicated along the abscissa. Levels of RNA are also shown for E12 retinas cultured for 3 hours without (-DEX) and with (+DEX) 50 nM dexamethasone.
Figure 15. Reversibility of GS induction after long-term exposure to glucocorticoids. Embryonic day 12 retinas were cultured with dexamethasone for three days, washed, and RNA was isolated at the times shown along the abscissa. Levels of RNA from retinas that were cultured for three days without (-DEX) and with (+DEX) 50 nM dexamethasone are also shown.
Figure 16. Reversibility of GS induction at the level of transcription. Embryonic day 12 retinas were cultured for 5 hours with or without 50 nM dexamethasone and with or without washing. CONTROL: Cultured without steroid. STEROID: Cultured with steroid. STEROID WASHED: Cultured with steroid for an hour; washed with steroid-containing medium; continued in culture with steroid for 3 hours. REVERSED: Cultured with steroid for an hour; washed to remove steroid; followed by three hours without steroid. The symbol "II" indicates hybridization of run-on material with pCRGSII; "IV" indicates pCRGSIV.
first order rate constant for GS mRNA degradation (the derivation is given in Figure 17). Both the experiments described above suggest a half-life of 1.5 to 2 hours (Figure 17).

These results corroborate another experiment I performed to examine the turnover of the mRNA which involved measuring the kinetics of induction. In this experiment, E12 retinas were cultured for varying times in the presence of dexamethasone and RNA samples were isolated and dot blotted. Figure 18 shows a synopsis of the data similar to that given for the reversibility experiments.

Two major conclusions can be drawn from the experiments discussed in this section. The first is that the half-life of embryonic chicken retinal GS is about 1.5 to 2 hours and that this turnover rate is not affected by treatment with glucocorticoids. The second conclusion is that the steroid-mediated induction of chicken retinal GS is a reversible phenomenon and that even long-term exposure to the hormone does not result in a persistent increase in expression. This result is consistent with the idea that glucocorticoids generally act as reversible modulators of transcription rather than as initiators of permanent changes in expression.

F. Inducibility as a Function of Embryonic Age

I stated at the beginning of this chapter that E12 retinas exhibit a 15- to 30-fold increase in GS mRNA when
\( k_s = \) zero order rate constant for synthesis of RNA
\( k_d = \) first order rate constant for degradation of RNA
\( X = \) concentration of RNA

\[ \frac{dX}{dt} = k_s - k_d \times X \]

\[ \frac{k_s}{k_d} = X_{eq} = \text{concentration of RNA at equilibrium} \]

\[ \frac{dX}{dt} = k_d \left( X_{eq} - X \right) \]

\[-\ln \left( X_{eq} - X \right) = k_d \times t + C \]

At \( t = 0, X = X_0 \)

\[ \ln \frac{X_{eq} - X}{X_{eq} - X_0} = -k_d \times t \]

**Figure 17.** Half-life analysis of GS mRNA. Top: Derivation of a formula that allows one to relate changes in mRNA levels to the turnover of the mRNA (see ref. 35). Bottom, left: Plot of the data from Figure 14. Bottom, right: Plot of the data from Figure 15.
Figure 18. Kinetics of induction of GS mRNA. Top: Embryonic day 12 retinas were cultured without (-DEX) 50 nM dexamethasone for 1 hour or with hormone for the number of hours shown along the abscissa. Bottom: Analysis of the data using the method described in Figure 17.
exposed to glucocorticoids. However, it has long been known that the extent of GS inducibility is not constant throughout development (37). Figure 19 shows data compiled from a dot hybridization experiment involving RNA from retinas of different ages cultured for 3 hours with and without 50 nM dexamethasone. From the data presented thus far, it appears that there are three stages of GS expression. The time prior to E8 constitutes the first stage, when GS mRNA is present in a very small amount and is not inducible. In the second stage, from approximately E8 through at least E14, GS is inducible and the basal level of expression slowly increases. As the basal level increases, the relative extent of induction decreases. The third stage begins at E16, when GS expression increases rapidly and inducibility is diminished. The nature of these stages (particularly the third stage) will be discussed in more detail in Chapter V.

The reason for the lack of inducibility early in development is not clear. Numerous studies have shown the presence of the glucocorticoid receptor in the retina throughout development (19, 22). Moreover, receptors isolated at E8 are able to effectively translocate labeled steroids into E13 nuclei, and vice-versa (46). Furthermore, receptors isolated at E7 are indistinguishable from E12 receptors with regard to several physical properties such as sedimentation coefficient and salt-extractability (50). Although the absence of a glucocorticoid response before E8 is
Figure 19. Inducibility as a function of embryonic age. Retinas obtained at the ages shown along the abscissa were cultured for three hours with (+dex) or without (-dex) 50 nM dexamethasone.
clearly not due to a lack of functional receptors, it may be
that the receptors are not present in the glioblasts at this
stage, only the neuroblasts. Another possibility is that
some other factor or some chromatin structure which arises
after E8 is required for inducibility. At present there is
insufficient data to resolve this issue.

G. Conclusion

I have used organ cultures of embryonic chicken retinas
to characterize the mechanism of the induction of GS by
glucocorticoids. I have found that glucocorticoids elicit a
rapid, primary, receptor-mediated induction in the level of
GS mRNA to the extent of 10- to 25-fold. This increase is
achieved primarily by an increase in the frequency of tran-
scription and is readily reversible. This increase falls
short of the 100- to 150-fold increase in GS mRNA seen
during development.

The GS gene appears to go through three stages of ex-
pression. From the time that the retina is first accessable
(E5) to E7, GS expression is very low and not glucocorticoid
inducible. During the second stage, from E8 to approximately
E16, GS mRNA and enzyme activity rise very slowly and the
gene acquires inducibility by glucocorticoids. After E16,
expression rises sharply and plateaus shortly after hatching.
During this third stage, the extent of inducibility
declines.
Chapter V

MECHANISM OF THE DEVELOPMENTAL RISE IN
GLUTAMINE SYNTHETASE EXPRESSION

A. Developmental Changes in Glutamine Synthetase Transcription

It has been suggested that the 100- to 200-fold rise in GS enzyme activity that occurs between E12 and the first week after hatching is triggered by an increase in the production of glucocorticoid hormones by the adrenal glands (36, 53, 27). This conclusion was based largely on the temporal coincidence of the rise in GS activity and the rise in glucocorticoid hormone levels around E16, coupled with the fact that GS can be precociously induced with steroids in organ culture. If it were true that these hormones were acting to initiate this aspect of cellular differentiation, it would represent a rather novel mode of steroid action. Most commonly, glucocorticoids act as modulators of gene expression rather than effectors of permanent changes in expression (60). Accordingly, I have investigated some aspects of the developmental rise in retinal GS activity.

The increase in GS enzyme during development can be largely accounted for by a corresponding increase in GS mRNA (see Figure 6). The increase in mRNA, however, can only be partially accounted for by an increase in the frequency of
transcription initiation. Figure 20 shows a representative nuclear run-on experiment comparing E12 retinas to H3 retinas. As noted in the figure, the average of three experiments shows that there is a 8.3-fold increase in GS transcription when using pCRGSII in the quantitation and about 6.6-fold when using pCRGSIV. Again, the difference is due to a lower level of detection by pCRGSII at E12 (see Chapter IV, section D). Clearly, the increase in mRNA is in excess of that predicted by the increase in transcription.

Since there is such a large quantity of GS mRNA in a hatchling retina, one might suppose that nuclei isolated from hatchling retina could be contaminated with GS message from the cytoplasm. If this were the case, these unlabeled molecules could compete with the labeled run-on material and the result would be an apparent decrease in the extent of induction. However, contamination is very unlikely since the nuclei are purified through a sucrose gradient (see Chapter II, section G). Moreover, the experiment shown in Figure 21 argues against this scenario. I isolated H15 nuclei and performed the run-on reaction. After quenching the reaction with guanidine isothiocyanate, I divided the material into three equal parts. One part received no addition, another received a three-fold excess of unlabeled E12 nuclei, and the third received a three-fold excess of unlabeled H15 nuclei. If the nuclei are contaminated with large amounts of RNA, then the sample that received H15 nuclei should contain
Figure 20. Developmental increase in GS transcription. Top: Example of a run-on measurement using El2 and H3 retinas. Bottom: Average and standard deviation of three run-on measurements using El2 and hatching retinas.
Figure 21. Effects of adding unlabeled nuclei to run-on material. Labeled nascent transcripts from H15 retina nuclei were mixed with nuclei from E12 (H+E) or from H15 (H+H) retinas and the extent of hybridization to pCRGSII (II) and pCRGSIV (IV) are compared with hybridization using unadulterated run-on material (H).
a large number of unlabeled GS transcripts, since the steady state level of GS mRNA is high at Hl5. This unlabeled RNA should compete with the run-on material during the hybridization, resulting in a lowered signal. The sample that received E12 nuclei should show less diminution in signal, since there is less GS mRNA at E12 than at Hl5. In fact, no reduction in signal is seen, suggesting that contamination is not a problem.

B. Attempts at Measuring Developmental Changes in Message Stability

Several mechanisms can be proposed to account for the non-transcriptional component of the developmental accumulation of GS mRNA. The primary transcript may be processed more efficiently in the hatchling retina than at E12. Another possibility is that there may be less effective transport of message from the nucleus at E12. Still another possible explanation is that GS mRNA turnover may be developmentally regulated. As I pointed out earlier, at E12 the half-life of the message is only 1 to 2 hours. It would seem plausible therefore that the stability of the mRNA may be greater after hatching.

Unfortunately, there are severe limitations on the types of experiments that can be performed on hatchling chick retinas due to the fact that the tissue cannot easily be kept alive in organ culture. Figure 22 shows the result of experiments in which retinas were cultured with tritiated
Figure 22. Determination of retina viability in organ culture. Retinas were cultured for the amount of time shown and were given labeled uridine during the last hour of culture. The relative cpm incorporated per microgram of DNA in the sample is reported, with the one hour time point set equal to 1. Left: Embryonic day 12 retinas. Right: Hatchling day 8 retinas.
uridine either immediately after dissection or after several hours in organ culture. Although the results show that embryonic retinas thrive in culture for several days, the amount of radioactivity that is incorporated by the hatching tissue shows a clear decrease after only a few hours. The existence of this alteration in RNA metabolism would cast doubt upon the interpretation of experiments involving the measurement of message stability.

Nevertheless, I considered it worthwhile to explore the possibility of measuring RNA turnover using actinomycin D, a drug which intercalates into double-stranded nucleic acid. Since the drug prevents transcription, measuring GS mRNA levels at various times after treatment should allow one to observe the rate of message turnover. However, Schwartz (51) has studied the action of actinomycin D in chick retina and has found that the drug has deleterious effects on translation, ATP pools, polyribosome content, and the histology of the tissue. Moreover, Reif-Lehrer (41, 42) has found that when E12 retinas are cultured for 18 hours with hydrocortisone, washed to remove the hormone, and then treated with actinomycin D, glutamyltransferase activity continues to increase two- to three-fold relative to retinas which are washed but do not receive the inhibitor. This effect is blocked by cycloheximide. Although there may be several interpretations of this experiment, the data is consistent
with the notion that actinomycin D might be acting to prolong the lifespan of GS mRNA.

Figure 23 shows the results of a test of the usefulness of actinomycin D in measuring GS mRNA half-life. I induced E12 retinas overnight with dexamethasone, washed the retinas, and continued culturing with or without 10 microgram/ml actinomycin D. In the absence of the drug, GS mRNA levels decline at roughly the same rate as in the previous experiments (see Chapter IV, section E), but the kinetics are noticeably different in the presence of actinomycin D. This apparent effect on RNA stability suggests that actinomycin D is not a good tool for measuring GS message turnover.

C. Steroid-Independent Rise in Glutamine Synthetase Message

Another aspect of GS regulation that I investigated was the role of glucocorticoids in the developmental rise in GS expression. Several researchers have stated their belief that the "increase in GS activity in the retina during embryonic development is triggered by endogenous adrenal corticosteroids" (53, and similar statements in refs. 36 and 27). It seemed that this standpoint needed re-evaluation in view of the fact that the effects of glucocorticoids at E12 are reversible and the hormonally-induced changes in mRNA levels are much smaller than those seen during development.

Figure 24 shows the results of one of several experiments I performed to determine whether the changes in GS
Figure 23. Effects of actinomycin D on GS mRNA turnover. Embryonic day 12 retinas were induced overnight with 50 nM dexamethasone and were then washed in the absence (-act D) or presence (+act D) of 10 microgram/ml actinomycin D.
Figure 24. Steroid-independent rise in GS mRNA. Embryonic day 12 retinas were cultured with (+DEX) or without (-DEX) 50 nM dexamethasone for the number of days shown along the abscissa. In tandem, retinas were harvested from embryos at E13 through E18 and RNA was isolated either immediately (NO CULTURE) or after 5 hours in culture with 50 nM dexamethasone (5 HOUR + DEX).
expression that occur during development can be observed in culture in the absence of steroids. I cultivated E12 retinas for up to six days in the presence or absence of 50 nM dexamethsone. In parallel with these cultures, I dissected retinas from E13 through E18 embryos. One retina from each chick was taken immediately for RNA, while the other was cultured for 5 hours with steroid. The results of RNA blotting show that there is an increase in the level of GS mRNA in the absence of glucocorticoids which is qualitatively similar in kinetics and magnitude to the rise that occurs in ovo. That this rise is not due to contamination of the medium by agents having glucocorticoid activity is shown by the time-course for the increase. As shown in Figure 18, the induction that occurs after placing retinas into steroid-containing medium is rapid, with a half-time of 1 to 2 hours. In contrast, the hormone-independent rise observed here occurs over a period of 2 to 3 days.

As additional proof that steroid activity is not responsible for this increase, I made use of the highly effective anti-glucocorticoid RU38486. This drug has been shown to be a potent glucocorticoid antagonist with essentially no agonist activity (25). To demonstrate that the drug is effective in the embryonic chick retina, I cultured E12 retinas for half an hour with or without 500 nM RU38486, followed by 3 hours with or without 50 nM hydrocortisone. Tissue treated without the drug but with hormone showed the
usual induction in GS mRNA. In contrast, not only was the induction completely blocked in the RU38486-containing cultures, but the basal level of expression was unaffected (see Figure 25). As shown in Figure 26, E12 retinas cultured for up to five days in the presence of RU38486 exhibited a 17-fold increase in GS mRNA, thus lending further credence to the notion that glucocorticoids are not required to trigger a rise in GS expression.

Although it is unequivocal that a significant non-hormonally-induced increase in GS expression occurs in culture, there is a formal possibility that this phenomenon is not the same one that occurs in ovo, i.e., that the retina is not continuing its program of differentiation in vitro, but rather is simply exhibiting anomalous expression of this one gene due to some aspect of the culturing conditions. This point of view is difficult to argue against, but some evidence to the contrary is presented in Figure 27. This experiment shows that in a qualitative sense, the phenomena of GS mRNA accumulation and glucocorticoid responsiveness do seem to advance through the same stages in vitro as they do in ovo. E6 retinas were given four hour treatments with or without 50 nM dexamethasone after 6 and 12 days in culture and their response was compared to those of retinas of equivalent chronological age. As the experiment in Chapter IV, section F showed, E6 retinas express very low levels of GS mRNA and are not glucocorticoid re-
Figure 25. Test of the efficacy of RU38486. Top: Embryonic day 12 retinas were cultured for one half hour with or without 500 nM RU38486 followed by 3 hours with or without 50 nM hydrocortisone (HC). Bottom: Structure of RU38486.
Figure 26. Steroid-independent rise in GS mRNA in the presence of RU38486. Embryonic day 12 retinas were cultured with (+RU38486) or without (-RU38486) 400 nM RU38486 for 2, 3, 4, and 5 days.
Figure 27. In vitro development of GS mRNA expression. Top, left: Retinas isolated at the ages shown were cultured for 4 hours with (+DEX) or without (-DEX) 50 nM dexamethasone. Levels of GS RNA are expressed relative to the E12 control. Top, right: Embryonic day 6 retinas were cultured for 6 or 12 days as indicated and then spent 4 hours with or without steroid. Bottom: Northern blot using 15 micrograms of RNA from the E6 retinas that were cultured for 4 hours with or without hormone.

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sponsive. But after 6 days in culture, E6 retinas appear to have followed their program of differentiation to the extent that they acquire a hormonal inducibility and basal expression comparable to that of E12 retinas. After an additional 6 days in culture, the basal level of mRNA rises still more and there is a corresponding drop in the relative extent of inducibility, again roughly mirroring the events that occur in the embryo. Thus it would seem that the retina is "pre-programmed" to undergo a set of changes in its expression of GS and that glucocorticoids are not required to trigger these events.

D. Conclusions

I have examined the nature of the 100- to 150-fold increase in the level of GS mRNA that occurs in the chick retina after E16 and have found that changes in transcription can account for only about 10 percent of this rise. Analysis of other factors, such as alterations of mRNA half-life, are hampered by the lack of viability of hatchling retinas.

The E16 rise in GS mRNA begins to occur in cultured retinas in the absence of steroids. As discussed below, this finding discredits previous speculation that glucocorticoids are responsible for initiating this phenomenon. Moreover, retinas in culture will pass through several stages of GS expression similar to the way they do in ovo. Initially, there is low basal expression and no steroid inducibility;
then inducibility is acquired while the basal level increases very slowly; and finally there is a large rise in expression and a concomitant decrease in inducibility.

The underlying events which bring about the progression of the retina through these stages are not known. It seems very unlikely that alterations in the glucocorticoid receptor or in levels of the receptor are responsible, for reasons discussed in Chapter IV section F. One possible explanation may lie in the notion of transcription factor recruitment, as put forth by Yamamoto (60). When steroids diffuse into a cell, they meet up with a receptor which then stimulates the expression of a gene by binding to a nearby sequence of DNA known as the hormone responsive element (HRE). Exactly how the binding of the receptor to the HRE activates nearby genes is not well understood, but one theory is the receptor/DNA complex provides an entry site for transcription factors that are required for initiation of transcription. If this scenario of factor recruitment exist in the chick retina, then perhaps the lack of inducibility before E8 can be explained in terms of the lack of a necessary transcription factor.

Several investigators have argued that the rapid rise in GS mRNA that occurs after E16 is triggered by an increase in the amount of glucocorticoids produced by the adrenal gland around this time (36, 27, 51). My research does not support this view. I have found that a substantial accumulation of
GS mRNA occurs in cultured retinas in the absence of steroids over a time course similar to the in ovo rise. It should be noted that in 1968, Reif-Lehrer observed a substantial rise in glutamyltransferase activity in retinas cultured without steroids for 8 days starting at E10 (40). Her interpretation was that glucocorticoids are not required to trigger the E16 increase. This finding was never addressed by the above-mentioned investigators, as is evidenced by the fact that none of them ever cited her paper (16).

Although the data that I have presented argues against the idea of a glucocorticoid requirement for the initiation of a developmental increase in GS expression, it is certainly possible that the role of the hormone is to enhance or accelerate the induction of this gene. This type of activity by glucocorticoids has been established in other systems. For example, the embryonic rat pancreas will develop normally in organ culture in the absence of steroids, but dexamethasone appears to accelerate or modulate certain differentiation events (38). Also, embryonic chick skin cultures exhibit more rapid accumulation of epidermal structural proteins when treated with hydrocortisone than they would otherwise (56).

Of course, the question of what does trigger the developmental activation of the GS gene remains unanswered. Also, the role of message stability and other post-
transcriptional events needs closer examination as soon as methods for maintaining hatchling retinas in culture are worked out. The work I have presented here will serve as a useful framework for further investigation in these areas.


A. Glutamyltransferase Assay

Freeze and thaw the retina and add 1 ml of sonication buffer (composition of solutions is given at the end of this section). Sonicate on ice using 3 or 4 bursts on the maximum setting for the microtip. Store the sonicant on ice.

Perform the assay in 12 x 75 mm tubes. Add 600 microliters of solution A and 180 microliters of solution B. Warm the tubes to 37 degrees and add 420 microliters of the sonicant. Cover with parafilm, swirl, and incubate at 37 degrees for one hour. For a spectrophotometric blank, prepare a tube that has the amounts of solutions A and B given above plus 420 microliters of sonication buffer. Also prepare at least four tubes with solutions A and B but with varying amounts of gamma-glutamylhydroxamic acid. The volumes of the assays and the standards should be the same, for example, if you wanted to make a 1 micromole standard from a 10 mM stock, you would add 100 microliters of 10 mM gamma-glutamylhydroxamic acid and 320 microliters of sonication buffer. You will usually want to have standards with 0.1, 0.25, 0.5, and 1 micromoles of gamma-glutamylhydroxamic acid.

When the incubation is completed, add 1.2 ml of stop solution and spin the samples in the tabletop centrifuge at full speed for 5 minutes. Read the \( OD_{500} \) of the samples and
calculate the micromoles of product formed per hour based on your standards.

Using a one hour incubation, an E12 retina should give a reading which is well within the range of the standards. If you are using an older retina, or one that has been induced with glucocorticoids, dilute a portion of the sonicant with sonication buffer. Alternatively, shorten the incubation time; but do not make it so short that the mixture does not warm up to 37 degrees after adding the cold sonicant.

Sonication buffer:

Solution A:
0.681 mg/ml imidazole at pH 6.6
240 mM glutamine
20 mM Na\(_2\)AsO\(_4\)
0.2 mM ATP
2 mM MnCl\(_2\)
93 mM imidazole
Adjust to pH 6.6. Store frozen in small aliquots. Note: MnCl\(_2\) causes a precipitate to form. Try to get as much in solution as possible.

Solution B:
200 mM hydroxylamine hydrochloride
Adjust pH to 6.6.

Stop solution:
8.75% TCA
0.875 M HCl
5.24% FeCl\(_3\) hexahydrate
To quantitate protein in the sonicant, warm 2.5 ml of Bradford's reagent to room temperature in a cuvette. Then add 5 to 10 microliters of sonicant (more if you are using an early retina) and mix very thoroughly. After 5 minutes, read the OD$_{595}$. The blank, to which you have added only sonication buffer, should have an OD of about 0.45. For standards, use 5, 10, 15, and 20 micrograms of bovine serum albumin.

B. Quantitating Nucleic Acids

Place the retina in 250 microliters of cold 0.5 M perchloric acid. Disrupt the tissue with a sealed off pipet and spin in a microfuge for 5 minutes. Discard the supernatant. Repeat two more times to insure removal of free nucleosides.

Incubate the pellet at 37 degrees for four hours with 500 microliters of 1 M NaOH to hydrolyze the RNA. Add 167 microliters of 5 M perchloric acid and incubate on ice for 1 hour to precipitate DNA. Spin in a microfuge, save supernatant, and wash the pellet with 333 microliters of 0.5 M perchloric acid. Spin and pool the supernatant with the previous supernatant for RNA determination.

Add 500 microliters of 0.5 M perchloric acid to the pellet and heat at 80 degrees for a half hour to hydrolyze the DNA. Precipitate the remaining macromolecules by chilling on ice for a half hour. Spin down the pellet and save the supernatant. Repeat with another 500 microliters and pool the supernatants for DNA determination.
In uridine labeling experiments, the radioactivity in the RNA fraction is determined by scintillation counting. To quantitate DNA and RNA spectrophotometrically, use the following formulae:

\[
\text{micrograms of RNA} = 5.61 \times (\text{OD}_{260} - \text{OD}_{286})
\]

\[
\text{micrograms of DNA} = 8.00 \times (\text{OD}_{268} - \text{OD}_{284})
\]

For a retina, a ten-fold dilution is necessary before measuring the optical densities.

C. Nuclear Run-on

To isolate nuclei, place a retina in a microfuge tube and add 600 microliters of lysis buffer (composition of buffers is given at the end of this section). After 2 minutes add 600 microliters of Nonidet buffer and dounce homogenize. Spin the nuclei at 500 x g for 5 minutes and discard the supernatant.

Resuspend the pellet in 0.5 ml of resuspension buffer and layer it over 1.5 ml of cold nuclear cushion. Spin at 23000 x g for 60 minutes at 4 degrees. Discard the supernatant and resuspend the pellet in 150 microliters of 1x NRB. Spin the nuclei at 300 x g for 3 minutes and resuspend them in a 1x NRB solution that contains 1 microcurie/micro-liter of labeled UTP; 0.5 mM ATP, GTP, and CTP; and 4 micromolar unlabeled UTP. Incubate at 27 to 28 degrees for 35 minutes with occasional swirling.

Isolate RNA by the guanidinium isothiocyanate/CsCl method. After recovering the RNA, ethanol precipitate it once to
remove the trace amount of unincorporated label which will be present.

<table>
<thead>
<tr>
<th>Lysis buffer</th>
<th>Nonidet buffer</th>
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<tbody>
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<td>0.6 M sucrose</td>
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<tr>
<td>4 mM MgCl₂</td>
<td>0.5 mM dithiothreitol</td>
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<tr>
<td>6 mM CaCl₂</td>
<td>0.2% Nonidet P-40</td>
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<tr>
<td>0.5 mM dithiothreitol</td>
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<tr>
<td>150 microgram/ml lysolecithin</td>
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<table>
<thead>
<tr>
<th>Resuspension buffer</th>
<th>Nuclear cushion</th>
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<td>2.0 M sucrose</td>
</tr>
<tr>
<td>10 mM Tris pH 8</td>
<td>10 mM Tris pH 8</td>
</tr>
<tr>
<td>5 mM MgCl₂</td>
<td>2 mM MgCl₂</td>
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<tr>
<td>1 mM dithiothreitol</td>
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<table>
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<th>2x NRB</th>
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<tbody>
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<tr>
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<td>4 mM dithiothreitol</td>
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<tr>
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</table>
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