FUNCTIONAL GENOMICS OF THE AVIAN CIRCADIAN SYSTEM

A Dissertation

by

MICHAEL JOSEPH BAILEY

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2004

Major Subject: Zoology

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ABSTRACT

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(December 2004)

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The genetic identification of molecular mechanisms responsible for circadian rhythm generation has advanced tremendously over the past 25 years. However the molecular identities of the avian clock remain largely unexplored. The present studies seek to determine candidate clock components in the avian species *Gallus domesticus*. Construction and examination of the transcriptional profiles of the pineal gland and retina using DNA microarray analysis provided a clear view into the avian clock mechanism. Investigation of the pineal and retina transcriptomes determined the mRNA profiles of several thousand genes over the course of one day in LD (daily) and one day in DD (circadian) conditions. Several avian orthologs of mammalian clock genes were identified and many exhibited oscillating patterns of mRNA abundance including several of the putative avian clock genes. Comparison of the pineal transcriptional profile to that of the retina revealed several intriguing candidate genes that may function as core clock components. Including the putative avian clock genes and several others implicated in phototransduction, metabolism, and immune response.

A more detailed examination of several candidate photoisomerase/photopigment genes identified from our transcriptional profiling was conducted. These include peropsin (*rrh*), RGR-opsin (*rgr*), melanopsin (*opn4*) and *cryptochrome 2* (*cry2*) genes. This analysis revealed several interesting patterns of mRNA distribution and regulation for these genes in the chick. First, the mRNA of all 4 genes is located within the Inner Nuclear Layer (INL) and Retinal Ganglion cell Layers (RGL) of the ocular retina, where circadian photoreception is present. Second, *opn4* and *cry2* mRNA is expressed in the photoreceptor layer of the chick retina where melatonin biosynthesis occurs. Lastly, the mRNA for all 4 candidate photopigment genes is regulated on a circadian basis in the pineal gland.

As a whole these data yield significant insight into the mechanisms of the avian circadian system and present several candidate genes that may function to integrate photic information, and/or regulate circadian rhythm generation in birds.

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TABLE OF CONTENTS

Page

CHAPTER Page

LIST OF FIGURES

FIGURE Page

CHAPTER I

INTRODUCTION

1. Formal Properties of Circadian Organization

The ability of organisms to survive on earth depends upon an adaptation to the surrounding environment. As the earth rotates on its axis on a daily and around the sun on a yearly basis, cyclical events occur. The environmental alterations include temperature, day length, magnetic fields and pressure. These changes present formidable challenges an organism must anticipate and adapt to. An innate ability to sense and prepare for these and other environmental tendencies confers a distinctive survival advantage to the organism. For example the eyes of fish require approximately 20 minutes for adaptation from nighttime lighting to daylight conditions. As such, an animal able to sense the coming environmental change and whose eyes are prepared for the onset of dawn will be able to spot its prey more readily and also avoid predators more efficiently. The ability to anticipate time and environmental change is determined by an endogenous biological clock. Biological clocks are fundamental properties of most living organisms, ranging from bacteria to multicellular plants and animals. The organism's biological clock(s) provides the mechanism responsible for anticipating environmental changes and regulating several biological processes in a rhythmic manner, including transcription, metabolism, and behavior (Pittendrigh 1993).

This dissertation follows the style of the Journal of Neuroscience.

Biological clocks are defined by three formal properties. 1) The regulation of rhythmic biological processes must be endogenous, with a period of approximately 24 hours. 2) The biological clock is entrainable to external cues. 3) The period of endogenous rhythmicity is temperature compensated (Menaker 1982). Specifically, endogenous rhythmicity must continue in the absence of external cues, wherein an organism shut off from the outside environment is still able to rhythmically regulate biological processes. The period (τ) of the rhythmically regulated biological processes is generally longer or shorter than 24 hours (T). Given the variation from exactly 24 hours, the biological clock is referred to as the circadian (meaning approximately a day) clock. The endogenous period of the circadian clock varies somewhat from species to species, and also within the species itself. The period of a particular organism is determined by placement in constant conditions (constant darkness or illumination) lacking any external cues the organism would use for entrainment. When placed in constant conditions an organism will "free run" reflecting its endogenous period, and indicating the existence of a biological clock and its characteristics (Menaker 1982).

As stated previously the ability of the clock to adjust to external light cycles is critically important for an organism's survival. This process is termed "entrainment". The mechanism(s) by which entrainment occurs is not completely understood. However, two hypotheses exist, the parametric and non-parametric models of entrainment. The parametric model of entrainment suggests that the clock is updated daily, via a change of its angular velocity to yield a period of 24 hours, meaning the clock speeds up and slows down at times during the day. The non-parametric model for entrainment states the clock

is updated daily by altering its angular position, or "phase", meaning the clock automatically moves ahead or back to adjust the clock to its approximately 24 hour period. A great deal of evidence obtained from several classic experiments supports the non-parametric model, wherein animals are placed under constant conditions and exposed to short pulses of light at particular times (phase, Φ) in the circadian day. As a result of these light pulses, the animals previously free running in constant conditions, shifted to a new phase the next day and began free running with their new phase instead of the previous one. These events are termed phase shifts (ΔΦ) and can occur as either advances or delays. Additionally, the size of the phase shift (advance or delay) is dependent upon multiple factors, including the phase at which the stimulus is applied and the magnitude of the applied stimulus (Pittendrigh and Daan 1976).

In order to fully determine the effects of the phase shifting stimulus the generation of a phase response curve (PRC) is required. This is accomplished by the application of light pulses applied to animals maintained in constant conditions and measuring the resulting phase shifts versus the circadian phase at which the light pulses were applied. The shape of this curve contains certain characteristics, representative of all organisms on which these experiments have been conducted. Characteristically, PRCs contain dead zones wherein no phase shifting effect is observed in response to the stimulus applied. Phase advances occur when a stimulus is applied in the early subjective night while phase delays occur when a stimulus is applied in the late subjective night. The resulting PRCs occur in two forms, a type 1 and type 0. A type 1 PRC is characteristic of an organism that exhibits small phase shifts in response to a

light pulse under constant conditions. A Type 0 PRC is representative of organisms that exhibit large phase shifting responses under constant conditions. The names of these curves correspond to the slope of the phase transition curve (PTC). The PTC is a plot of the organisms previous phase versus the new phase when given a stimulus in constant conditions (Pittendrigh and Daan 1976).

To say that the circadian clock is temperature compensated means that it is capable of maintaining a steady period over a varying range of temperatures, neither slowing down nor speeding up. Presumably at the heart of the biological clock is a mechanism controlled by a subset of biochemical reactions. These reactions themselves are not temperature compensated, but together comprise a temperature compensated biological clock. Typically the rate of enzymatic reactions increases as temperature increases and vice-versa. The relationship of the reaction rate versus the temperature is referred to as the Q10. Generally this value is equal to 2, which indicates that for every 10°C change in temperature the enzymatic reaction rate changes 2-fold. As one would expect a circadian clock that existed with these same principles would function at an elevated rate on hot days and slow on cold days. This type of clock would not be beneficial. The circadian clock however is temperature compensated over a wide range of varying temperatures and maintains a Q10 of approximately 1.1. Much like the mechanisms of entrainment, the process governing the circadian clock's ability to be temperature compensated is largely unknown (Pittendrigh et al., 1973).

2. The Avian Circadian System

From these founding principles and several additional significant historical studies, a generalized model of circadian organization is constructed and used to investigate particular aspects of the circadian clock. It includes an input mechanism, whereby an external cue is collected and transmitted to the circadian clock. The information received from the input mechanism is used to entrain the circadian clock. The clock integrates this information on multiple levels and then orchestrates overt circadian rhythms in behavioral, physiological, and biochemical processes.

This generic model can be applied to study all circadian systems, including birds. However, the avian circadian system is a unique system for the study of circadian rhythm generation. It is a multi-oscillatory system, comprised of oscillators in the retina, pineal gland, and suprachiasmatic nucleus (SCN). The pineal gland was the first avian circadian oscillator discovered, and possesses all the properties of a circadian oscillator both *in vivo* and *in vitro*. The pineal's integral role in the avian system is evidenced by surgical removal, wherein pinealectomy disrupts and/or abolishes circadian rhythms in several avian species (Gaston and Menaker 1968; Ebihara and Kawamura 1981; Gwinner et al., 1987; Pant and Chandola-Saklani 1992; Underwood 1994). Further, the avian pineal gland contains a circadian oscillator to drive circadian rhythms in the biosynthesis of the indoleamine hormone melatonin, a commonly measured circadian output (Deguchi 1979; Kasal et al., 1979). The rhythm in melatonin biosynthesis is such that high levels are produced during the night and secreted into the blood for circulation

throughout the body. The rhythmic release of melatonin influences several aspects of physiology and also affects overt circadian rhythm generation.

The oscillatory and biosynthetic mechanism(s) generating melatonin rhythms within the pineal gland occurs within a single cell type, the pinealocyte (Nakahara et al., 1997). The molecular mechanisms regulating the rhythmic release of melatonin in birds have been studied extensively (Cassone 1998; Bernard et al., 1999; Ganguly et al., 2002). Briefly, tryptophan is taken up and converted to 5-hydroxytryptophan (5HTP) by tryptophan hydroxylase (TrH; E.C. 1.14.16.4). Then, aromatic amino acid decarboxylase (AADC; E.C.4.1.1.28) converts 5HTP to serotonin (5HT). During the night, 5HT is converted to N-acetylserotonin (NAS) by arylalkylamine-N-acetyltransferase (AANAT; E.C. 2.3.1.87). NAS is the primary substrate for hydroxyindole-O-methyltransferase (HIOMT; E.C.2.1.1.4), which converts NAS to melatonin. In the chicken, TrH, AANAT and HIOMT are regulated on a circadian basis by both transcriptional and by post-transcriptional mechanisms, including proteosomal proteolysis (Gastel et al., 1998; Bernard et al., 1999; Ganguly et al., 2002). Placed in organ and/or cell culture conditions, the pineal of several species of birds express rhythmic melatonin biosynthesis under constant conditions of continuous darkness or dim red light (Takahashi et al., 1980; Zatz et al., 1988; Murakami et al., 1994).

In addition to the pineal gland, the avian retina also contains a circadian oscillator. Not surprisingly, the retina possesses several of the same properties as the pineal gland *in vivo* and *in vitro*. Loss of the retinal clock in turn disrupts and/or abolishes output processes in certain species of birds (Underwood and Siopes 1984).

The retinal clock rhythmically synthesizes and releases melatonin. The retinal clock also functions at a local level regulating several processes including rod outer-segment disk shedding, cGMP channel sensitivity (Ko et al., 2001; 2003), electroretinogram (ERG) responses (McGoogan and Cassone 1999), melatonin, and dopamine biosynthesis (Doyle et al., 2002). Lastly, an important function of the retina is the integration and relay of photic information to the SCN via retinal ganglion cells (RGC) and the retinohypothalamic tract (RHT). This tract serves as the major pathway for entrainment of the SCN in mammals (Cassone et al 1988; Moore et al., 2002).

A third avian oscillator is located in the avian homolog of the mammalian SCN. The SCN of mammals is the master pacemaker regulating all aspects of circadian rhythm generation including electrical activity, metabolism, and peptide release *in vivo* and *in vitro* (Green and Gillette 1982; Earnest and Sladek 1987; Rusak 1989; Newman et al., 1992). Surgical ablation of the SCN eliminates circadian rhythm generation. Experimental evidence suggests that the avian SCN also rhythmically regulates biological processes albeit at a more subdued level. Several rhythmically regulated processes are controlled by the avian SCN including metabolic activity in house sparrows and chickens (Lu and Cassone 1993, Cantwell and Cassone 2002). In the Japanese quail, circadian rhythms of electrical activity recorded from the SCN *in vitro* exhibit a rhythmic pattern throughout the course of the day (Juss et al., 1994). Similar to the situation in mammals, lesions of the SCN in avian species, including the house sparrow, Java sparrow, and Japanese quail, abolishes circadian rhythms in locomotor activity (Ebihara and Kawamura 1981; Simpson and Follett 1981; Takahashi and

Menaker 1982). Furthermore discrete lesioning studies of a specific area of the SCN, the visual suprachiasmatic nucleus or vSCN, abolish activity in norepinephrine (NE) turnover in the pineal gland (Cassone 1990).

Another interesting feature of circadian systems is extraocular photoreception. Several non-mammalian vertebrates are known to possess deep photoreceptive capability (Menaker 1968; Foster 1998). In enucleated chicks, rhythms of locomotor activity can be synchronized to a LD cycle, indicating that the communication of light to the clock still occurs in blind birds (Nyce and Binkley 1977). The exact nature and location of the deep brain photoreceptors is unknown, however, the examination of several opsin based photopigments, including melanopsin and the cryptochrome genes, provides candidates which may perform such a function.

As a whole, evidence clearly exists to indicate that the avian circadian system is comprised of multiple oscillators forming a dynamic timing mechanism. To some extent, the avian oscillators function in a feedback loop, capable of influencing the oscillation of one another. The ability of one oscillator to influence the other varies from species to species (Cassone and Menaker 1984). Nonetheless a model depicting this interaction has been hypothesized and termed the neuroendocrine loop model (Cassone and Menaker 1984). Within the model each oscillator is referred to as a damped oscillator. The retina receives input from the surrounding environment and relays this signal to the SCN. The SCN is capable of influencing the pineal via sympathetic innervation. The pineal produces melatonin in a rhythmic fashion with high levels occurring at night. This rhythmic production of hormone functions at one level by

influencing the SCN oscillator via the sympathetic nervous system and the release of norepinephrine.

3. Molecular Mechanisms of Biological Clocks

Drosophila has been the subject of circadian clock research for decades. Initial studies by Pittendrigh revealed one of the formal properties of circadian clocks in temperature compensation (Pittendrigh 1954). Wherein, he demonstrated circadian rhythms in elcosion of flies at varied temperatures leading to the use of flies as a genetic model for the study of circadian clocks. Further mutagenesis screens by Konopka and Benzer (1971) resulted in the discovery of the first of several genes critical for the generation of circadian rhythms in flies, the period (*per*) gene. Similar genetic approaches uncovered several additional clock genes including timeless (*tim*), cycle (*cyc*), doubletime (*dbt*) and cryptochrome (*cry*). Genetic approaches toward clock gene discovery were successfully applied to other model organisms, identifying mutants with disrupted circadian rhythm generation in cyanobacteria, *Neurospora*, *Arabidopsis*, hamsters, mice, rats, and fish (Dunlap 1999). Specifically, in mammals, an extensive mutagenic screen administering N-ethyl-N-nitrosourea (ENU) to mice was performed. As a result of this mutagenesis a mutant strain of mice was identified which exhibited a lengthening in free running period by four hours and then became arrhythmic after approximately two weeks in constant conditions. The gene was subsequently cloned and shown to encode a bHLH-PAS domain protein, labeled clock (*clock*). This type of

experimental analyses and the proposed functional conservation of clock genes across species were used to discover orthologs of the *Drosophila* clock genes in other systems.

As a result of the oscillating mRNA and protein abundances of these genes and their interactions, an interlocking transcriptional/ translational feedback loop model has been proposed to describe circadian rhythm generation. The loops consist of "negative elements" and "positive elements" and are the current general model applied to study molecular circadian systems (Dunlap 1999; Shearman et al., 2000; Glossop and Hardin 2002; Cyran et al., 2003). In mammals the positive elements including clock (*clock*) and the bmals (*Bmal1, 2*) are transcribed, dimerize and enter the nucleus. Once inside the nucleus, the CLOCK/BMAL dimers initiate the transcription of the negative elements and other genes via an e-box promoter element. The negative elements comprise the period genes (*per1-3*) and cryptochromes (*cry1, 2*). These negative elements in turn are translated, oligomerize and re-enter the nucleus where they inhibit their and other genes' transcription. There are, of course other clock genes involved in this mechanism. One such is casein kinase I epsilon (*CK1E*), encoding a kinase that targets period and timeless proteins for degradation.

As one would expect clock genes and their respective proteins reside in several clock tissues. However, they also exist outside core clock structures in the periphery. Not only do clock genes reside in several peripheral tissues including the liver, heart, and lungs many clock genes exhibit rhythmic oscillations within these peripheral tissues. For example, various rat tissues express rhythmic oscillations of clock gene regulation, exhibiting oscillation in mRNA and protein abundance. This is also true *in vitro*, as

explanted rat tissues and immortalized cell lines derived from fetal SCN tissue, exhibit oscillations for several days of clock genes expression (Allen and Earnest 2002; Yoo et al., 2004). Further, immortalized SCN cells are capable of inducing clock gene rhythms in previously arrhythmic fibroblasts (Allen et al., 2001). The analysis of clock proteins within known clock and peripheral tissues *in vitro* provide distinct advantages, since there are easily manipulated, monitored and verifiable *in vivo*.

The surge forward in the genetic determination of molecular circadian components has rapidly advanced the circadian clock field in the last 20 years. If elements of circadian clocks are evolutionarily conserved among organisms then one would expect that similar molecular elements to that of flies and mammals exist in birds. This aspect of the avian circadian system has not been thoroughly examined even though much is known about avian physiology and the interactions of the circadian oscillators in birds. Therefore we propose to determine candidate molecular elements of the avian system including photopigments and their signal transduction cascades, the molecular mechanisms involved in the circadian rhythm generation itself, and the coupling of this rhythm to pineal clock output. Birds present a complex challenge, since it is not readily manipulated through the same genetic and molecular approaches used to discover components of the *Drosophila* and mammalian circadian systems. Therefore alternate experimental approaches must be pursued to identify candidate molecular components of the avian circadian system. One such approach is the examination of an entire transcriptome of an organism via DNA microarray analyses. This procedure allows the determination of mRNA profiles of several thousand genes simultaneously.

By investigating the transcriptome at different times throughout the course of the day an entire circadian profile of mRNA activity can be produced. This type of analyses would likely yield many intriguing clock controlled genes and perhaps a core clock components. Further, application of microarray analysis to specific oscillators in the avian system would permit the further dissection of the inner workings of an avian oscillator itself and also provide the means to directly compare the circadian profiles of two oscillators within the same system.

Several experimental hypotheses are tested in this thesis, specifically, to determine candidate avian clock components within the avian pineal and retina. 1) Do similar molecular clock components present in mammals exist in the chick circadian system? 2) Are these genes regulated on a circadian basis within the retina and pineal gland, and do they exhibit similar patterns of mRNA abundance? 3) What are the expression patterns of these genes and do they exist in similar cell types and structures? The results indicate that several, if not all, the clock genes of mammals exist in birds and several oscillate on a circadian basis. A detailed microarray analysis of both the retina and pineal transcriptome performed here has provided several candidate molecular components of the avian clock and also provided insight into the interaction and/or function in the avian circadian system.

CHAPTER II

TRANSCRIPTIONAL PROFILING OF THE CHICK PINEAL GLAND, A PHOTORECEPTIVE CIRCADIAN OSCILLATOR AND PACEMAKER*

1. Introduction

The avian pineal gland is a critical component of the birds' biological clock and is an important model system for the study of circadian rhythm generation in general. The gland is important for overt circadian organization, since surgical removal of the pineal gland disrupts and/or abolishes circadian rhythms in several species of birds (Gaston and Menaker 1968; Ebihara and Kawamura 1981; Gwinner et al., 1987; Pant and Chandola-Saklani 1992; Underwood 1994). Further, the avian pineal gland contains both a circadian oscillator and pacemaker to drive circadian rhythms in the biosynthesis of the indoleamine hormone melatonin and photoreceptors to synchronize that rhythm to environmental lighting (Deguchi 1979; Kasal et al., 1979). When placed in organ and/or cell culture, the pineal glands of several avian species express at least 4 circadian cycles of melatonin biosynthesis under constant conditions of continuous darkness or dim red light (Takahashi et al., 1980; Zatz et al., 1988; Murakami et al., 1994). Further, chick pineal glands *in vitro* respond to environmental light in at least three mutually exclusive (Zatz et al., 1988).

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ways: 1) phashift of the circadian cycle, 2) acute suppression of melatonin biosynthesis and release, and 3) decrease in rhythm damping (increase in amplitude)

The cellular sites of the oscillatory and biosynthetic mechanism(s) generating melatonin rhythms within the gland reside in a single cell type, the pinealocyte (Nakahara et al., 1997), and the molecular mechanisms for the biosynthesis of melatonin within those pinealocytes have been thoroughly worked out (Figure 1A).
However, the details of the molecular regulation of rhythmic melatonin biosynthesis vary among species (Cassone 1998). In the chick, TrH, AANAT and HIOMT are regulated on a circadian basis by both transcriptional and by post-transcriptional mechanisms, including proteosomal proteolysis (Gastel et al., 1998; Bernard et al., 1999; Ganguly et al., 2002).

As stated above, the chick pineal gland is photoreceptive and responds to light in several ways. Several opsin-based photopigments and two cryptochromes have been isolated and characterized from this gland (Okano et al., 1994; Yamamoto et al., 2001; Bailey et al., 2002). Further, many of the known opsin-based signal transduction mechanisms are present in the gland (Kasahara et al., 2000; Kasahara et al., 2002). However, the roles these putative photopigments play in each of these light-based processes or the second messenger systems underlying their role are not known.

In spite of the fact that much is known about pineal physiology, the molecular mechanisms by which melatonin is synthesized and the identity of photopigments and their signal transduction cascades, the molecular mechanisms involved in the circadian rhythm generation itself, and the coupling of this rhythm to pineal clock output are

A. B.

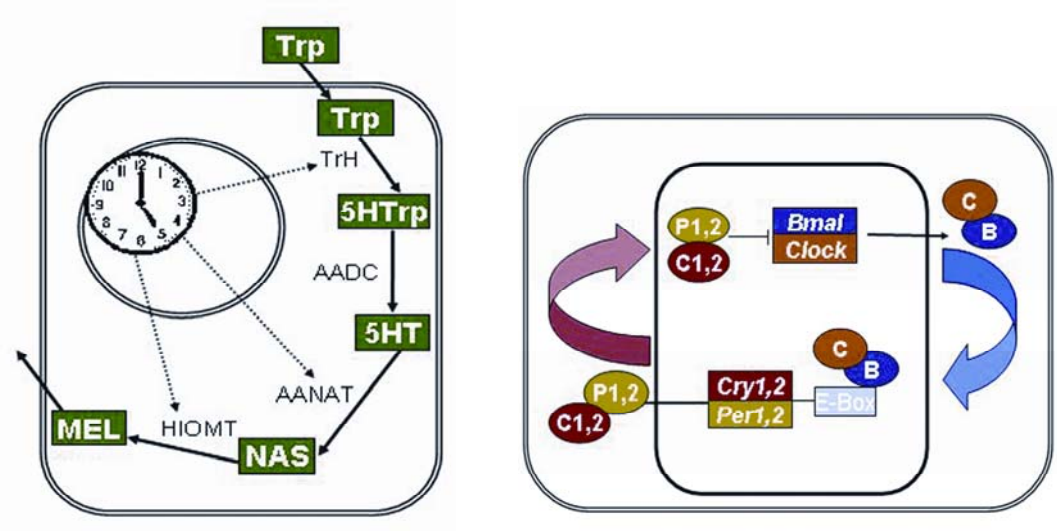


Figure 1. The melatonin biosynthesis pathway and simplified transcriptional/translational feedback model of circadian rhythm generation in mammals. **A.** The amino acid tryptophan (TrP) is taken up by the cell and converted to 5-hydroxytryptophan (5HTP) by tryptophan hydroxylase (TrH). Then, dopa decarboxylase (AADC) converts 5HTP to serotonin (5HT). During the night, 5HT is converted to N-acetylserotonin (NAS) by arylalkylamine-N-acetyltransferase (AANAT). Hydroxyindole-O-methyltransferase (HIOMT) converts NAS to melatonin. **B.** The negative elements in mammals comprise the period genes (green box) and cryptochromes (red box). These genes are transcribed in response to the dimerization of the positive elements, which include clock (orange oval) and Bmals (blue oval), and their subsequent binding to an E-box in the promoter regions of several genes. The negative elements in turn are translated (red, green ovals), oligomerize in the cytoplasm and re-enter the nucleus to inhibit their and other genes' transcription.

completely unknown. Analysis of the molecular mechanisms responsible for circadian rhythm generation in other model systems has advanced rapidly. Initiated with the discovery of the period gene in *Drosophila* (Konopka and Benzer 1971), the molecular clock model has now expanded into a network of "clock genes" that form interlocking transcriptional/ translational feedback loops of "negative elements" and "positive elements" (Figure 1B) (Dunlap 1999; Shearman et al., 2000; Glossop and Hardin 2002; Cyran et al., 2003).

Although several studies have utilized high-density oligo and cDNA microarrays with the goal of uncovering genes involved in the regulation of clock function and elements under clock control (Harmer et al., 2000; McDonald and Rosbash 2001; Akhtar et al., 2002; Duffield et al., 2002; Kreps et al., 2002; Lewis et al., 2002; Wiechmann 2002), none have comprised arrays derived from cDNAs isolated from a specialized clock tissue itself and none have focused on the chick pineal gland, a unique model system for the study of biological clocks. Our study utilizes the uniqueness of the pineal gland model and cDNA microarray technology to identify candidate molecular components involved in the pineal clock, including core oscillatory elements as well as components of the input pathway (photopigments) and outputs (clock-controlled genes). We validated the transcriptional profile generated from the pineal cDNA microarrays via northern analysis and have identified several interesting candidate pineal clock components that further our understanding of circadian biology and avian physiology.

2. Methods and Materials

Animals. Chicks were obtained from Hyline International (Bryan Tx.) and housed under a lighting schedule of 12 hours of light and 12 hours of darkness (LD 12:12) for 7 days with food and water continuously available. For cDNA library production chicks (N=25/per timepoint) were sacrificed by decapitation and their pineal glands pooled. For microarray hybridizations chicks (N=5/timepoint/experiment) were sacrificed by decapitation and their pineal glands pooled. Three separate pineal RNA sampling cycles were performed for array hybridizations. All DD dissections were performed using an infrared viewer. Animals were treated in accordance with PHS guidelines, these procedures have been approved by the Texas A&M University Laboratory Animal Care Committee (AUP #2001-163).

cDNA Library Production. Chicks were housed in a LD 12:12 light cycle for one week. On the eighth day at midday (ZT-6) and midnight (ZT-18), chicks were sacrificed, decapitated and their pineal glands removed and pooled. mRNA was extracted using a MicroPolyA Pure Kit (Ambion). The mRNA was subsequently used to generate two cDNA libraries using a Lambda Zap cDNA Library Synthesis Kit (Stratagene). Approximately 5,000 clones from each library were selected, sequenced and organized into a database, and subjected to PCR amplification for microarray printing. The cDNA clones were stored in 384 well plates and were propagated in four 96 well plates for plasmid production. Simplified alkaline lysis plasmid minipreps were conducted in each 96 well plate, and plasmids obtained therein were used as templates for DNA sequencing reactions. Sequencing reactions were carried out using SK primer

and Big Dye Terminator Cycle Sequencing Ready Reactions (Applied Biosystems) in AB9700 96 well thermocyclers (Applied Biosystems). Reaction products were analyzed on ABI 377 automated DNA sequencers. The resulting sequence chromat files were then processed through the EST pipeline. The EST data pipeline consisted of a Dell PowerEdge 2400 running Linux 7.3, networked to the campus LAN/Internet and to an internal, private LAN, consisting of 3 shuttle cubes networked to the private LAN. The system ran the following software: 1) "Phred" read the DNA sequence trace, called bases and assigned quality values to the bases. 2) "Cross-match" created a vector-masked version of the sequence. 3) "Phrap" constructed a contig sequence as a mosaic of the highest quality reads, and 2) "BLAST" was run on vector-masked ESTs using a local copy of the NCBI protein database.

Microarray Production. The cDNA microarrays were prepared based upon approximately 4,000 PCR products from each of the two pineal cDNA libraries constructed, for a total of 7,988 cDNAs present on the array. The cDNA in plasmids from each library were PCR amplified using flanking primers (SK and T7), purified by ethanol precipitation using ammonium acetate, and placed in the wells of 96 well plates at a concentration of 50 ug/ml in 3X SSC. A GeneMachines OmniGrid microarrayer equipped with 8 Telechem SMP3 pins was used to spot the samples onto poly-L-lysine coated slides (CEL Associates). Duplicate 100 micron diameter spots were placed at intervals of 190 microns (center to center). Slides were printed in batches of 50-100. Printing was accomplished at 70^{o} C and 60 % humidity. The OmniGrid's operating software used information on the position of the individual clones within the 96 well

sample plates and the order in which the plates were used to create a file describing the position of each cDNA clone on the array for later analysis. Slides were stored desiccated at room temperature until their subsequent use. Prior to hybridization, the dried spots were hydrated gently over a steaming water bath, snap dried, and the DNA was UV cross-linked using a Stratalinker (Stratagene).

Hybridizations. To determine a rhythmic transcriptional profile of the pineal gland, total RNA was harvested using Trizol Reagent (Gibco-Brl) every four hours for one day in a light dark cycle of 12 hours light and 12 hours darkness (LD12:12; lights on 0600 CST; lights off 1800 CST) and for one day in continuous darkness (DD). Times of sampling began two hours after lights on (Zeitgeber Time, ZT2) and continued every four hours hence (ZT6, ZT10, ZT14, ZT18, ZT22). When lights would have normally turned on, the timer was disabled, and birds were placed in DD. They were then collected again every 4 hours, and designated Circadian Time (CT) CT2, CT6, CT10, CT14, CT18 and CT22. This sampling procedure was performed on two separate occasions. A total of 4 experimental microarray hybridizations were conducted for each time point, two from each respective biological sampling procedure. The total RNA samples were then amplified to produce aRNA using a MessageAmp Kit (Ambion). Randomly primed fluorescent probes were produced from aRNA samples using Genisphere 3DNA Array 350RP expression array detection kit. The fluorescent dye on probes derived from the experimental aRNA was Cy5, while the dye on control probes was Cy3. In all experiments, the control sample was derived from pineal glands harvested at midnight (ZT18) under LD conditions. Hybridizations and washes were

conducted as suggested by Genisphere. The labeled arrays were scanned in an Affymetrix 428 array scanner, and tif images were made of both Cy5 and Cy3 specific fluorescence on the array. The tif images were subsequently analyzed by GenePixPro software (Axon Instruments). Information from the gal file and the tif image were then combined to determine the Cy5 and Cy3 fluorescence of each spot as well as the corresponding background fluorescence. The program also generated a false color image of the combined fluorescence. Cy5 fluorescence is colored red, Cy3 green and an equal mix of each is yellow.

Data Analysis. The fluorescence values and background calculated by the GenePix program were saved as gpr files. These files were then further analyzed by GeneSpring 5 (Silicon Genetics). GeneSpring allows the data to be normalized in a variety of ways, allows the assignment of parameters and interpretations, and then allows the data to be filtered to determine differential expression. The current analysis used intensity dependent LOWESS normalization. Intensity dependent normalization is just one technique used to eliminate dye-related artifacts in two-color experiments such as this. At each time point, the results for each gene were reported as an average obtained from 4 microarrays. The data were reported as the normalized ratio of Cy5 (experimental) to Cy3 (control at ZT18). Thus, the data is reported for each time point relative to the abundance of the same gene at midnight (ZT18). Rhythmic genes were defined as those transcripts with a 2.0 fold or higher amplitude change in mRNA levels over a 24-hour period relative to the genes' respective mRNA abundance level at ZT18.

3. Results

Chick pineal EST analysis

To optimize our chances of identifying important rhythmic transcripts in the chick pineal that would be under-represented in public databases, we began our analysis by creating a pineal EST database accessible through our Center for Biological Clocks homepage (http://www.tamu.edu/clocks/index.html), and used it as a source for generating high density microarrays. The clones used for this database were chosen at random from two pineal cDNA libraries we constructed. One library was prepared from cDNA derived from RNA isolated from pineal glands harvested at midday (ZT-6), the other from cDNA generated from RNA obtained from pineal glands harvested at midnight (ZT-18).

We conducted one-pass sequence analysis from the 5' end of approximately 10,000 cDNAs. Approximately 5000 ESTs were sequenced from the ZT-6 and ZT-18 libraries, respectively. Sequences were filtered, trimmed and analyzed on a local BLAST server to assign potential gene IDs to the ESTs (Altschul et al., 1990). 9,867 sequences were submitted for BLAST analysis; of these, 54 % (n=5284) had a BLAST hit. We have used several approaches to estimate the prevalence distribution and complexity of the pineal libraries. For example, contig assembly using phrap (http://www.phrap.org) suggests that only about 20% of the cDNAs in the library occurs at least twice. However, this is likely an underestimate. A more realistic estimate was

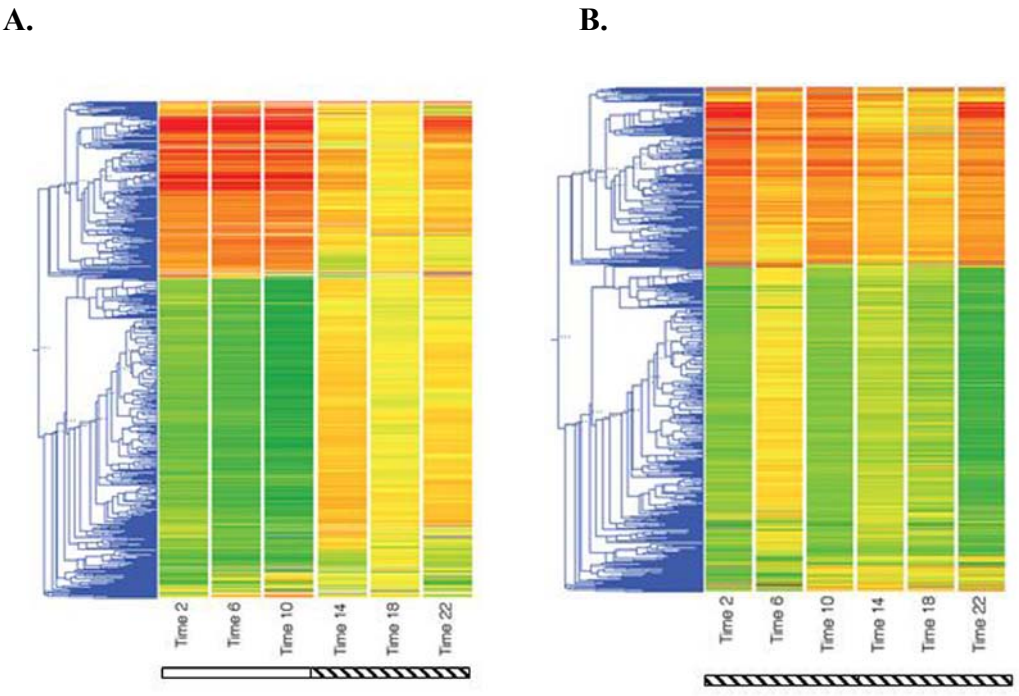


Figure 2. Gene trees of pineal gland transcripts oscillating in a LD and DD cycle **A.** Pineal transcripts rhythmically expressed under LD conditions were organized into a gene tree to visualize transcript abundance patterns. Increased transcript abundance is represented by red fluorescence, decreased by green, and non changing transcript abundance by the color yellow. All fluorescence values are relative to respective values obtained at ZT18. Of the approximately 8,000 pineal ESTs present on the microarray 1,797 oscillate with at least a 2-fold amplitude change in a light dark cycle. This data set includes 382 unique classified genes; while 902 transcripts had no significant BLAST score. **B.** Pineal transcripts rhythmically expressed under constant darkness conditions were also organized into a gene tree as in A. The number of rhythmic pineal transcripts observed was 682, with 128 of these transcripts being unique classified genes, while 254 transcripts exhibited no significant BLAST score.

obtained by determining the frequency at which the same annotated genes occurred in the library. Here, roughly 50% of the ESTs with a BLAST hit occurred two or more times. However, 75% of the cDNAs are represented five or fewer times indicating the chick pineal mRNA population is complex. It is noteworthy that 8% of the cDNAs in the library are represented by only 20 genes. The most abundant is transthyretin, representing 2.8% of all cDNAs sequenced in the chick pineal database. The microarrays used in these experiments were produced when the database contained approximately 8,000 entries. Currently, approximately 10,000 entries have been made in the chick pineal database.

Transcriptome analysis

Using our method of hybridization and analysis, consistent, very high signal to noise ratios were obtained. Of the approximately 8,000 pineal cDNAs represented on the microarray, 1,797 oscillate with 2-fold amplitude or greater change in a light dark cycle as indicated via a gene tree (Figure 2A). The most abundant transcripts that were oscillating rhythmically in LD were transthyretin precursor (prealbumin; n=184), cystatin (n=34), HIOMT (n=13), glyceraldehyde-3-phosphate dehydrogenase (G3PDH; n=12), group III secreted phospholipase C (n=9), actin (n=7), AANAT (n=6) and TrH (n=5). Upon correction and removal of transcripts for redundancy (n=518) and unknown/unclassified transcripts (n=902) this data set is reduced to 377 unique classified genes. (This gene list and all subsequent genomic data in the following chapters are available by visiting http://www.tamu.edu/clocks/index.html).

In DD, the number of rhythmic pineal transcripts observed was 682 as indicated via a gene tree (Figure 2B). The most abundant cDNAs on the microarray that were oscillating rhythmically in both LD and DD were again transthyretin (n=118), HIOMT (n=12), cystatin (n=7), actin (n=5), G3PDH (n=5), and TrH (n=5). AANAT was expressed 3 times. Upon correction and removal of transcripts for redundancy (n=302) and unknown/unclassified transcripts (n=254), this data set is narrowed to 126 unique classified genes. The fact that the largest single category of genes that were rhythmic was ESTs that had not returned a significant BLAST hit should not be construed as there being a significant pineal specific transcriptome, since our sequencing was only 1 pass from the 5 prime ends. Thus, many of the "unknown" genes may be identified with additional sequencing. We have recently been able to place some of these genes into contigs. Preliminary BLAST analysis of the contig consensus has allowed tentative identification of some of the unknowns. Of the 254 unknown rhythmic cDNAs under both LD and DD, about 21 appear to be additional transthyretin genes, 4 are likely TrH, 3 match cystatin, 2 with AANAT and one each with EURL, G3PDH, purpurin precursor and CATRO cytochrome P450. At least 7 fall into a single contig that has no BLAST match.

It was not surprising to see components of the melatonin biosynthesis pathway among the most abundant rhythmic transcripts in the pineal gland. The pineal gland is best known for its production of this hormone. However, it was surprising to observe the very large number of transcripts corresponding to transthyretin, which has been

A.

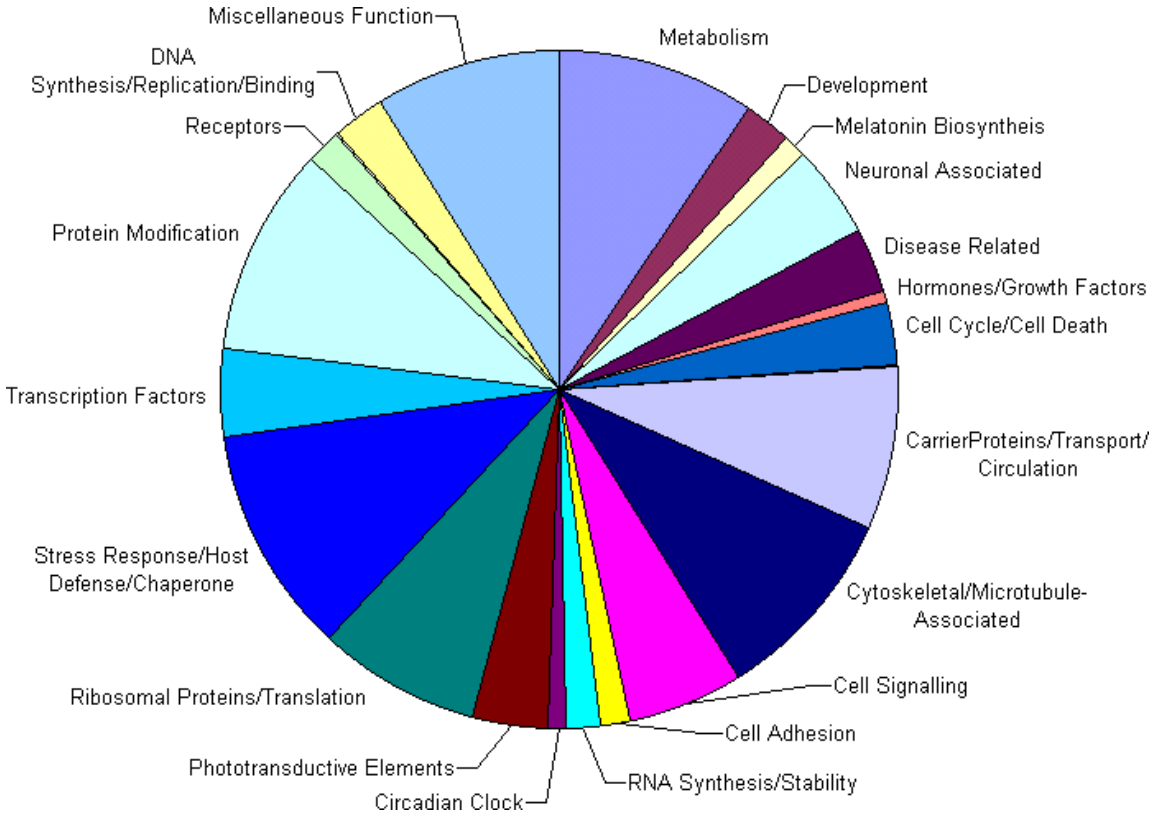


Figure 3. Clustering of oscillating pineal gland transcripts via proposed function. Rhythmically expressed transcripts under LD conditions were also clustered according to proposed function into a pie chart, revealing a wide variety of applications. First, the entire melatonin generating enzymatic cascade was represented on the array, as were many chick orthologs associated with circadian clock function ("clock genes"). In addition, cluster analysis revealed transcripts in several broad categories were also rhythmically expressed in the chick pineal gland.

B.

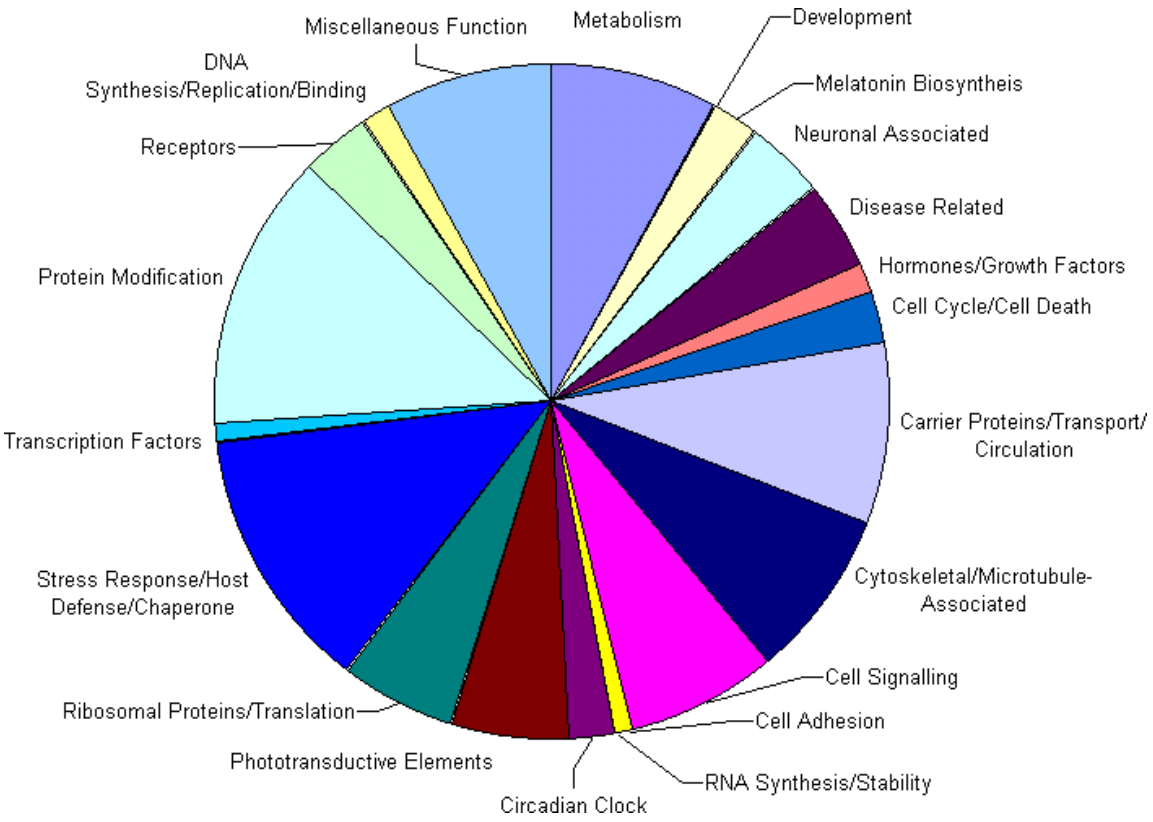


Figure 3 Continued. Clustering of oscillating pineal gland transcripts via proposed function.

B. The functional categorization method used in (A) was also applied to transcripts oscillating under conditions of constant darkness.

primarily associated with the choroid plexus. Since the pineal gland is connected to choroid plexus in many species, including chickens, it is possible that some choroid tissue may have been included in our dissections. Although the method used during pineal dissection excluded choroids tissue, it is possible some choroid cells reside within the pineal parenchyma. It is more likely, in view of the high number of transcripts and their rhythmic expression, that pineal cells themselves produce this binding protein. Transthyretin is linked to thyroid hormone binding, as a vitamin A carrier via an interaction with retinol binding protein, lipid transport, and as a marker of nutritional fitness (Robbins 2002). Preliminary data from our laboratory indicates that transthyretin binds melatonin with reasonably high affinity. Perhaps, pineal transthyretin binds melatonin in a heretofore-unrecognized release mechanism for melatonin. It has always been assumed that melatonin diffuses from the pineal gland following biosynthesis due to its very high lipid solubility. However, the high pineal melatonin content observed by many authors, is not consistent with the diffusion theory, and may be due to an intra-pineal melatonin binding protein such as transthyretin.

Functional categories of rhythmically expressed transcripts

Oscillating pineal gland transcripts in LD and DD were classified according to proposed function, revealing a wide variety of potential biological activities. First, the entire melatonin generating enzymatic cascade was represented, as were many chick orthologs associated with circadian clock function ("clock genes"). Further, cluster analysis revealed transcripts in several broad categories that were also rhythmically expressed in

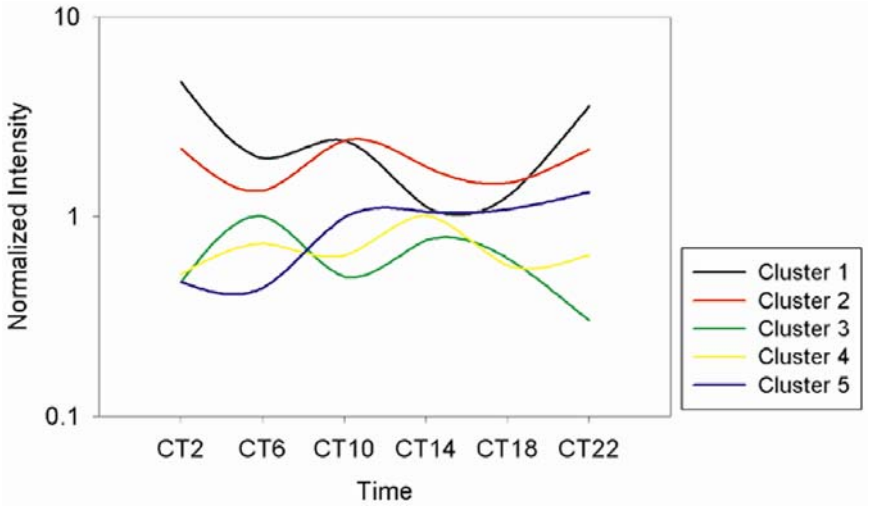


Figure 4. Phase cluster analysis of rhythmic pineal gland transcripts in DD

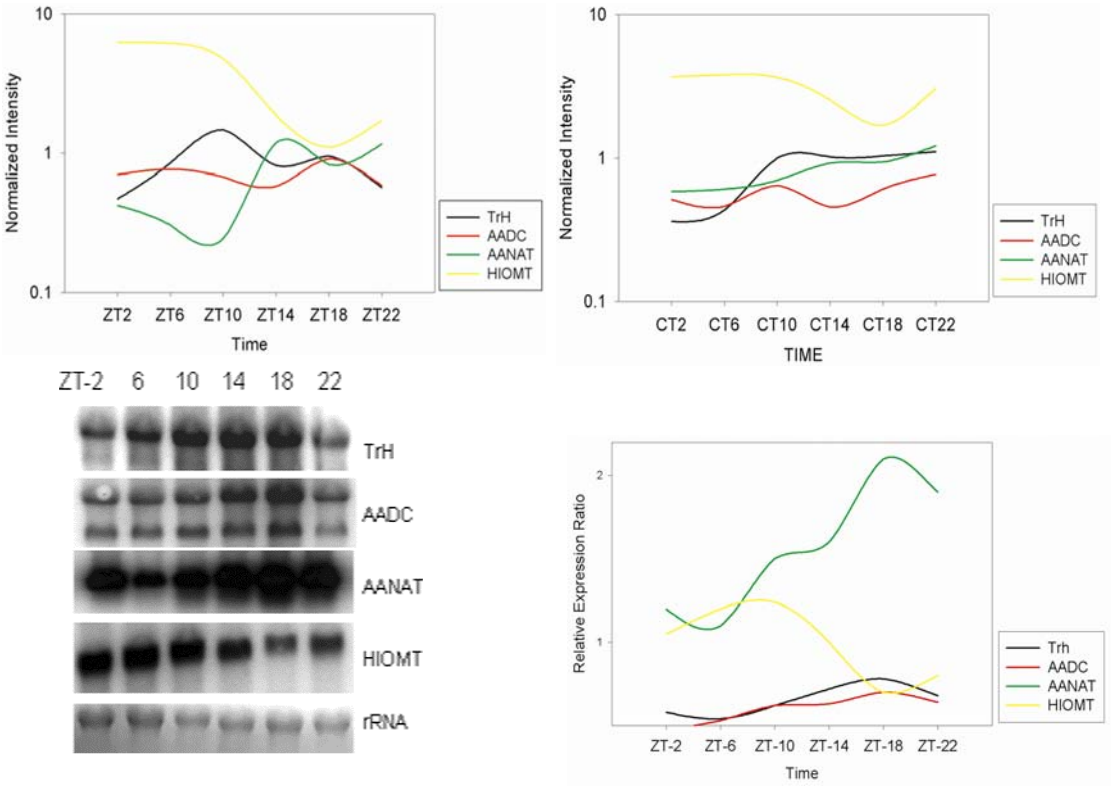
Pineal transcripts rhythmically expressed under conditions of constant darkness were clustered using a K-means clustering algorithm (GeneSpring), according to phase of mRNA abundance. The clustering analysis revealed a diverse pattern of mRNA phasing, with 5 clusters of peaking transcript abundance across the entire circadian day. For each cluster a representative trace is shown indicating the average profile off all oscillating transcripts in each respective cluster. Cluster 1 is indicative of transcripts peaking at onset of subjective dawn CT-22, CT-2, cluster 2 at late subjective day CT-10, cluster 3 at mid subjective day CT-6, cluster 4 at early subjective night CT-14, and finally cluster 5 at late subjective night CT-18, CT-22.

the chick pineal gland. Categories examined include transcription factors, ribosomal and translation factors, hormones and growth factors, carrier and transport proteins, components of cell signaling/adhesion, metabolic components, retinal and phototransduction elements, and stress response and host defense elements (Figure 3). The percentage of rhythmic transcripts represented in each category is also given. The stress response cluster includes immune function genes which under LD and DD had the highest number of oscillating pineal transcripts, 40 and 16 respectively. These functional clusters as well as others are indicated in Figure 2A, and 2B, and also Table 1, and 2.

Daily and circadian phase analysis

Initial analysis of daily and circadian phase revealed rhythmic transcripts that were either accumulating or peaking during the day and those peaking during the night in the LD cycle**.** However, pineal transcripts rhythmically expressed in DD were clustered using a K-means clustering algorithm (GeneSpring), according to phase of mRNA abundance. The clustering analysis revealed a diverse pattern of mRNA phasing, with 5 clusters of peaking transcript abundance across the entire circadian day (Figure 4). For each cluster a representative trace is shown indicating the average profile off all oscillating transcripts in each respective cluster. Cluster 1, which comprises 10% of the unique DD transcript set, is indicative of transcripts peaking at approximately the onset of subjective dawn CT-22, CT-2. Cluster 2, 26% of the unique DD transcripts, at late subjective day CT-10. Cluster 3, 50% of the unique DD transcripts, at mid subjective day CT-6. Cluster 4, 6% of the unique DD transcripts, at early subjective night CT-14.

A. B.



C. D.

Figure 5. Validation of microarray analysis examining the melatonin biosynthesis pathway in the chick pineal gland **A.** TrH and AADC mRNA oscillate such that transcript abundances were highest in the early night. AANAT mRNA was highest during the late night, while HIOMT mRNA peaked during the day. **B.** These patterns corresponded with northern blot data. **C.** The rhythm in TrH, AANAT and HIOMT persisted in DD with similar phase angles, while AADC mRNA was not significantly rhythmic in DD. **D.** Normalization of blots in C.

Cluster 5, 6% of the unique DD transcripts, peaked at late subjective night CT-18, CT-22. This is very similar to previous findings of other groups (Harmer et al., 2000, Duffield et al., 2002). Every pineal gland transcript regulated on a circadian basis was represented in one of the above mentioned phase clusters and is indicated in Table 2.

Melatonin biosynthesis mRNAs

As has been previously shown by several authors, the mRNAs encoding several of the enzymes involved in melatonin biosynthesis are rhythmic on a daily and circadian basis. Under LD conditions the microarray pattern indicated a low amplitude rhythm in TrH and AADC mRNA such that levels were highest in the early night. AANAT mRNA was highest during the night, while HIOMT mRNA peaked during the day (Figure 5A). These patterns corresponded favorably with northern blot data employing radioactive probes derived from the microarray cDNAs corresponding to each respective gene (Figure 5B). One difference was that the HIOMT rhythm amplitude was significantly greater in the microarray data than in the northern analyses. The rhythm in TrH, AANAT and HIOMT persisted in DD with similar phase angles, while AADC mRNA was not significantly rhythmic in DD (Figure 5C).

Clock genes

Several mRNAs encoding putative orthologs of genes associated with circadian clock function were identified in the cDNA libraries and represented on the microarray. These included the putative positive elements *Clock*, *Bmal1* and *Bmal2* and sequences

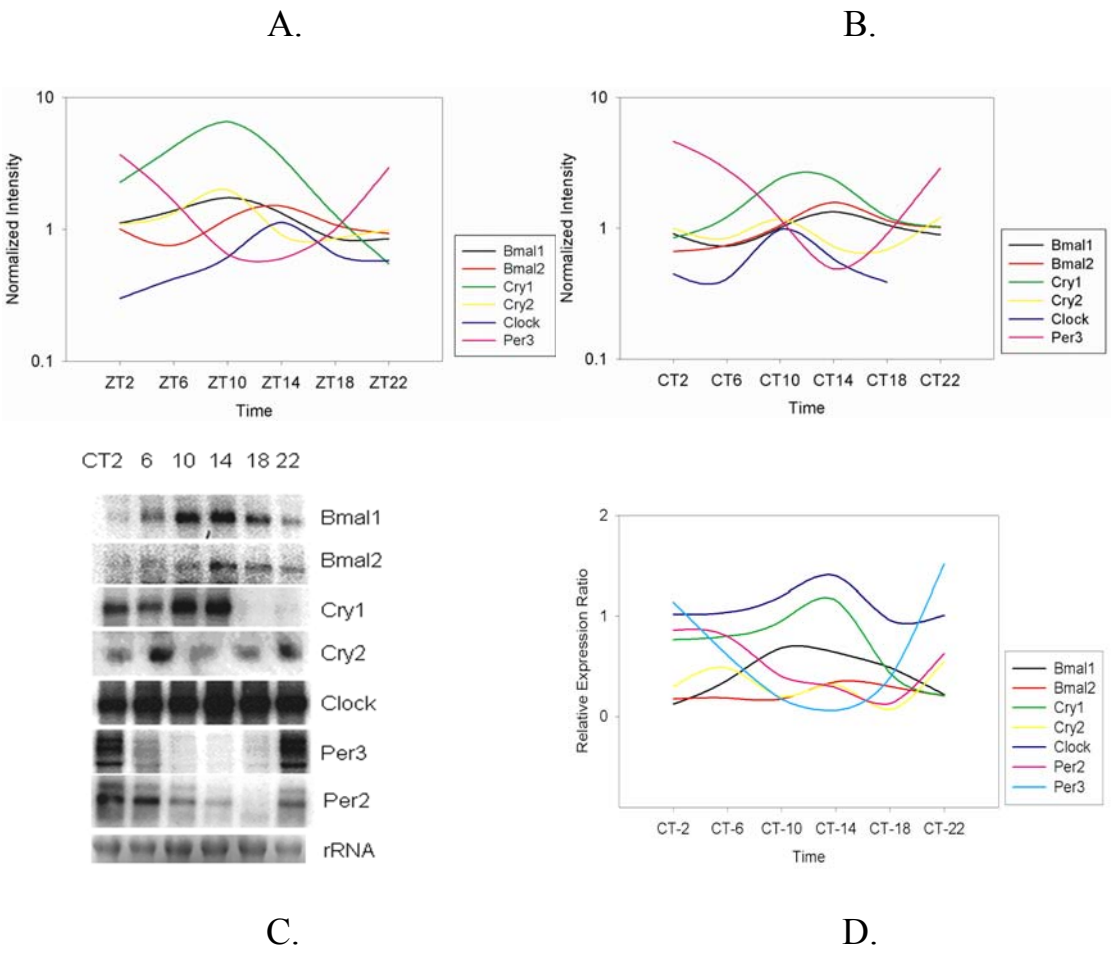


Figure 6. Validation of microarray analysis examining chick pineal clock genes

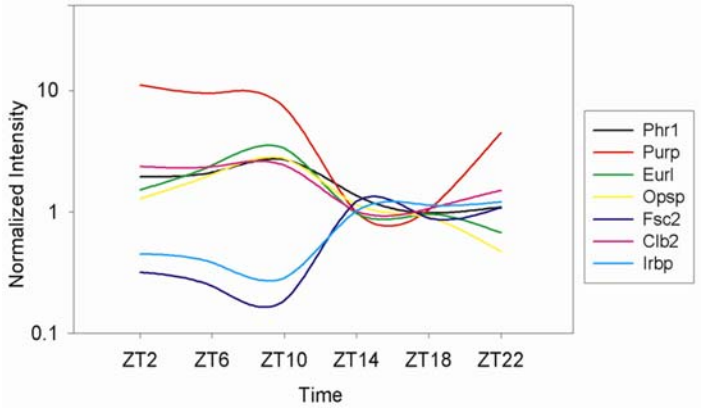
A. Orthologs of the mammalian clock gene mRNAs examined were expressed rhythmically in LD. **B.** This rhythm persisted in DD, although with overlapping phases. **C.** These circadian rhythms and their phases were confirmed by northern blot analyses. **D.** Normalization of blots in C.

corresponding to the putative negative elements cryptochrome 1 (*Cry1*), cryptochrome 2 (*Cry2*) and period 3 (*Per3*). Consistent with their putative function and consistent with published data, these mRNAs oscillated rhythmically in both LD and DD, although with overlapping phases (Figure 6A, 6B). These circadian rhythms and their phases were confirmed by both northern analysis (Figure 6C) and *in situ* hybridization (data not shown). In addition, period 2 (*Per2*), timeless (*Tim*) Acc. #AY046570, and doubletime (*Dbt*) Acc. # AY046571, which is encoded by casein kinase I epsilon in mammals, were examined on the array. *Per2* yielded an inconsistent or no signal on the array and therefore was arrhythmic in our study, although our own northern, *in situ* data and published data (Okano et al., 2001) indicate *per2* to be rhythmic in the chick pineal gland with a peak in mRNA amplitude at ~CT2-6. This also was the case for *tim,* which yielded an inconsistent or no signal on the array and was arrhythmic. The inconsistent or no signal results for these transcripts are likely an underestimation in the amount of PCR product spotted on the array for these transcripts, given the number of hybridizations performed in the current experiment. *Dbt*, however, gave a clear and consistent signal and was found to be arrhythmic in the chick pineal gland (data not shown).

Retinal and phototransduction transcripts

Several components of the photoreceptive/phototransduction pathway were examined on the microarray, yielding many rhythmic profiles under either a light dark cycle or conditions of constant darkness (Figure 7A, 7B). Those examined here include pinopsin

A.



B.

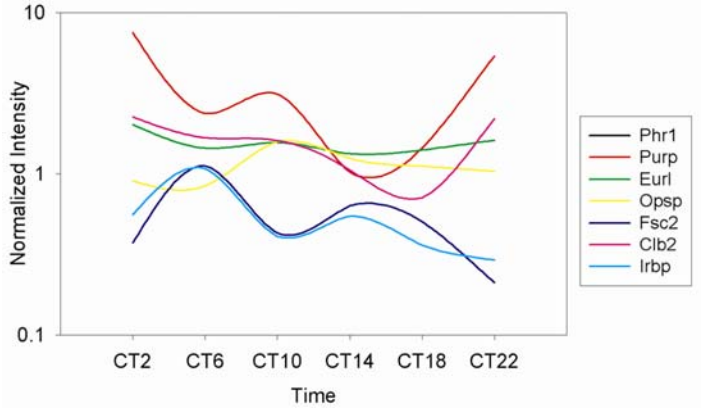


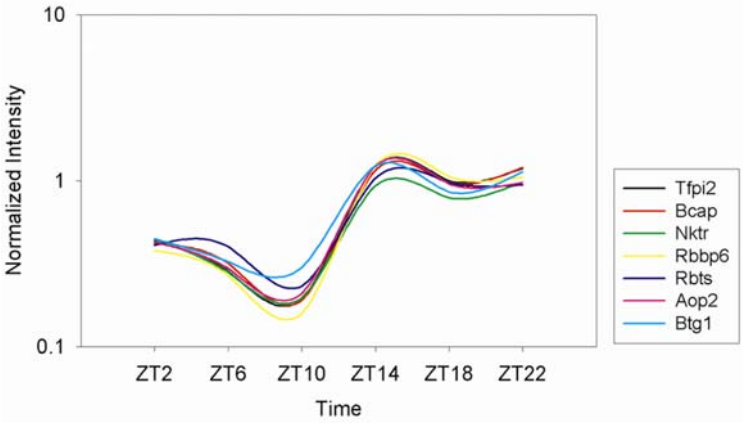
Figure 7. Identification of photoreceptive/phototransduction elements oscillating in the chick pineal. **A.** Photoreceptive/phototransduction pathway elements shown include pinopsin (*Opsp*), fascin 2 (*Fsc2*), plekstrin homology domain receptor 1 (*Phr1*), interphotoreceptor retinoid-binding protein (*Irbp*), calretinin (*Clb2*), early-undifferentiated retina and lens gene (*Eurl*), and chick purpurin (*Purp*). **B.** These transcripts were also examined under conditions of constant darkness.

(*Opsp*) (Okano et al., 1994), fascin2 (*Fsc2*) (Saishin et al., 1997), plekstrin homology domain receptor 1 (*Phr1*) (Xu et al., 1999), interphotoreceptor retinoid-binding protein (*Irbp*) (Yan and Wang 2000), calretinin (*Clb2*) (Rogers 1987), early-undifferentiated retina and lens gene (*Eurl),* Acc. #AF162861, and chick purpurin (*Purp*) (Berman et al., 1987). Additional potential elements of phototransduction examined but not significantly rhythmic under our criteria include transducin gamma subunit (Akhmedov et al., 1998), photoreceptor outer segment all-trans retinol dehydrogenase (Rattner et al., 2000), retinaldehyde-binding protein (CRALBP) (Crabb et al., 1991), and retinal short-chain dehydrogenase/reductase 2 (Haeseleer and Palczewski 2000). Two peropsin (Sun et al., 1997) clones were also examined, but each oscillated in antiphase to each other and was left out of further analysis.

Immune function

Included in the three cell types comprising the chick pineal gland (Korf 1994) are B-lymphocytes, which function in immune response (Cogburn and Glick 1981; 1983). We examined several components of immune function responses and determined all to be rhythmic under a light dark cycle and constant conditions (Figure 8A, 8B). These include tissue factor pathway inhibitor 2 (*Tfpi2*) (Hisaka et al., 2002), B-cell associated protein 2 (*Bcap2*) (Montano et al., 1999), natural killer tumor recognition factor (*Nktr*) (Anderson et al., 1993), retinoblastoma binding protein 6 (*Rbbp6*) (Witte and Scott 1997), retinoblastoma tumor suppressor (*Rbts*) (Wagner et al., 2001), antioxidant protein 2 (*Aop2*) (Fisher et al., 1999), and B-cell translocation gene 1 (*Btg1*)

A.



B.

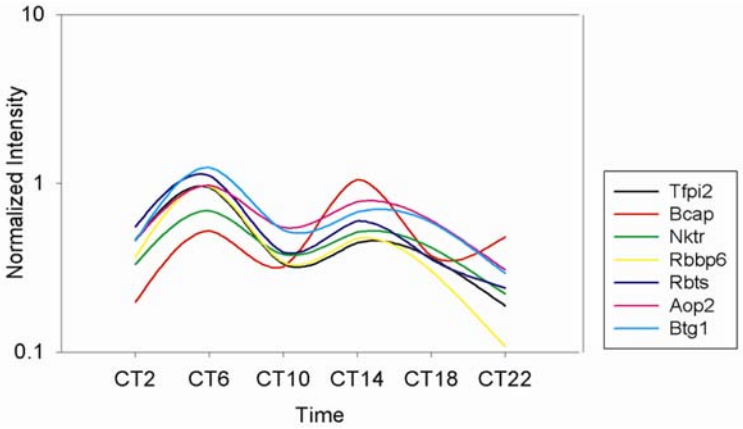


Figure 8. Identification of immune function responses oscillating in the chick pineal **A.** Tissue factor pathway inhibitor 2 (*Tfpi2*), B-cell associated protein 2(*Bcap2*), natural killer tumor recognition (*Nktr*), retinoblastoma binding protein 6 (*Rbbp6*), retinoblastoma tumor suppressor (*Rbts*), antioxidant protein 2 (*Aop2*), and B-cell translocation gene 1 (*Btg1*). **B.** These transcripts were also examined under conditions of constant darkness. They all exhibited peak mRNA accumulation at the light dark transition (ZT-12) and remained in similar phases under conditions of constant darkness.

Acc. # NM_173999. These genes all exhibited peak mRNA expression at the light dark transition (ZT-12) and remained in similar phases under conditions of constant darkness.

Protein synthesis and turnover

Many mRNAs encoding ribosomal proteins, proteins involved in protein synthesis and elongation and proteins involved in protein turnover, including those involved in ubiquitinylation are expressed on a daily and circadian basis. Presumably, some of these are involved in the biosynthesis of the enzymes that produce rhythmic melatonin, and others are involved in the proteosomal proteolysis of at least AANAT (Zatz et al., 2000; Iuvone et al., 2002; Klein et al., 2002). It is known, for example, AANAT is synthesized on a daily and circadian schedule such that *de novo* transcription and translation are required each circadian cycle. Further, it is known that proteosomal proteolysis is largely responsible for AANAT degradation at the end of the subjective night and in response to acute illumination. It is likely that these processes are similarly involved in the *de novo* biosynthesis of transthyretin, clock gene products, photopigments, and immune function proteins.

4. Discussion

It has generally been assumed that different organisms use their clocks to control species specific events; thus, similarities within output pathways in organisms from different kingdoms would not have been predicted *a priori*. The recent widespread use of microarrays to profile rhythmic genes in distinct species and tissues has demonstrated

the incredible diversity of clock regulated functions. As predicted, in overall outline there is very little overlap between cycling genes in different organisms and even among tissues; however several surprises with regards to conservation of clock-controlled genes and processes are beginning to emerge from the data. For example, comparisons of the clock-controlled transcriptome between chicks and the filamentous fungus *Neurospora crassa* reveals several genes that are regulated in both organisms (Table 3), including genes involved in protein synthesis, metabolism, and protein processing. The observation that several ribosomal protein-encoding genes are under clock control in these as well as other organisms confirms the significance of translational regulation in clock function. This influence may occur at both the levels of output genes and central clock components. Similarly, the role of ubiquitin-mediated protein turnover in clock function is well established (Naidoo et al., 1999). However genes involved in metabolism and other cellular functions that are common to both organisms, such as the putative senescence associated gene, provide important clues for determining key pathways that are regulated by clocks in diverse organisms.

The emerging picture is that the avian pineal gland orchestrates a large array of rhythmic transcriptional events. These, of course, include the mRNAs involved in melatonin biosynthesis, which is the only known function of the gland. The presence of rhythmic mRNAs of chick orthologs of clock genes previously identified in *Drosophila* and several mammalian species raises the real possibility that these genes and their products are either directly or indirectly involved in the generation of the circadian

Table 1.
Conserved clock elements

A. Gene ID	B. Peak amplitude in mRNA *N. crassa*	C. Peak amplitude in mRNA *G. domesticus*
ribosomal protein L15	midnight	midnight
ribosomal protein L4	midnight	midday
elongation factor1-a	midnight	midday
glyceraldehyde-3-phosphate dehydrogenase	dawn	midday
cytochrome c oxidse subunit	dawn	midnight
ubiquitin-ribosomal protein fusion	dusk	midday
putative senescence-associated protein	midnight	midday

Table 1. Comparison of chick and *Neurospora* conserved clock controlled elements.

Column A: Gene ID.

Column B: Peak of mRNA accumulation of gene for *Neurospora crassa*.

Column C: Peak of mRNA accumulation of gene for *Gallus domesticus*.

pattern of melatonin biosynthesis. However, more physiological research on this aspect of pineal function will be required to confirm this.

More surprising is the profoundly rhythmic expression of phototransduction sequences, protein biosynthetic sequences and mRNAs involved in stress responses, such as the heat shock proteins, and immune function. Since it has been shown that the chick pineal gland is a lymphopoietic organ (Cogburn and Glick 1981; 1983), it is likely that the local regulation of lymphoid activity translates into both daily and seasonal regulation of lymphatic and immune activity in this species. Several studies on rodent immune function have similarly pointed to daily and seasonal changes in B- and T-lymphocyte activity (Brock 1983; Planelles et al., 1994; Haldar and Singh 2001). These changes have been associated with melatonin's actions on these cells in the blood (Demas and Nelson 1998; Drazen et al., 2000; Drazen et al., 2001). The present data suggest that the clock directly regulates pineal lymphocytes, either through local melatonin action and/or by other paracrine signals.

In summary, the present study confirms and extends the dynamic regulation of melatonin biosynthetic and clock gene expression using a cDNA microarray technique, thereby validating our microarray as an accurate reflection of pineal activity. This microarray approach will therefore be a very useful tool in analysis of pineal clock function in the future. However, it raises the possibility that the clock(s) present in the avian pineal gland are responsible for more rhythmic processes than has been previously appreciated. These processes may serve as springboards for further physiological research on the holistic avian and vertebrate circadian organization.

CHAPTER III

TRANSCRIPTIONAL PROFILING OF CIRCADIAN PATTERNS OF mRNA EXPRESSION IN THE CHICK RETINA*

1. Introduction

Circadian biological rhythms are a pervasive property of most multicellular organisms, most eukaryotic microorganisms and at least some prokaryotic taxa. It has been known for some time that circadian rhythms are genetically determined (Pittendrigh 1993). Circadian traits such as free-running periods (τ) and phase-angle (Ψ) to entraining light cycles can be selected for, and mutations for these traits have been identified, leading to the isolation and characterization of molecular clock components (Young et al., 2001; Rosbash et al., 2003).

In vertebrates, circadian organization also relies on discrete neuroendocrine structures that drive and/or entrain downstream rhythms of biochemistry, physiology and behavior. These structures include the hypothalamic suprachiasmatic nucleus (SCN), the pineal gland and the retina. In mammals, the SCN serves as a master pacemaker, responsible for coordinating all downstream oscillators and rhythmic processes. In birds and many other non-mammalian vertebrates, however, the pineal

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gland, retina and the avian homologue of the SCN are equally important in regulating overt rhythmicity. The avian pineal gland and retina share several key processes: (1) both are directly photoreceptive, (2) both contain circadian oscillators, and (3) both synthesize and release the indoleamine neurohormone melatonin on a rhythmic basis. The retinal clock regulates many local processes including rod outer-segment disk shedding, cGMP channel sensitivity (Ko et al., 2001; 2003), electroretinogram (ERG) responses (McGoogan and Cassone 1999), melatonin and dopamine biosynthesis (Doyle et al., 2002), and clock gene regulation (Zhuang et al., 2000; Bailey et al., 2002; Haque et al., 2002; Chong et al., 2003). Several of these processes that are controlled by the retinal clock also persist *in vitro*, wherein retinas grown in culture continue to generate oscillations under constant conditions (Besharse and Iuvone 1983; Tosini and Menaker 1996; Chong et al,. 1998; Tosini and Mennaker 1998).

At the systems level, the retina functions integrally with the pineal gland and the SCN, such that these oscillatory structures feedback and influence each other (Underwood and Siopes 1984; Underwood et al., 1988; Yamazaki et al., 2002; Beaule and Amir 2003). Under several experimental circumstances loss of the retinal clock in turn disrupts and/or abolishes the organism's clock or output processes (Cassone and Menaker 1984; Stokkan et al., 2001; Reppert and Weaver 2002; Dudley et al., 2003). The retina also is responsible for integrating and relaying photic information to the SCN via retinal ganglion cells (RGC) and the retinohypothalamic tract (RHT). This tract serves as the major pathway for entrainment of the SCN in mammals (Cassone et al 1988; Moore et al., 2002), and has been an area of great focus in the search for the

photopigment(s) existing in RGCs that serve as the entraining photoreceptor of the system.

Our previous transcriptome analyses (Bailey et al., 2003) identified candidate molecular components of the avian pineal clock. Here, we determine candidate retinal clock transcripts under light dark and constant darkness conditions to better understand the molecular organization of the avian system. Subsequently, a comparative transcriptome analysis of the two data sets determined candidate molecular machinery regulating the circadian system of chicks within both pacemakers and circadian oscillators.

2. Methods and Materials

Animals. Chicks were obtained from Hyline International (Bryan, TX.) and housed under a lighting schedule of 12 hours of light and 12 hours of darkness (LD 12:12) for 7 days with food and water continuously available. For microarray hybridizations chicks (N=3/timepoint/experiment) were sacrificed by decapitation and their retinas dissected and pooled. Two separate retinal RNA sampling cycles were performed for array hybridizations. All DD dissections were performed using an infrared viewer. Animals were treated in accordance with PHS guidelines; these procedures have been approved by the Texas A&M University Laboratory Animal Care Committee (AUP #2001-163).

Microarray Production and Hybridization. The cDNA microarrays were prepared as previously described (Bailey et al., 2003) based upon approximately 4,500

PCR products from each of the two pineal cDNA libraries constructed, for a total of ~9,000 ESTs. Briefly, the cDNA in plasmids from each library were PCR amplified using flanking primers (SK and T7), purified by ethanol precipitation using ammonium acetate, and placed in the wells of 96 well plates at a concentration of 50 ug/ml in 3X SSC. A GeneMachines OmniGrid microarrayer equipped with 8 Telechem SMP3 pins was used to spot the samples onto poly-L-lysine coated slides (CEL Associates) in duplicate. Slides were stored desiccated at room temperature until their subsequent use. Prior to hybridization, the dried spots were hydrated gently over a steaming water bath, snap dried, and the DNA was UV cross-linked using a Stratalinker (Stratagene).

Total RNA was harvested using Trizol Reagent (Gibco-Brl) every four hours for one day in a light dark cycle of 12 hours light and 12 hours darkness (LD12:12; lights on 0600 CST; lights off 1800 CST) and for one day in continuous darkness (DD). Times of sampling began two hours after lights on (Zeitgeber Time, ZT2) and continued every four hours hence (ZT6, ZT10, ZT14, ZT18, ZT22). When lights would have normally turned on, the timer was disabled, and birds were placed in DD. They were then collected again every 4 hours, and designated Circadian Time (CT) CT2, CT6, CT10, CT14, CT18 and CT22. This sampling procedure was performed on two separate occasions. A total of 4 experimental microarray hybridizations were conducted for each time point, two from each respective biological sampling procedure. The total RNA samples were then amplified to produce aRNA using a MessageAmp Kit (Ambion). Randomly primed fluorescent probes were produced from aRNA samples using a Genisphere 3DNA Array 900MPX expression array detection kit. The fluorescent dye

on probes derived from the experimental aRNA was Cy5, while the dye on control probes was Cy3. In all experiments, the control sample was derived from retina harvested at midnight (ZT18) under LD conditions. Hybridizations and washes were conducted as suggested by Genisphere. The labeled arrays were scanned in an Affymetrix 428 array scanner. The images obtained were subsequently analyzed by GenePixPro software (Axon Instruments).

Data Analysis. Data files were then further analyzed by GeneSpring 6.1 (Silicon Genetics). GeneSpring allows the data to be normalized in a variety of ways, allows the assignment of parameters and interpretations, and then allows the data to be filtered to determine differential expression. The current analysis used intensity dependent LOWESS normalization. Intensity dependent normalization is just one technique used to eliminate dye-related artifacts in two-color experiments such as this. At each time point, the results for each gene were reported as an average obtained from 4 slides. The data are reported as the normalized ratio of Cy5 (experimental) to Cy3 (control at ZT18). Thus, the data reported for each time point are relative to the abundance of the same gene at midnight (ZT18).

The criteria used to define rhythmic genes were those transcripts with a 2.0 fold or higher amplitude change in mRNA levels over a 24-hour period relative to the genes' respective mRNA abundance level at ZT18, in addition to exhibiting this oscillation in 3 out of 4 hybridizations performed. Analysis at the 1.5 fold level was also performed but not to the extent of examination as the 2.0 folds data set. Those gene sets that were determined to achieve the 1.5 and 2.0 fold criterions were analyzed statistically by one

way ANOVA, with $p<0.05$ considered significant. Only statistically significant changes will be reported here. However, the entire dataset is available on our website (http://www.tamu.edu/clocks/index.html).

3. Results

Transcriptome Analysis

Of the approximately 9,000 cDNAs represented on the microarray, 546 classified transcripts oscillated with 1.5 fold amplitude, and 175 classified transcripts oscillated with 2-fold or greater amplitude in a light dark cycle. Upon examination of these transcripts under conditions of constant darkness, the number of oscillating transcripts observed was reduced to 383 at 1.5 fold and to only 49 at 2.0 fold or better (http://www.tamu.edu/clocks/index.html). This percentage of rhythmic transcripts is similar to those found in the chick pineal gland (Bailey et al., 2003) and other experimental analyses of rhythmic processes using cDNA and/or oligonucleotide arrays (Harmer et al., 2000; McDonald and Rosbash 2001; Akhtar et al., 2002; Duffield et al., 2002). We restricted our most detailed analyses to those transcripts that varied by 2.0 fold or more.

Functional categories of rhythmically expressed transcripts

Oscillating retina transcripts in LD and DD were classified according to proposed function. Components of the melatonin generating enzymatic cascade were represented, as were many chick orthologs associated with mammalian circadian clock function.

A. B.

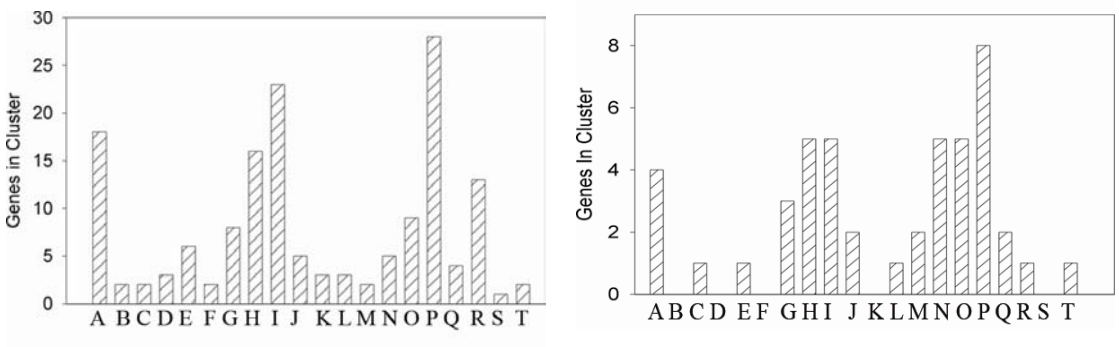


Figure 9. Functional classification of rhythmic retinal transcripts. A. Rhythmically expressed transcripts under LD conditions were classified according to proposed function. This revealed oscillating transcripts in several broad categories including elements of melatonin biosynthesis in addition to chick orthologs of mammalian clock genes. Metabolism=A, Development=B, Melatonin Biosynthesis=C, Neuronal Associated=D, Disease Related=E, Hormones/Growth Factors=F, Cell Cycle/Cell Death=G, Carrier Proteins/Transport/Circulation=H, Cytoskeletal/Microtubule-Associated=I, Cell Signaling=J, Cell Adhesion=K, RNA Synthesis/Stability=L, Circadian Clock=M, Phototransductive Elements=N, Ribosomal Proteins/Translation=O, Stress Response/Host Defense/Chaperone=P, Transcription Factors=Q, Protein Modification=R, Receptors=S, DNA Synthesis/Replication/Binding=T, Miscellaneous Function=U. The number of rhythmic transcripts in each proposed functional category name is also given in the supplemental tables. B. This functional categorization method was also applied to transcripts oscillating in DD.

Further, functional analysis revealed transcripts in several broad categories that were also rhythmically expressed. The stress response/chaperones and cytoskeletal classes under LD conditions had the highest number of oscillating transcripts with 28 and 23 respectively. In DD the stress response/chaperones again had the largest classification with 8, followed by the cytoskeletal, carriers, phototransduction, and ribosomal clusters with 5 members in each. These functional classifications are indicated in Figure 9A and 9B.

Circadian phase analysis

Initial analysis of daily and circadian phase relationships revealed rhythmic transcripts that exhibited a diverse set of phases in the LD cycle. However, retinal transcripts rhythmically expressed in DD either accumulated primarily during the day or peaked during the night. These were clustered using a K-means clustering algorithm (GeneSpring), according to phase of mRNA abundance. The clustering analysis revealed 3 primary clusters of peaking transcript abundance at CT22-2, CT6, and CT14 (Figure 10). For each cluster a representative trace is shown indicating the average profile of all oscillating transcripts in each respective cluster: Cluster 1, which comprised 20% of the unique DD transcript set, is indicative of transcripts peaking at approximately the onset of subjective dawn CT-22-CT-2, Cluster 2, 49% of the unique DD transcripts, at mid subjective day CT-6, and Cluster 3, 31% of the unique DD transcripts, at early subjective night CT-14. This distribution of phases is similar to previous findings of

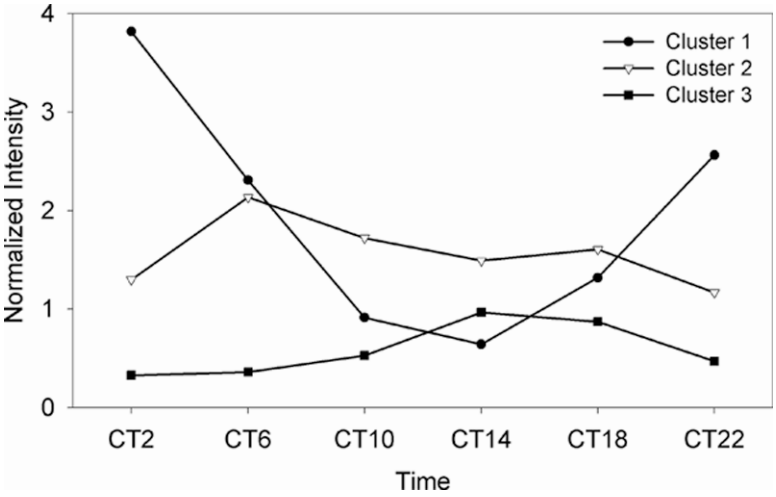


Figure 10. Phase cluster analysis of rhythmic retina transcripts in DD

Transcripts rhythmically expressed under conditions of constant darkness were clustered using a K-means clustering algorithm (GeneSpring), according to phase of mRNA abundance. For each cluster a representative trace is shown indicating the average profile off all oscillating transcripts in each respective cluster. Cluster 1 is indicative of transcripts peaking at onset of subjective dawn CT-22/2, cluster 2 at mid subjective day CT-6, cluster 3 at early subjective night CT-14.

other groups (Harmer et al., 2000; McDonald and Rosbash 2001; Akhtar et al., 2002; Duffield et al., 2002).

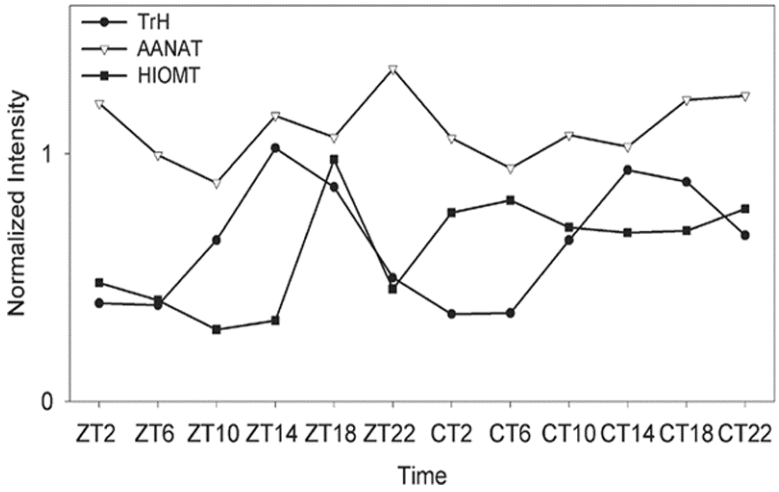
Melatonin biosynthesis

As has been previously shown, the mRNAs encoding enzymes involved in melatonin biosynthesis were rhythmically expressed on a daily and circadian basis. Under LD conditions the data indicated a rhythm in TrH mRNA such that levels were highest in the early night (ZT14) and HIOMT was highest at mid-night (ZT18). AANAT mRNA exhibited a low amplitude rhythm that was highest during the night as well (Figure 11A). These patterns corresponded favorably with northern blot data employing radioactive probes derived from the microarray cDNAs corresponding to each respective gene (Figure 11B). The rhythm in TrH persisted in DD with similar phase angles, while HIOMT and AANAT mRNA were not significantly rhythmic in DD at the 2.0 fold criterion (Figure 11C).

Clock genes

Several mRNAs encoding putative orthologs of genes associated with circadian clock function were identified in the dataset. These include putative negative elements cryptochrome 1 (c*ry1*), cryptochrome 2 (*cry2*), period 2 (*per2*) and period 3 (*per3*) (Figure 12A). Under both LD and DD conditions *cry1* and *per3* exhibited rhythmic mRNA profiles, such that peak *cry1* mRNA levels occurred during the midday (ZT6) in LD and mid-subjective day (CT6) in DD, and peak *per3* mRNA levels occurred during

A.



B.

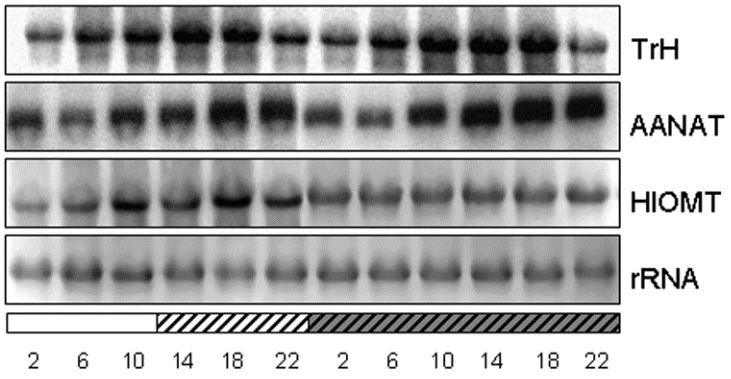


Figure 11. Examination of the melatonin biosynthetic pathway in the chick retina **A.** LD conditions on the microarray indicated a robust rhythm in TrH and HIOMT mRNA such that transcript abundances were highest in the early night. AANAT mRNA was highest during the late night although with a lower amplitude. The rhythm in TrH, AANAT persisted in DD with similar phase angles, while HIOMT mRNA was not significantly rhythmic in DD. **B.** These patterns corresponded to northern blot data for each respective gene.

the late (ZT22) night and subjective night (CT22). Although neither *cry2* nor *per2* mRNA levels were rhythmic in LD, *per2* mRNA levels expressed a modest increase at CT10 in DD. These profiles and their phases were confirmed by northern blot analyses (Figure 12B). The putative positive elements *clock*, *bmal1*, and *bmal2*, as well as casein kinase 1 epsilon (*ck1E)* were examined. Analysis of the putative positive elements yielded a low amplitude rhythm in LD for *bmal1* and *bmal2*, peaking in the early night, ZT14, while *clock* was not rhythmically expressed. In addition no rhythm in *ck1E* mRNA abundance was observed. In DD, *clock*, *bmal1*, and *bmal2* were rhythmically expressed, reaching peak mRNA abundance at CT10-14 (Figure 12C).

Retinal and phototransduction transcripts

Several components of the photoreceptive/phototransduction/visual cycle pathway were examined on the microarray, yielding many rhythmic profiles under LD and DD (Figure 13). Since the microarray was produced from cDNA libraries derived from chick pineal gland, it is important to point out here that retina-specific sequences (genes expressed in the retina but not the pineal gland), such as rhodopsin, are not represented here. Nonetheless, several genes associated with visual transduction were analyzed including melanopsin (*Opn4*) (Provencio et al., 1998), rod gamma subunit phosphodiesterase 6 (*Pde6*) (Morin et al., 2001), as well as opsins and binding proteins associated with visual cycle function, such as peropsin (*Rrh*) (Sun et al., 1997), Retinal G-protein coupled receptor opsin (RGR-Opsin) (Jiang et al. 1993), and retinal pigmented epithelium 65 (*Rpe65*) (Hinterhuber et al., 2004). The mRNA for all of these genes was expressed

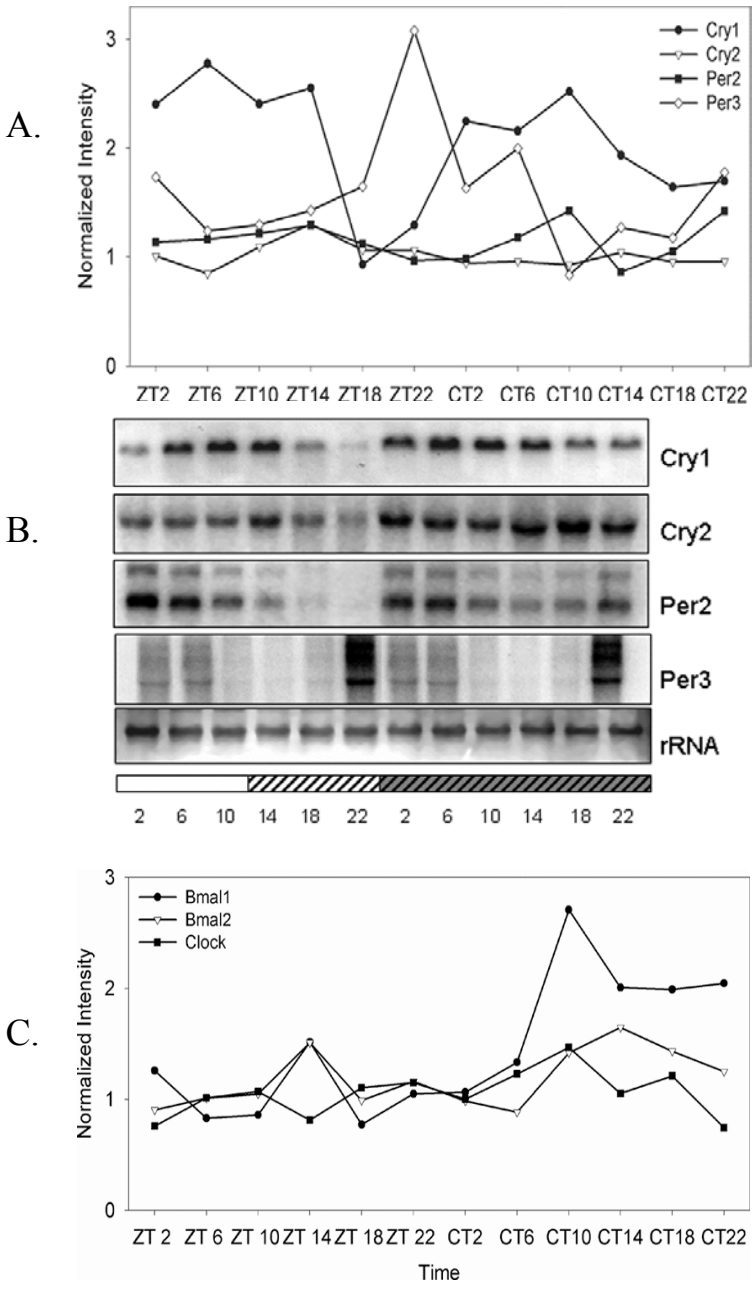


Figure 12. Analysis of chick retina clock genes **A.** Orthologs of the mammalian negative element clock genes were examined on the microarray under LD and DD conditions. **B.** Northern blot analyses confirming microarray results. **C.** Microarray analysis of the putative positive elements was also performed.

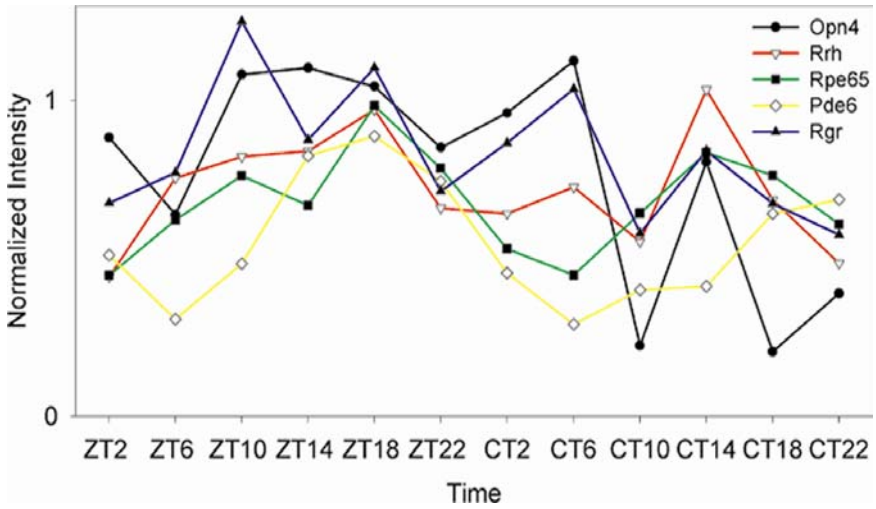


Figure 13. Microarray analysis of photoreceptive/phototransduction elements in the retina. Several photoreceptive/phototransduction pathway elements were examined including melanopsin (*Opn4*), peropsin (*Rrh*), retinal pigmented epithelium 65 (*Rpe65*), Retinal G-protein coupled receptor opsin (*RGR-Opsin*), and rod gamma subunit phosphodiesterase 6 (*Pde6*).

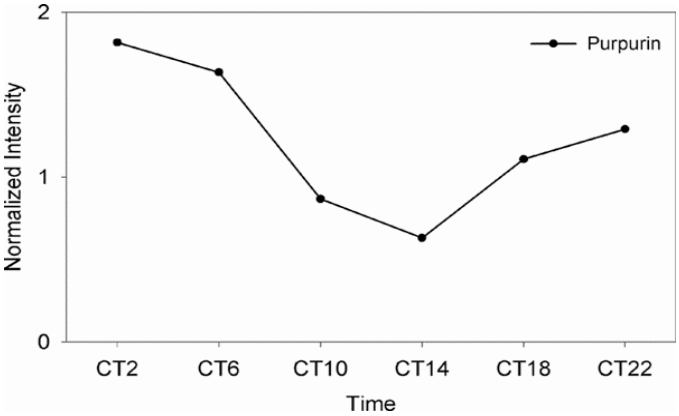
rhythmically under both LD and DD conditions with the exception of melanopsin. Although melanopsin was expressed predominantly in the late day through night in LD, there was no apparent circadian pattern of expression in DD. Additional potential elements of phototransduction and visual cycle function that were examined but found to be not significantly rhythmic under our criteria include transducin gamma subunit Akhmedov et al., 1998), photoreceptor outer segment all-trans retinol dehydrogenase (Rattner et al., 2000), cellular retinaldehyde-binding protein (CRALBP) (Crabb et al., 1991), and retinal short-chain dehydrogenase/reductase 2 (Haeseleer and Palczewski 2000).

Retinal transport

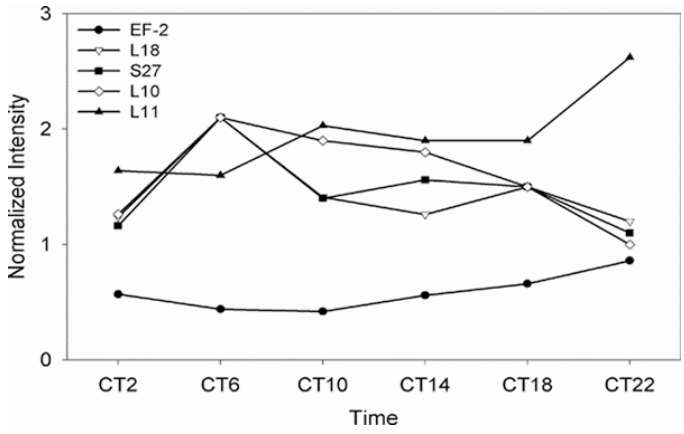
The vertebrate visual cycle process involves at least three different retinal cell-types, the photoreceptors, pigmented epithelial cells and Muller glia. Thus, the problem of transported retinaldehyde, and by-products from one compartment to another and from the periphery into the retina is likely solved by transport proteins. One of these is purpurin (Berman et al., 1987), whose mRNA is rhythmically expressed in the retina. Purpurin was expressed highly during the early day and late night in LD and early subjective day and late subjective night in DD (Figure 14A).

Another retinal transport molecule is transthyretin which was found to be rhythmically expressed in the pineal gland (Bailey et al., 2003), was not consistently rhythmic in the retina under constant conditions.

A.



B.



C.

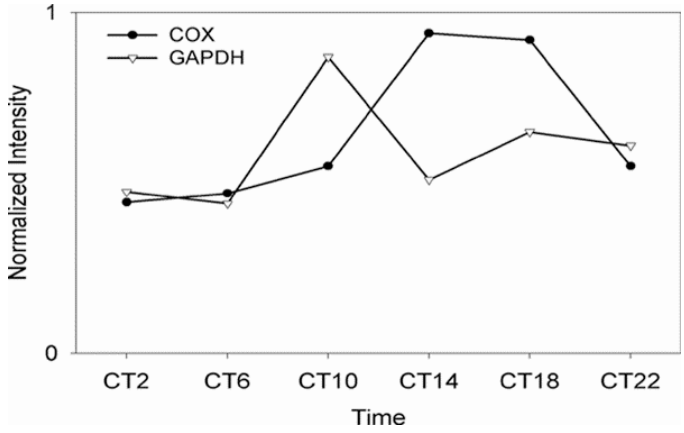


Figure 14. Microarray analysis of select functional groups in the retina

A. Retinal transport- Purpurin mRNA is rhythmically expressed in the retina with peak accumulation during the late night in LD and late subjective night in DD

B. Ribosomal Proteins- S27, L10, and L18, in addition to chick elongation factor 2 (EF-2) and L11 were expressed rhythmically. **C.** Intermediary Metabolism- GAPDH and cytochrome c oxidase subunit II were also expressed rhythmically.

Translational control and protein trafficking/modification

A number of ribosomal and ribosome-associated proteins were rhythmically expressed in both LD and DD. The 40S ribosomal proteins S3, S5, S18 and S27 as well as 60S ribosomal proteins L10, and L18 were expressed predominantly during the day with a 2.0 fold rhythm. Of these, S27, L10, and L18 were expressed rhythmically with a 2.0 fold or higher amplitude such that expression was highest during subjective day in DD (Figure 14B). In addition chick elongation factor 2 (EF-2) and ribosomal protein L11 were expressed rhythmically in both LD and DD such that mRNA was highest in late day in LD and late subjective night in DD.

A large number of mRNA species associated with proteolytic proteosomal activity were rhythmically expressed in LD. These included several ubiquitin sequences as well as sequences associated with ubiquitin modification and several protein phosphatases and carboxypeptidase M. Of these, only carboxypeptidase M persisted in its rhythmicity in DD at the 2.0-fold criterion, peaking at CT14 in the early subjective night. However, most of the ubiquitin-associated sequences persisted in DD at the 1.5-fold criterion, peaking during the subjective day.

Intermediary metabolism

Many mRNA species associated with glycolytic, lactate fermentation and electron transport processes were expressed rhythmically in LD at the 2.0-fold criterion. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphoglucose isomerase and lactate dehydrogenase (LDH) were all expressed predominantly in the mid to late day

(ZT6-10). Additionally, cytochrome b and NADH dehydrogenase subunit 4 were expressed highly at midday (ZT-6). ATP synthase beta subunit, ATP synthase H+ transporter, and NADH dehydrogenase (ubiquinone) 1 alpha were expressed predominantly at early day (ZT2) and cytochrome c oxidase subunit II was expressed at early night (ZT14). In DD several of these oscillations failed to persist although GAPDH and cytochrome c oxidase subunit II were expressed rhythmically at the 2.0 fold criterion, with the former expressed in the late subjective day and the latter expressed in the early to late subjective night (Figure 14C). However, most of the electron transport system genes were expressed rhythmically in DD at the 1.5-fold criterion.

Stress response/host defense/chaperone

Many mRNA species associated with stress response were found to be rhythmic in LD cycles. These included cathepsin and cystatin, cytochrome p450, the aryl hydrocarbon receptor, several heat shock proteins, and several genes associated with histocompatibility. Of these, cytochrome P450, 3 heat shock proteins (HSP8, HSP 90 and a 70kD cognate heat shock protein ATPase), 3 antigen identification genes (beta2 microglobulin, BM88 antigen, MHC class II associated invariant chain proteins) and the aryl hydrocarbon receptor persisted in DD at the 2.0 fold criterion.

4. Discussion

Both the retina and pineal gland have been identified as circadian pacemakers in the avian circadian system. Of course, both are photoreceptive structures, containing both common and tissue-specific photopigment and phototransductive cascades. For example, both tissues express melanopsin, peropsin, RGR-opsin, cryptochromes, transducins, and cGMP phosphodiesterase, among other transducins, and cGMP phosphodiesterase, among other phototransductive sequences. Further, photoreceptor cells in both tissues rhythmically synthesize and, at least in some species, release the indoleamine hormone melatonin, such that melatonin is synthesized during the night in LD cycles, and during the subjective night in DD in both tissues. This circadian secretion of melatonin accounts for most, if not all, of the pineal and retina's system level functions. However the relative roles of the pineal and retina clock vary among avian species. For example, in oscine passerine birds such as the house sparrow, *Passer domesticus*, pinealectomy abolishes circadian patterns of locomotor activity and of physiological functions, such as body temperature (Gaston and Menaker 1968; Binkley et al., 1971), while complete enucleation has little effect on either entrainment or circadian patterns of activity (Menaker 1972). Conversely, pinealectomy of juvenile and adult domestic fowl has little effect on overt behavioral rhythms of activity and feeding (Mcgoogan and Cassone 1999), while enucleation abolishes locomotor rhythms (Nyce and Binkley 1977). Even so, although pinealectomy has no effect on overt behavioral rhythms, the surgery abolishes circadian patterns of electroretinogram (ERG) a- and b-wave function (Mcgoogan and Cassone 1999), and administration of exogenous

melatonin affects both ERG and visually evoked potentials in chicks and pigeons, *Columba livia* (Lu et al., 1995; Mcgoogan and Cassone 1999; Wu et al., 2000). In some species, the retina and pineal contribute to overt rhythms equally. Neither pinealectomy nor enucleation alone abolishes locomotor rhythms in Japanese quail, *Coturnix japonica*, and pigeons, but the two surgeries combined abolish behavioral rhythms in both species (Ebihara et al., 1984; Underwood and Siopes 1984). Thus, both the pineal gland and retina are photoreceptive, circadian oscillators that affect behavioral and physiological rhythms via the rhythmic production of melatonin. In chicks, therefore, they represent two sets of circadian pacemakers that reside in the same organism, which one would expect to employ identical molecular mechanisms to generate and regulate their rhythmic functions.

As stated above, melatonin production is localized in photoreceptor cells in both tissues (Zawilska and Iuvone 1992; Cahill and Besharse 1993; Bernard et al., 1997; Niki et al., 1998), and its biosynthesis is reasonably well understood (Underwood et al., 1988; Iuvone et al., 1997; Tosini and Menaker 1998; Haque et al., 2003). However, the specifics of the molecular regulation of rhythmic melatonin biosynthesis vary among species (Cassone 1998) and between tissues in the same species. In the chick pineal gland, TrH and AANAT mRNA are expressed rhythmically, such that levels are high during the night, and low during the day in LD, while HIOMT mRNA is expressed higher during the day than during the night. All three of these rhythms persist in DD (Bernard et al., 1997; Bailey et al., 2003). In contrast, in the retina, TrH, AA-NAT, and HIOMT mRNA are regulated on a daily basis such that TrH and HIOMT are high at

early to late night and AANAT is high at mid-night (Bernard et al., 1997; Iuvone et al., 1997). In DD, HIOMT becomes arrhythmic while TrH and AANAT mRNA continue to accumulate with similar phases as in the LD cycle, albeit with a lower amplitude (Bernard et al., 1997; Tosini and Menaker 1998). These patterns of expression are confirmed in the present study in both the microarray data and in northern blot analyses of the same mRNA extracts (Figure 11A, 11B), demonstrating the validity of this microarray approach and punctuating the observation that, at the mRNA level, melatonin biosynthesis is regulated differentially in the chick pineal and retina.

The mechanism by which rhythmic melatonin biosynthesis is regulated has been assumed to include the transcription, translation and post-translational activation of chick orthologs of mammalian clock genes. Many of these orthologs have been identified and characterized, at least within the pineal gland (Okano et al., 2001; Natesan et al., 2002; Bailey et al., 2003; Chong et al., 2003; Haque et al., 2003;). These include putative negative elements *per2, per3, cry1,* and *cry2,* and positive elements *clock, bmal1,* and *bmal2* as well as *ck1e.* Further, heterologous expression of positive element *clock* in COS7 cells has been shown to activate the chick AANAT expression and enzyme activity (Chong et al., 2000). In the present study, retinal *bmal1* and *bmal2* are expressed rhythmically in LD and DD such that expression peaks at early night (ZT14/CT14), while *clock* does not appear rhythmically expressed. Of the putative negative elements in this tissue, only *per3* and *cry1* are expressed rhythmically, such that *per3* is expressed predominantly in the late night/early day (ZT22-2) and late subjective night to early subjective day (CT22-6), while *cry1* is expressed throughout

the day in LD and subjective day in DD (Figure 12A). The amplitudes of these rhythms are 2-3 fold. None of the other orthologs negative elements were found to be rhythmic, including *per2* and *cry2,* nor is *ck1e* expressed rhythmically.

The temporal distributions of the mRNA for these clock proteins are interesting in several respects. First, there is no strict antiphase of positive and negative elements that has been observed in *Drosophila* and mammalian models. Here, both *bmal*s are expressed coincidentally with *cry1*, and, of the rhythmic negative elements, *cry1* and *per3* are 180^{o} in antiphase. This temporal pattern of clock gene mRNA abundance is similar, but not identical, to that found in the pineal gland (Bailey et al., 2003). In the pineal gland, *bmal1, bmal2, cry1, cry2, per2* and *per3* are all expressed rhythmically with high amplitudes, and *clock* is expressed rhythmically in LD and DD with a lower amplitude of 1.5 fold, all of which were confirmed by northern analysis. The exception is the temporal distribution of retinal *cry2*, which is expressed bimodally in the pineal gland with peak mRNA values primarily during the late subjective day (CT10) and late subjective night (CT22). In the retina, *cry2* is not rhythmically expressed. Of course, we do not, at this stage, have any data concerning either the level or sub-cellular localization of the proteins that these genes encode, but the similarities in temporal distribution between the chick retina and pineal gland, and the differences between the temporal distributions of these mRNA species in these chick tissues and those of mammals and flies strongly suggest that other rhythmic mechanisms are in place that regulate molecular rhythms among these model systems. Further, if clock gene expression regulates melatonin biosynthetic rhythms in these two tissues, one would

predict that the downstream processes between clock function and its melatonin output differ between retina and pineal gland.

One might therefore ask, what are the other common elements between rhythmic retinal mRNA species in the present study and pineal rhythms in our previous work? First, in both tissues, mRNA encoding phototransduction and retinaldehyde transport are rhythmically expressed. This is not surprising, since both tissues are photoreceptive. Secondly, in both tissues, GAPDH and cytochrome c oxidase are expressed rhythmically such that GAPDH is expressed predominantly at CT10 and cytochrome oxidase is expressed highly from CT14-18. Thirdly, several ribosomal proteins, and elongation factor 2 are expressed rhythmically in both tissues, suggesting protein synthesis and modification may play a role in regulating circadian function in these two tissues.

Several recent studies have pointed to rhythmic processes involving metabolic activity that are more or less independent of clock gene function. First, induction of clock gene rhythms does not necessarily induce metabolic rhythmicity in cultured cells (Allen et al., 2001). We have found that induction of robust clock gene rhythms in fibroblast lines by serum shock has no effect on glucose utilization in these cells, even though rhythms of glucose utilization are accompanied by clock gene expression rhythms in SCN cells. Secondly, induction of metabolic rhythms in cultured astrocytes does not necessarily result in rhythmic clock gene expression, since glucose utilization rhythms can be induced in chick astrocytes with cycles of melatonin (Adachi et al., 2002) but clock genes are not responsive to the hormone (Peters et al., 2003). Recent studies by McKnight and others have shown that redox state in *in vitro* systems can

determine the affinity of negative elements such as the cryptochromes for positive element activation of clock gene promoter regions (Rutter et al., 2002). Both GAPDH and cytochrome oxidase have the potential of altering redox state via the reduction of NAD+. Thus, it is possible that the rhythms in components of intermediary metabolic pathways we observe here in the retina and in the pineal gland elsewhere (Bailey et al., 2003) may underlie a semi-independent oscillatory mechanism. Current research in our laboratory is focused on this issue.

It is interesting that several mRNA species involved in regulation of translation and protein trafficking are regulated on a circadian basis in the chick retina. Both 40S and 60S ribosomal proteins are regulated at the mRNA levels in this tissue in LD and DD, as are several sequences associated with proteosomal proteolysis, suggesting a global regulation of many processes by the clock through the regulation of the translational machinery. This is a feature that is shared with the pineal gland as well (Bailey et al., 2003). While many researchers in the field have focused on rhythmic regulation of mRNA as a central mechanism in biological clock function (including the present study), translational control of clock components and their outputs that is independent of transcriptional regulation have been described in many model systems, including vertebrates. For example, in the filamentous fungus *Neurospora crassa*, circadian regulation of the clock gene *frq* and its protein FRQ can be separated by entrainment to long and short photoperiods, suggesting independent regulation of these two components (Tan et al., 2004). In *Gonyaulax*, circadian regulation of GAPDH levels and activity, a common clock-regulated protein (Morre et al., 2002), appears to be

independent of mRNA levels (Fagan et al., 1999), and, while AANAT activity is rhythmically regulated in the sheep pineal gland, no rhythm in mRNA levels can be determined (Coon et al., 1999). This post-transcriptional regulation of key clock components and their outputs may be the result of circadian regulation of either rhythmic protein synthesis and/or proteolysis. Whether this post-transcriptional regulation is globally regulated or contains features that specifically target some protein regulation is not known at this stage.

In summary, the chick retina, an important component of the biological clock in birds, shares with the pineal gland many rhythmic mRNA species. These include components of the melatonin biosynthetic pathway as well as several "clock genes". However, the details of their regulation are both similar and different, raising the reasonable possibility that the "clocks" in each of these tissues in the same organism are regulated differentially. Among the common elements between the rhythmic transcriptome in these tissues are key components of intermediary metabolism and protein synthesis and degradation, which should be considered candidates for clock regulation and its output.

CHAPTER IV

OPSIN PHOTOISOMERASES IN THE CHICK RETINA AND PINEAL GLAND: CHARACTERIZATION, LOCALIZATION AND CIRCADIAN REGULATION*

1. Introduction

Several opsin photopigments have been identified and described in cells and tissues beyond the traditionally accepted retinal photoreceptors, the rods and cones, in several vertebrate species (Foster and Hankins 2002; Berson 2003; Van Gelder 2003). These tissues include the pineal gland, parapineal structures and deep tissues in the hypothalamus but also within the inner nuclear (INL) and retinal ganglion cell (RGL) layers of the retina itself. Sequence comparisons suggest there are several classes of vertebrate opsins. The photosensory classes include the rod and cone opsins, a vertebrate ancient opsin class (Soni and Foster 1994) and a pinopsin class (Okano et al., 1994). The other classes include exo-rhodopsin, (Mano et al., 1999) parapinopsin, (Blackshaw and Snyder 1997) tmt-opsin, (Moutsaki et al., 2003) RGR-opsin, (Jiang et al., 1993) melanopsin, (Provencio et al., 1998) peropsin, (Sun et al., 1997) and encephalopsin (Blackshaw and Snyder 1999). Characteristically, all of these photopigments and their related retinal proteins are 35–55 kDa membrane proteins that consist of a single opsin

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and its retinaldehyde chromophore. The chromophore is bound to a lysine residue located in the seventh helix of the opsin through a protonated Schiff base linkage (Bownds 1967). Additionally, vertebrate opsins contain seven transmembrane-helical domains connected by three extracellular and three cytoplasmic loops. These domains are the primary motifs of G protein-coupled receptor (GPCR) superfamily (Wald 1968).

The precise functions of the non-visual opsins are not completely understood. Several of these that have been isolated in pineal tissue (Okano et al., 1994; Max et al., 1995; Blackshaw and Snyder 1999), have been implicated in photic regulation of the pineal hormone melatonin (Klein et al., 1999) by virtue of the fact that they are expressed in photoreceptive pinealocytes (Foster and Hankins 2002) Recently, melanopsin expression in the RGL of mammals has been implicated in circadian photoentrainment and in non-image forming responses such as pupillary responses to bright light (Berson 2003; Panda et al., 2003; Rollag et al., 2003).

In addition to photosensory opsins, several opsin-based molecules have been identified as photoisomerases, which catalyze the regeneration of photosensory opsins by converting the all-*trans*-retinal to produce the chromophore 11-*cis* retinal in the process known as the "visual cycle" (Wald 1968). Of these, RGR-opsin has been found in retinal pigmented epithelium (RPE) and glial Mueller cells (Jiang et al., 1993), and is capable of functioning as a photoisomerase generating 11-*cis*-retinal in the retinal pigmented epithelium (Hao and Fong 1999). Peropsin (*Rrh*) was originally identified in human RPE (Sun et al., 1997) and likely functions as a photoisomerase as well (Koyanagi et al., 2002).

Recently, as part of an extensive functional genomics analysis of the chick pineal gland (Bailey et al., 2003) we confirmed the rhythmic regulation of mRNAs involved in melatonin biosynthesis, confirming the microarrays' validity, and demonstrated circadian variation of mRNAs corresponding to a wide variety of other processes, including orthologs of mammalian "clock genes", sequences associated with immune function and photoreception. Among the sequences associated with photoreception, genes that encode two opsin-like photopigments, RGR-opsin and peropsin, stood out because these proteins have been primarily associated with the RPE, which is typically not found in the pineal gland (Jiang et al., 1993; Sun et al., 1997).

Since the photopigment(s) and its photoisomerase(s) required for photic entrainment are unknown, we have investigated these two classes of opsins within the pineal gland and retina of chicks. In this study chick orthologs of mammalian RGR-opsin and peropsin were isolated and characterized in the pineal gland and retina. The mRNA of both opsins is expressed in cells that have been associated with both melatonin biosynthesis and circadian entrainment. Further, both are regulated on a circadian basis. Whether these function as photosensors and/or photoisomerases is discussed in the context of their distribution and sequence analysis.

2. Methods and Materials

Animals. White leghorn cockerels were obtained from Hy-Line International (Bryan, TX) and maintained for three weeks in a light:dark (LD) cycle of 12 h:12 h [lights on Zeitgeber time (ZT) 0-12] with food (Purina Starteena) and water ad libitum. Animals

maintained in conditions of constant darkness were sacrificed using an IR viewer. Animals were treated in accordance with PHS guidelines, these procedures have been approved by the Texas A&M University Laboratory Animal Care Committee (AUP #2001-163). These experiments were conducted in accordance with the guidelines of the Association for Research on Vision and Ophthalmology.

*Isolation of gRgr and gRrh.*The cDNAs encoding full length clones were isolated from a chick pineal cDNA library, as part of our chick pineal EST sequencing project previously described (Bailey et al., 2003). Briefly, the cDNA library was constructed from pineal mRNA collected at ZT-18 using a Lambda Zap II cDNA Synthesis Kit (Stratagene). Clones were selected from the library at random for sequencing. Two clones for each gene were initially isolated and then sequenced in sense and antisense orientations to confirm identities. Sequences were deposited into Genbank database, *gRrh* (accession no. AY339626) and *gRgr* (accession no. AY339627).

Bioinformatic analysis. Sequence alignments were performed using Clustal W (1.75) software (*Thompson* et al., 1994). Accession numbers used included AH001149: *B.taurus* rhodopsin, U15762: *G. gallus* pinopsin, AY036061: *G. gallus* melanopsin, NM-010098: *M. musculus* encephalopsin, AB035277: *D. rerio* VA opsin, AF028014: *I. punctatus* parapinopsin, M62903: *G. gallus* red cone opsin, AF109372 *D. rerio* blue opsin, PMRHOD1: *P. marinus* rhodopsin, AF402774: *T. rubripes* tmt-opsin, X57143: *T.pacificus* retinochrome AF012270: *H. sapiens* peropsin, NM_021340: *M. musculus* RgR-Opsin, GGU31821: *G. gallus* Mel_{1C}, AH001026: *D. melanogaster* rhodopsin, and M76446: Human alpha-1A-adrenergic receptor.

Following sequence alignments, cladistic analysis was performed using the neighbor joining (NJ) method. The NJ method works on a matrix of distances among all pairs of sequence to be analyzed. These distances are related to the degree of divergence among the sequences. The phylogenetic tree was generated using output data of CLUSTAL W as an input for the TreeView program, (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html) (Page 1996). The units for this scale depend on those used to construct the tree. For example, most tree-building programs output distances as numbers of substitutions per site. For such trees Tree View typically displays a scale of "0.1", meaning 0.1 nucleotide substitutions per site. The actual value depends on the branch lengths in the tree. The chick Mel_{1C} melatonin receptor and the human ά-1A adrenergic receptors, both non-opsin 7-transmembrane domain receptor proteins, were used as an outgroup for cladistic analysis.

RNA analysis. Total RNA was isolated from tissues using Trizol solution (Invitrogen, Carlsbad, CA) as described by the manufacturer. Northern blots were performed as previously described (Bailey et al., 2003). Unless otherwise stated, total (10 μg each lane) was fractionated on 1.5% agarose/0.66 M formaldehyde gel, and probed for *gRgr*, and *gRrh.* Probes were labeled with [α-^{32}P] dATP by random priming (DECA Prime II kit, Ambion, Austin, TX). Typically, blots were first hybridized with the *gRgr* probes and subsequently stripped (2 x 15 min in boiling water) before hybridization with *gRrh* probe. The final wash was at 42 ^{o}C in 0.1 X SSC containing 0.1 % SDS for 30 min. Blots were exposed to X-ray film (Biomax MS, Kodak, Rochester, NY) for 2 days and their images scanned. Transcript sizes were estimated by

comparison with ribosomal RNA bands. Data were normalized for variation in RNA loading and transfer efficiency by comparison to the 18S ribosomal band, which was obtained by digital photography of the blot after transfer. Transcript abundance was determined using Scion Image (www.scioncorp.com). Data shown is the average of two blots/gene.

In situ hybridization (ISH). Animals were sacrificed by decapitation; brains and eyes were removed and rapidly frozen in -40ºC isopentane. In situ hybridization techniques were carried out as previously described (Bailey et al., 2003). Following fixation, deproteination, and acetylation, slides were hybridized with sense and antisense cRNA encoding the open reading frame and 3'UTR. Probes were transcribed *in vitro* in the presence of [α-^{33}P] dUTP with T3 and T7 RNA polymerases for sense and antisense probes, respectively. Sections were incubated overnight at 53°C and then subsequently washed in SSC and then dehydrated in 100% ethanol. Sections were exposed to BioMax MS film (Kodak), for 2 days. Digoxigenin-labeled probes were synthesized encoding the sense and antisense sequences of both genes using a DIG RNA Transcription Kit (Roche). Following prehybridization, sections were incubated with the RNA probe (200 nmol/ml) in hybridization buffer at 42ºC for 16 hrs, and subsequently washed three times in 60% formamide/0.2X SSC. To visualize the hybridization a color reaction was then performed 8-14 hours.

A.

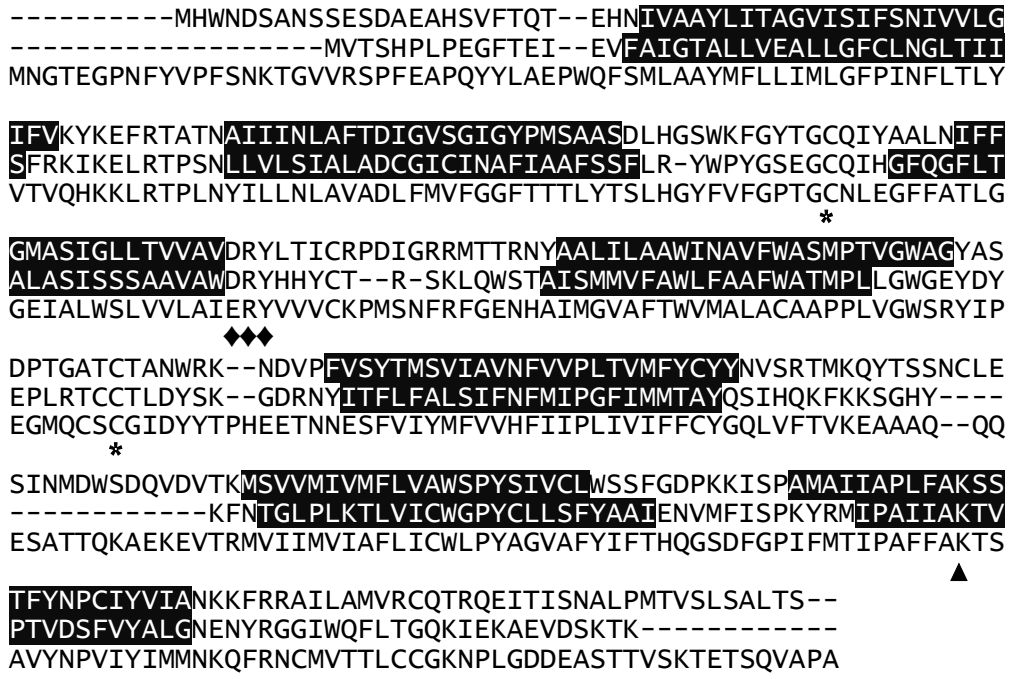

Figure 15. Bioinformatic and cladistic analyses of RRH and RGR. Sequence alignment of RRH and RGR-Opsin vs. *Bos taurus* rhodopsin, performed using CLUSTAL W analysis. Shaded areas indicated the seven transmembrane domains, * indicates cysteines in the first and second extracellular loop necessary for stabilization of the tertiary structure via disulfide bridge formation, ▲ indicates the lysine in the 7th transmembrane domain, corresponding to 284K of peropsin and 255K of RGR-opsin. This amino acid has been shown to be responsible for chromophore binding. ♦ indicates the DRY sequence motif next to transmembrane domain three, which is necessary for rhodopsin to activate G-proteins and transducin.

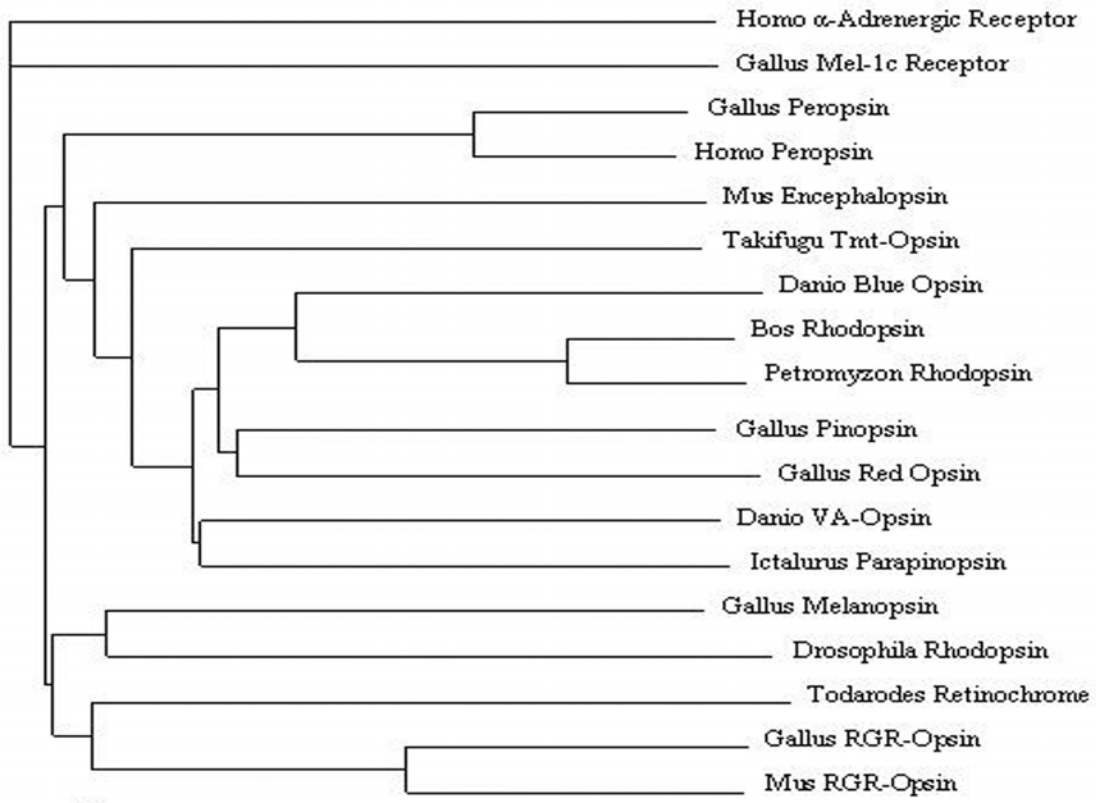

Figure 15 Continued. Phylogenetic analysis of gRRH and gRGR-opins. **B:** Analysis was performed using the TreeView program. Proteins used in analysis are as follows: AH001149: *B.taurus* rhodopsin, U15762: *G. gallus* pinopsin, AY036061: *G. gallus* melanopsin, NM-010098: *M. musculus* encephalopsin, AB035277: *D. rerio* VA opsin, AF028014: *I. punctatus* parapinopsin, M62903: *G. gallus* red cone opsin, AF109372 *D. rerio* blue opsin, PMRHOD1: *P. marinus* rhodopsin, AF402774: *T. rubripes* tmt-opsin, X57143: *T.pacificus* retinochrome AF012270: *H. sapiens* peropsin, NM_021340: *M. musculus* RgR-Opsin, GGU31821: *G. gallus* Mel_{1C}, AH001026: *D. melanogaster* rhodopsin, and M76446: Human alpha-1A-adrenergic receptor.

3. Results

Bioinformatic analysis of *gRrh* and *gRgr*

Sequence analysis indicates that mRNA encoding each chick opsin protein is very similar to mammalian opsins of the same class. The predicted amino acid sequence from the open reading frame (ORF) indicates that *gRrh* is 77% identical to human peropsin, while *gRgr* is 59% identical to human RGR-opsin. Each of the opsins contains 7 transmembrane regions indicative of GPCR superfamily and also contains a lysine in the 7^{th} transmembrane domain, corresponding to 284K ofperopsin and 255K of RGR-opsin (Figure 15A). This amino acid residue has been shown to be responsible for chromophore binding (Bownds 1967), leading to the assumption that both of these genes probably encode photosensitive proteins. *gRrh* and *gRgr* also contain a cysteine in the first and second extracellular loop necessary for stabilization of the tertiary structure via disulfide bridge formation (Karnik et al., 1988). The projected protein sequence of gRRH and gRGR contain the DRY sequence motif next to transmembrane domain three, which is necessary for rhodopsin to activate G-proteins and transducin (Ernst et al., 1995; Yamashita et al., 2000). Additionally, there are several serine/threonine residues present in the C-terminus, which may function as potential phosphorylation sites for kinases (Chen et al., 1995, Figure 15A).

Cladistic analysis of *gRgr* and *gRrh* and several other opsins, using the chick Mel_{1C} melatonin receptor (Reppert et al., 1995) and human $\tilde{\alpha}$-adrenergic receptor (Bruno et al., 1991), both 7-transmembrane domain, G-protein associated receptors, as out-group molecules, clearly indicates that *gRgr* and *gRrh* belong within the opsin clade.

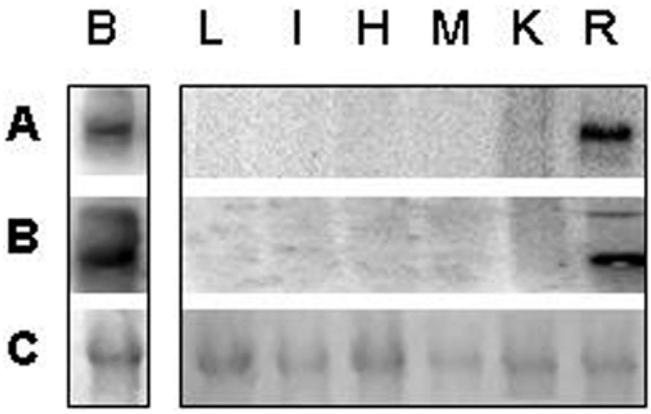

Figure 16. Northern blot analysis of *gRrh* and *gRgr* message. B=brain without pineal tissue, L=liver, I=intestine, H=heart, M=skeletal muscle, K=kidney, R=retina. **A:** mRNA expression for *gRrh* reveals one transcript ~2 kb was present in total retinal and brain RNA (10 µg). **B:** Expression of *gRgr* revealed one major transcript ~2kb and a minor transcript ~4kb. **C:** 18S ribosomal RNA. All RNA samples were isolated from the indicated tissues dissected at ZT-12. The blots were repeated with similar results on independently obtained samples.

However, they represent different sub-groups than either the visual opsins or the many putative extra-ocular opsins such as pinopsin, parapinopsin, encephalopsin, tmt-opsin and melanopsin. The sequence of *gRrh* belongs in a separate peropsin class of opsins with human peropsin, while *gRgr* belongs to the RGR-opsin class, closer to members of retinochrome (Hara and Hara 1991) and retinochrome-like opsins than to members of other classes of vertebrate opsins (Figure 15B).

Spatial and temporal distribution of *gRrh* and *gRgr* mRNA

Multiple tissue northern analysis at high stringency revealed that *gRrh* and *gRgr* mRNA are not widely expressed in the chicken, with high levels of expression visualized only in the brain and retina. The *gRrh* probes hybridized to one transcript at approximately 2.0kb in the retina and brain tissue excluding the pineal gland. Likewise, *gRgr* probes revealed two transcripts in the retina and brain, a major one occurring at approximately 2kb, and minor transcript at ~4Kb. No expression was visualized in heart, liver, skeletal muscle, intestine and kidney (Figure 16). *In situ* hybridization (ISH) of chick brain, using radioactive-labeled probes, indicated very strong expression of both *gRgr* and *gRrh* in the pineal gland, confirming our initial observation from pineal cDNA libraries, but also lower levels of expression throughout the brain (Figures 17A, 17B). This expression was specific; because corresponding sense controls were completely blank (data not shown).

Non-radioactive digoxigenin *in situ* hybridization of *gRrh* and *gRgr* mRNA revealed expression in both the chick retina and pineal gland. In the pineal gland,

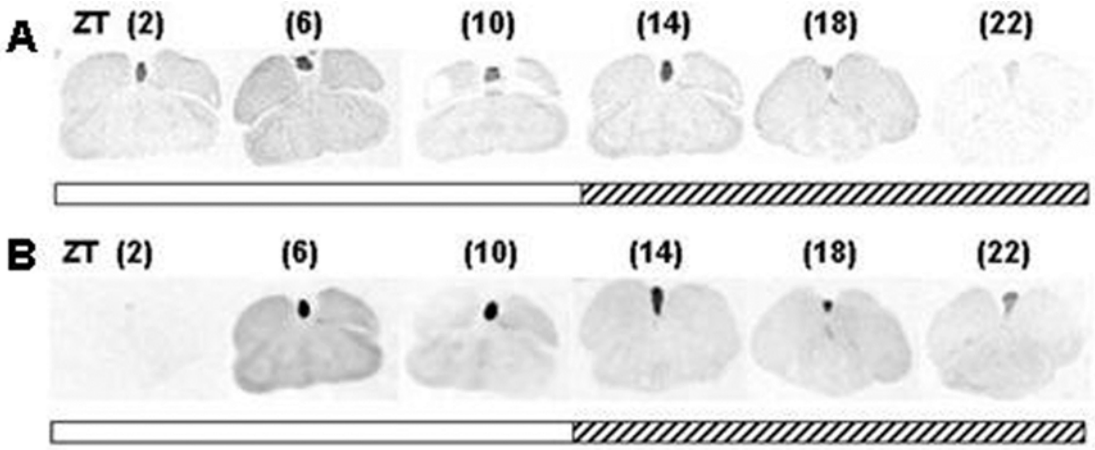

Figure 17. Radioactive ISH of *gRrh* and *gRgr* mRNA expression in the chicken brain. **A:** *gRrh* mRNA expression is rhythmic in the pineal gland with increases mRNA expression in the light phase, similar to northern analysis. **B:** *gRgr* mRNA expression is rhythmic in the pineal gland with increases mRNA expression in the light phase, similar to that of *gRrh* and northern analysis. Note high expression localized to the pineal gland and relatively low levels of expression are found elsewhere for both genes. Corresponding sense controls were blank (not shown). Open box indicated lights on period; dashed box indicates lights off period.

expression of *gRgr* message was primarily observed in para-follicular cells (PFC) broadly interspersed among pinealocytes (black arrow) but not within the pinealocytes themselves (PIN, open arrow) (Figure 18A). These cells appeared to be astrocytes, although this is very difficult to determine using *in situ* hybridization. Nonetheless, the distribution of *gRgr* mRNA was different from the distribution of either arylalkylamine-N-acetyl transferase (AANAT) or pinopsin in the chick pineal gland, both of which are localized in follicular pinealocytes (Okano et al., 1994; Max et al., 1995), so it is unlikely that they are expressed by follicular pinealocytes. Sense controls were blank (Figure 18B). Similarly, *gRrh* mRNA was expressed in both parafollicular (PFC, black arrow) cells surrounding pinealocytes and in "interstitial" cells (INT, open arrow), although the *gRrh* signal was more widespread than that of *gRgr* (Figure 18C). Sense controls were blank for *gRrh* as well (Figure 18D).

In the retina, the relative distributions of *gRgr* and *gRrh* differ from their distribution in pineal in that retinal *gRgr* appears more widespread than *gRrh*, although the northerns do not indicate differences in level of expression. Retinal *gRgr* mRNA predominated in the inner nuclear layer (INL, open arrow) and the ganglion cell layer (GCL, black arrow) (Figure 18E), while retinal *gRrh* mRNA expression was observed primarily in the ganglion cell layer (black arrow) (Figure 18G). In both cases, expression in the GCL appeared to reside within the retinal ganglion cells themselves, while expression in the INL could be of either glial (Mueller cells) or neural (eg. amacrine or bipolar) origin. Little, if any expression was seen in the sense controls for each gene (Figure 18F, 18H). We could not exclude expression in the RPE, since digoxigenin

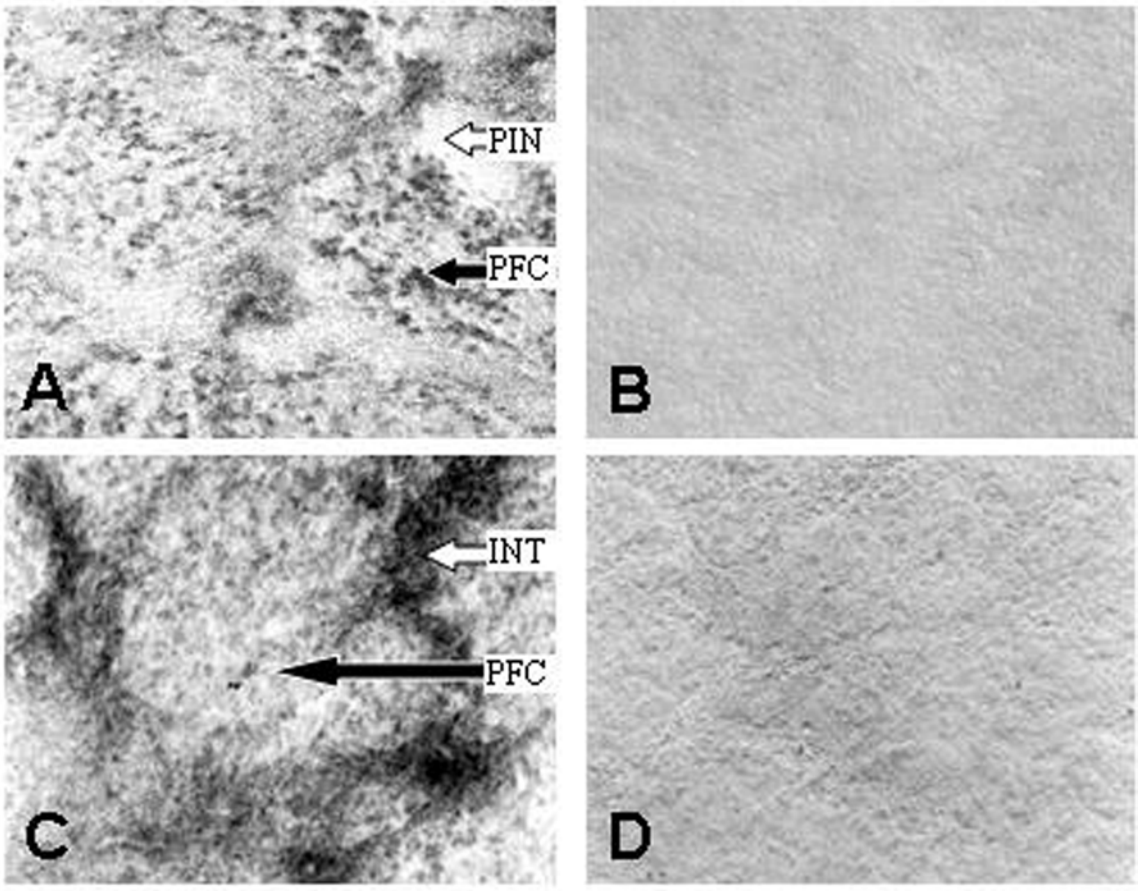

Figure 18. Non-radioactive digoxigenin *in situ* hybridization of *gRrh* and *gRgr* mRNA in the chick retina and pineal gland. Expression of *gRgr* message in the pineal was observed in para-follicular cells (PFC, black arrow) broadly interspersed among pinealocytes but not within the pinealocytes (PIN, open arrow) (Fig. 18A). *gRrh* mRNA was expressed in both parafollicular cells (PFC, black arrow) surrounding pinealocytes "interstitial" cells (INT, open arrow) (Fig. 18C). Sense controls were blank (Fig. 18B, D).

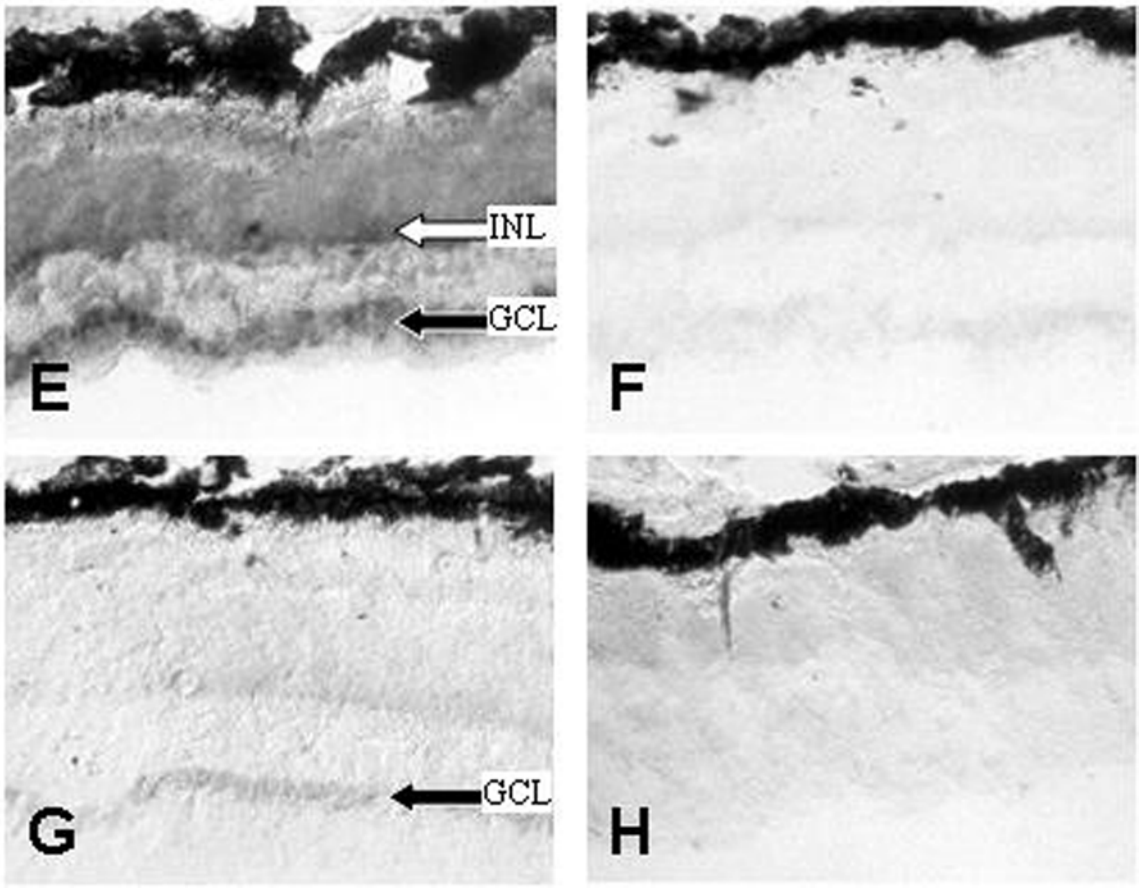

Figure 18 Continued. Non-radioactive digoxigenin *in situ* hybridization of *gRrh* and *gRgr* mRNA in the chick retina and pineal gland. Retinal *gRgr* mRNA expression was more widely expressed, with message predominating in the inner nuclear layer (INL, open arrow) and the ganglion cell layer (GCL, black arrow) (Fig. 18E), while retinal *gRrh* mRNA expression was observed primarily in the ganglion cell layer (GCL, black arrow) (Fig. 18G), Little, if any expression is seen in the sense controls for each gene (Figs. 18F, H).

precipitate would be obscured by the pigment, and background hybridization with radioactive probes was very high.

Expression of *gRrh* and *gRgr* mRNA was rhythmic in the pineal gland (Figures 17, 19). *In situ* hybridization of both *gRrh* (Figure 17A) and *gRgr* (Figure 17B) revealed optical densities that were higher during the day than during the night. Northern analysis of pineal tissues confirmed and extended this observation, using RNA prepared from pineal gland tissues collected across one day of LD and one day of DD (ZT 2-ZT 22, CT 2-CT 22). In the pineal gland, expression of *gRrh* mRNA oscillated on a 24 h basis in a LD and DD cycle such that *gRrh* mRNA exhibited high levels during the light phase of the day and reduced mRNA abundance in the nighttime (Figure 19). *gRgr* mRNA in the pineal gland also oscillated on a 24 hr.basis in a LD and DD cycle such that *gRgr* exhibited high levels during the light phase of the day similar to that of *gRrh* (Figure 19). Additionally, northern blot analysis of retina also showed a rhythm of *gRrh* and *gRgr* mRNA, which oscillated on a 24 hr. basis in LD and DD similarly to the pattern visualized in the pineal gland. Each gene exhibited high levels during the light phase of the day and reduced mRNA levels at late night (Figure 20).

4. Discussion

Nearly all organisms ranging from bacteria to humans possess an endogenous circadian clock that permits the organism to temporally regulate its internal physiology to the surrounding environment. In mammals the biological clock that controls behavioral, physiological and biochemical rhythms resides in the suprachiasmatic

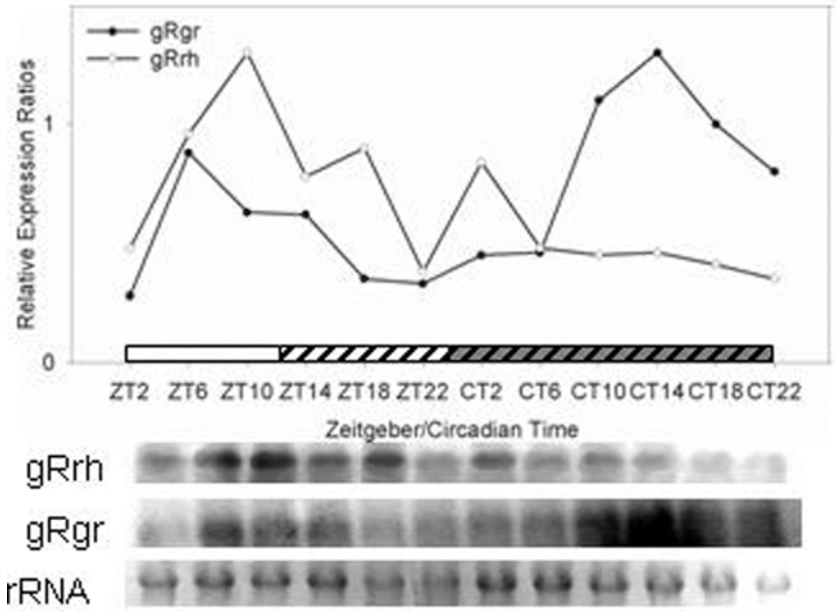

Figure 19. Circadian regulation of *gRrh* and *gRgr* message in the pineal gland. Open circles indicate a rhythm in *gRrh* mRNA under LD conditions that persists in DD in chicken pineal gland. Levels are high during daytime and decreased in the night. Filled circles indicate rhythm in *gRgr* mRNA, that persists in LD and DD in chicken pineal gland. Levels are high during daytime and decreased in the night. The 18S ribosomal RNA was used to normalize blots. Data shown is for the average of two blots. Open box indicates lights on period, open dashed boxes indicate lights off period, grey dashed boxes indicate continuous darkness.

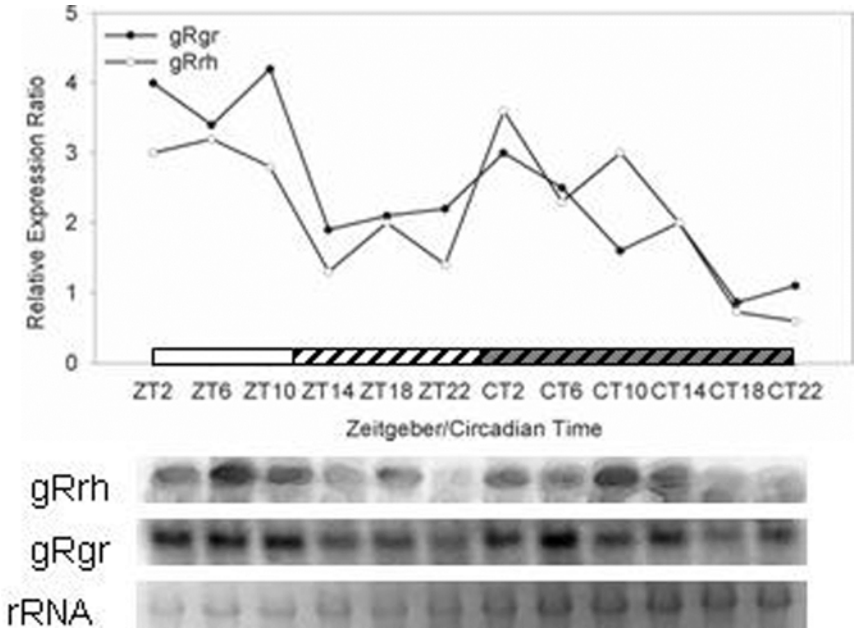

Figure 20. Circadian regulation of *gRrh* and *gRgr* message in the chick retina **A:** Open circles indicate a rhythm in *gRrh* mRNA under LD conditions that persists in DD in chick retina. Levels are high during daytime and decreased in the night. Filled circles indicate rhythm in *gRgr* mRNA, that persists in LD and DD in chick retina. Levels are high during daytime and decreased in the night. The 18S ribosomal RNA was used to normalize blots. Data shown is for the average of two blots. Open box indicates lights on period, open dashed boxes indicate lights off period, grey dashed boxes indicate continuous darkness.

nucleus (SCN). The localization of a master pacemaker to a single structure, as seen in mammals, varies depending on the species in question. For example, in birds it comprises a complex system of multiple oscillators coupled together. These oscillators are located in the ocular retinae, pineal gland and in the avian homologue of the mammalian SCN (Cassone and Menaker 1984; Gwinner and Brandstatter 2001; Underwood et al., 2001). Previous studies have shown that all three are critical components of the birds' biological clock, wherein surgical removal of the pineal gland, retina, or SCN disrupts and/or abolishes circadian rhythms in several species of birds (Gaston and Menaker 1968; Ebihara and Kawamura 1981; Gwinner et al., 1987; Pant and Chandola-Saklani 1992; Underwood 1994). In addition, the avian pineal gland and retina both contain a circadian oscillator and pacemaker to drive circadian rhythms in the biosynthesis of the indoleamine hormone melatonin and photoreceptors to synchronize that rhythm to environmental lighting (Deguchi 1979; Kasal et al., 1979; Hamm and Menaker 1980; Underwood et al., 2001). This hormone serves a vast array of physiological functions, is under direct regulation of the circadian clock, and is an important circadian marker of the system.

The avian pineal gland is a photosensitive structure but lacks both traditional rod and cone-type photoreceptor cells and a pigmented epithelium (Cassone and Natesan 1997). It is, however, capable of acutely responding to light by suppressing biosynthesis of the hormone melatonin (Zatz et al., 1988; Klein et al., 1999), entrainment of its endogenously generated circadian rhythm of melatonin biosynthesis to an LD cycle *in vivo* and *in* vitro (Takahashi et al., 1980; Cassone and Menaker 1983) and phase shifting

that rhythm to pulses of light *in vitro* (Zatz et al., 1988). The chick pineal gland contains essentially three cell-types (Korf 1999); the photoreceptive pinealocyte, which produces melatonin, astrocytes, and "interstitial" cells, which are predominantly B-lymphocytes and endothelial cells. There is no pigmented epithelium and no cells corresponding to Mueller cells that could be responsible for visual cycle regeneration of photopigment. Interestingly, the phase-shifting effects of light by the chick pineal gland, but not the acute effects, are remarkably resistant to Vitamin A deprivation (Zatz 1994), suggesting that the two effects of light are mediated by at least two different photopigments..

The molecular components that comprise biological clocks have been identified in diverse animal species ranging from *Drosophila melanogaster* to several species of mammals (Dunlap 1999; Lee et al., 2000; Glossop and Hardin 2002; Cyran et al., 2003). For example, in *Drosophila*, pacemaker cells in the brain, retinae, and other tissues express rhythmic patterns of transcription and translation of positive elements and negative elements regulate a feedback loop. In mammals, this autoregulatory loop is believed to be very similar and to be entrained to light dark cycles (LD) via the action of both opsin-based photopigments, including melanopsin and, perhaps, the flavin-based blue-light photopigment *cryptochrome* (*cry*) (Bailey et al., 2002; Bellingham and Foster 2002; Panda et al., 2003; Sancar 2003). However, the mechanisms by which pineal and retinal photopigments transduce photic information to these molecular clockworks are not fully understood (Van Gelder 2003).

Presuming that opsin-based photopigments, whether they are melanopsin, pinopsin or other pineal specific photopigments, mediate photoentrainment of circadian

rhythms and/or regulation of melatonin biosynthesis, the identity of molecules responsible for regeneration of the 11-*cis* opsin chromophore have not been identified. We believe the presence of two putative photoisomerases within pineal tissues and in retinal layers associated with biological clock function provides two candidates for such a function. Both g*Rgr* and *gRrh* are expressed within the pineal parenchyma, where melatonin biosynthesis is known to occur, and in the INL and RGL of the ocular retina, where circadian photoreception is also present. Intriguingly, both of these putative photoisomerases are also expressed throughout the brain. Since it is known that, like most non-mammalian vertebrates, birds possess intracranial photoreceptors that are sufficient for circadian entrainment and photoperiodic time measurement, it is tempting to suggest that these photoisomerases may mediate regeneration of extraocular opsin involved in these processes.

Conversely, the presence of a DRY motif on the third intracellular loop of each projected protein (Figure 15A) suggests a second messenger system may be associated with these two proteins. Whether these motifs function as part of an as yet unidentified second messenger system involved in chromophore regeneration or a heretofore unknown signaling function for these opsins is at this point unknown. Clearly further work will be necessary to delineate the functions of these two molecules in the visual cycles of the circadian clock.

Perhaps, the most interesting feature of the present study is the incomplete overlap of distribution of *gRgr* and *gRrh* with the several putative sensory photopigments that have been proposed for photoentrainment of rhythmic processes in

the pineal gland, retina and brain. For example, while both *gRgr* and *gRrh* are present in the pineal gland, they do not directly co-localize with cells known either to produce melatonin or to contain either iodopsin or pinopsin, reputed pineal photosensory photopigments. Both AANAT (Bernard et al., 1997) and the two photopigments are localized exclusively within follicular pinealocytes surrounding the follicular lumens. Conversely, both *gRgr* and *gRrh* are localized in the RGC, which have been shown to express melanopsin in mammals, teleost fish and chicks (Provencio et al., 1998; Drivenes et al., 2003; Rollag et al., 2003) suggesting that visual cycle processes may reside in the RGC themselves. Even so, it is important to point out that co-localization of photosensory photopigments and photoisomerases is not necessary for visual cycle function, since neither *gRgr* nor *gRrh* are present in the photoreceptor layers of chicks (Figure 18) or the several mammalian retinas in which they have been studied (Jiang et al., 1993, Sun et al., 1997). More surprising is the brain expression of both of these mRNA signals, which are generally expressed above sense background. We do not know which cell-types express these mRNA species *in vivo*, but preliminary data in our laboratory strongly suggests that astrocytes express these and other opsin photopigments *in vitro* (Peters et al., 2003). Perhaps, extraocular photosensitivity in avian brain is more pervasive than has been previously appreciated.

CHAPTER V

CHICKENS' CRY2: MOLECULAR ANALYSIS OF AN AVIAN CRYPTOCHROME IN RETINAL AND PINEAL PHOTORECEPTORS*

1. Introduction

The biological clock(s) that control the wide variety of behavioural, physiological and biochemical circadian rhythms in vertebrates are now believed to reside in multiple photoreceptive and oscillatory tissues (Allada et al., 2001; Krishnan et al., 2001; Ripperger et al., 2001). Nowhere has this multiplicity of circadian function been more apparent than in birds (Cassone and Menaker 1984; Underwood 1988; Cassone 2000; Ivanchenko et al., 2001). Circadian oscillators are located in the ocular retinae, pineal gland and in the avian homolog of the mammalian suprachiasmatic nucleus (SCN). Photoreceptors capable of entraining these oscillators have been localized in the retinae, pineal gland and several brain structures, in the septum and tuberal hypothalamus (Cassone and Menaker 1984; Underwood et al., 1988; Ivanchenko et al., 2001). Based on cross-species comparisons of gene sequence, mutation analysis and in vitro data, a homologous autoregulatory transcriptional/translational feedback loop comprised of gene products with remarkable similarity to those demonstrated in

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Drosophila has been postulated as the underlying mechanism in mammals (Allada et al., 2001; Krishnan et al., 2001; Ripperger et al., 2001 Hastings and Maywood 2000; Shearman et al., 2000). According to the current mammalian model, the positive elements are *clock* and *bmal1*, while the negative components are a quartet of genes comprising period 1 (*per1*), period 2 (*per2*) and the two cryptochromes (*cry1*) and (*cry2*). In the mouse, *Mus musculus*, *mCrys* are expressed in retina, brain and peripheral tissues (Thresher et al., 1998; Cashmore et al., 1999; Miyamoto and Sancar 2000; Sancar 2000). Mice lacking both *mCry1* and *mCry2* are behaviourally arrhythmic (Thresher et al., 1998; Vitaterna et al., 1999). It is interesting to note that, in mammals, the cryptochromes play a central role in the oscillation itself, co-opting the function of timeless, while in Drosophila, cryptochrome acts both as a photopigment (Ripperger et al., 2001) and in oscillator functions, at least in some tissues (Allada et al., 2001; Ivanchenko et al., 2001 Krishnan et al., 2001). These data indicate that *Crys* are key components of the circadian system in both Drosophila and mammals. However, their function as circadian photoreceptors in mammals is still under debate (Sancar 2000; Zordan et al., 2001).

Recent studies have reported the cloning and initial characterization of several avian clock factors including *clk*, *bmal1* and *per* genes (Larkin et al., 1999; Noakes et al., 2000; Yoshimura et al., 2000; Brandstatter et al., 2001, Bailey et., 2003). However, very little is known about their contribution to avian physiology, although in vitro evidence has strongly suggested that chicken clock gene heterodimers can directly activate the gene for chicken arylalkylamine N-acetyltransferase (AANAT), a crucial

enzyme in the biosynthetic pathway for the hormone melatonin (Chong et al., 2000). In addition, the sequences of all the known genes involved in melatonin biosynthesis in the chick pineal gland are well characterized (Takahashi et al., 1989; Klein et al., 1997). In order to examine further the molecular clock and photoreceptor components of the avian circadian clock, we determined the presence and regulation of this gene in the chicken pineal gland. We report here the cloning of a mammalian ortholog of *Cry2* from the chicken pineal gland, designated *gCry2* (GenBank accession number AY046568), and have characterized its expression. The data are consistent with the notion that *gCry2* is an evolutionarily conserved member of the animal cryptochrome family and plays a crucial role in avian circadian organization. The question whether *gCry2* serves as a photopigment and/or clock component will be discussed.

2. Methods and Materials

Animals. White leghorn cockerels were obtained from Hy-Line International (Bryan, TX, USA) and maintained for 2 weeks in a LD cycle of 12:12 h (lights on Zeitgeber time (ZT) 0–12) with food (Purina Startena) and water ad libitum. Thereafter, the lighting cycle was altered as described in the figure legends.

Isolation of gCry2. A fragment of mCry2 corresponding to bases ~700–1200 of the coding region was used to screen a chick pineal cDNA library. The cDNA library was constructed from pineal mRNA collected at ZT-18 using a Lambda Zap II cDNA Synthesis Kit (Stratagene). A positive clone, ~1.5 kb, was isolated and sequenced to confirm identity. The cDNA fragment shared high sequence similarity to mCry2, and

was therefore screened against a chicken bacterial artificial chromosome (BAC) library (HGMP Human Resource Centre, UK). Positive clone 64m7 was obtained from the Medical Research Centre HGMP Human Resource Centre (UK). BAC DNA isolation was performed using a Large Construct Isolation Kit (Qiagen). Direct BAC clone sequencing in the presence of Thermofidelase (Fidelity Systems) was performed using an ABI 377 sequencer under the following cycling conditions: 95°C for 5 min, followed by 100 cycles of: 95°C for 30 s, proper annealing temperature for 20 s, and extension at 60°C for 4 min.

Bioinformatic analysis of gCry2 sequence. Cladistic analysis was performed using the neighbor joining (NJ) method in the Vector Nti Molecular Biology analysis software (Informax). The NJ method works on a matrix of distances among all pairs of sequence to be analyzed. These distances are related to the degree of divergence among the sequences. The phylogenetic tree is then calculated after the sequences are aligned.

Further, homology modelling of chicken cryptochrome (*Cry2)* was conducted, based on the high sequence similarity between gCRY2 and DNA photolyase from two bacterial sources, *Escherichia coli* and *Synechococcus elongatus* (nee *Anacystis nidulans*) for which high resolution crystal structures are available (Tamada et al., 1997). Three-dimensional protein structural modelling for gCRY2 was performed in QUANTA/CHARM (version 2000, Accelrys) molecular modeling environment using a UNIX Silicon Graphics O2 workstation. The structural coordinates of the bacterial photolyase proteins (1DNP, 1QNF) were extracted from Protein Data Bank (http://www.rcsb.org/pdb/), and modeling was performed using the primary structure

alignment between gCRY2 and the bacterial photolyases using Clustal W (Thompson et al., 1994). The raw alignment result was manually refined using iterative alignment tools in the Protein Design module of QUANTA. Statistical significance of the pair-wise sequence similarities was evaluated by an alignment-independent program PRSS, which calculates the probability of similarities of randomly shuffled and unshuffled sequences using the distance matrix Monte Carlo procedure (Pearson et al., 1988). The analysis was carried out by setting the gap-opening penalty as 12 and gap-extending penalty as 2, and by performing 1000 global shuffling iterations using the BLOSUM62 scoring matrix.

After alignment, the template proteins were matched and superimposed. The coordinates of the aligned amino acid residues were averaged and copied to the modelled sequences. The newly defined coordinates were refined with a structural regularization tool. The connecting loop sequences were not modelled at this time. Cryptochromes are known to share the same chromophores, pterin (5, 10-methyl-6, 7, 8-trihydrofolic acid, MTHF) and flavin (flavin adenine dinucleotide, FAD), as does the photolyase from E. coli. The coordinates of the chromophores from E. coli were thus transferred directly to the cryptochrome protein model. The overall raw structure was energy minimized using the CHARMm procedure. The hydrogen-bonding pattern of the constructed PSII model was calculated on the Protein Design module and the secondary structure of the cryptochrome protein was derived.

RNA analysis. Total RNA was isolated from tissues using RNA Aqueous Midi-Kit (Ambion) as described by the manufacturer. Poly (A) + RNA was isolated from total

RNA using a MicroPure PolyA Kit (Ambion). Northern blots were performed as previously described (Bernard et al., 1997; Chong et al., 1998). Unless otherwise, total (10ug each lane) or Poly (A) + (2ug) RNA was fractionated on 1.5% agarose/0.66 M formaldehyde gel, and probed for gCry2. Probes were labelled with [α-^{32}P]dATP by random priming (DECA Prime II kit, Ambion). Typically, blots were first hybridized with the gCry2 probes (1 kb 3′-UTR) and subsequently stripped (2×15 min in boiling water) before hybridization with actin probe. The final wash was at 55°C in 0.1×SSC containing 0.1% sodium dodecyl sulfate for 30 min. Blots were exposed to X-ray film (Biomax MS, Kodak) for 2 to 3 days and their images scanned and analyzed using the Image software (Scion Image). Transcript sizes were estimated by comparison with standard RNA markers (Roche). Data were normalized for variation in RNA loading and transfer efficiency by probing the Northern blots with β-actin cDNA.

In situ hybridization (ISH). Animals were sacrificed by decapitation; brains and eyes were removed and rapidly frozen in isopentane at −40°C. ISH techniques were carried out as previously described (Bernard et al., 1997; Chong et al., 1998). Following fixation, deproteination, and acetylation, slides were hybridized with sense and antisense cRNA probes for gCry2. Probes encoding the 3′-UTR of gCry2 were generated in the presence of [α-^{33}P]dUTP, in vitro with T3 and T7 RNA polymerases for sense and antisense probes, respectively. Sections were incubated overnight at 50°C and then subsequently washed in SSC and then dehydrated in 100% ethanol. Sections were exposed to BioMax MS film (Kodak) for 36 h. Digoxigenin-labelled probes were synthesized encoding the antisense of the 3′-end of gCry2 and for the corresponding

sense sequences using a DIG RNA Transcription Kit (Roche). Following prehybridization, sections were incubated with the RNA probe (200 pmol/ml) in hybridization buffer at 50°C for 16 h. To visualize the hybridization a color reaction was then performed overnight.

3. Results

Bioinformatic analysis of gCry2

The *Cry2* gene isolated from the chicken BAC library corresponds very closely to the mammalian *Cry2* (human and mouse). Cladistic analysis of the CRY protein indicates that the gCRY2 sequence belongs within the general animal *Cry* family of genes (Figure 21A). It is important to note that gCRY2 is closer to CRY2 sequences of other taxonomic groups than it is to the CRY1 of other species, or to gCRY1indicating that this set of proteins represents separate and very ancient lineages, certainly preceding the divergence of amniotes from anamniote species. Genomic sequence indicates that the open reading frame (ORF) of the *gCry2* gene is spread across at least 8 kb of genomic DNA, consisting of at least five exons and six introns (data not shown).

The predicted amino acid sequence from the ORF of the cDNA sequence indicated that gCRY2 is 86% identical to human and mouse CRY2. Remarkably, the sequence is 29.5% identical and 59.6% similar to the 6-4 DNA photolyase in *S. elongatus* (P=7.3×10^{-53}) and 21.7% identical and 58.0% similar to the homologous *E. coli* enzyme (P=2.9×10^{-41}). The predicted amino acid sequence of *gCry2* contains a probable FAD-binding site, a MTHF (pterin)-binding domain and a DNA photolyase

A.

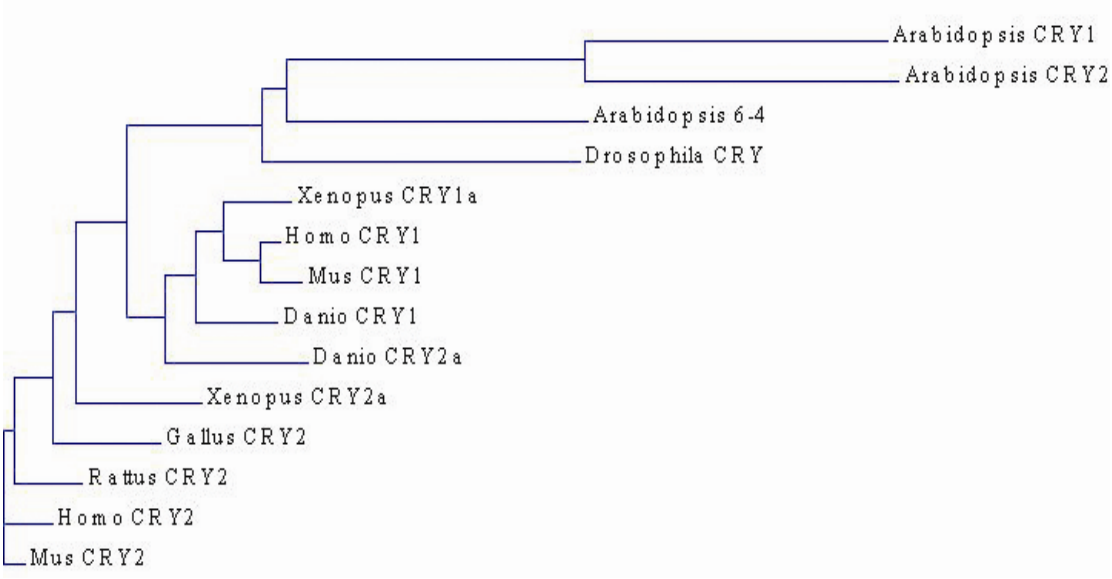


Figure 21. Bioinformatic and cladistic analysis of chicken CRY2. **A:** Cladogram indicating that *gCry2* is a phylogenetically conserved member of the animal cryptochromes and is more closely related to other vertebrate *Cry2* than to either *Cry1*or plant 6-4 photolyases or cryptochromes.

B.

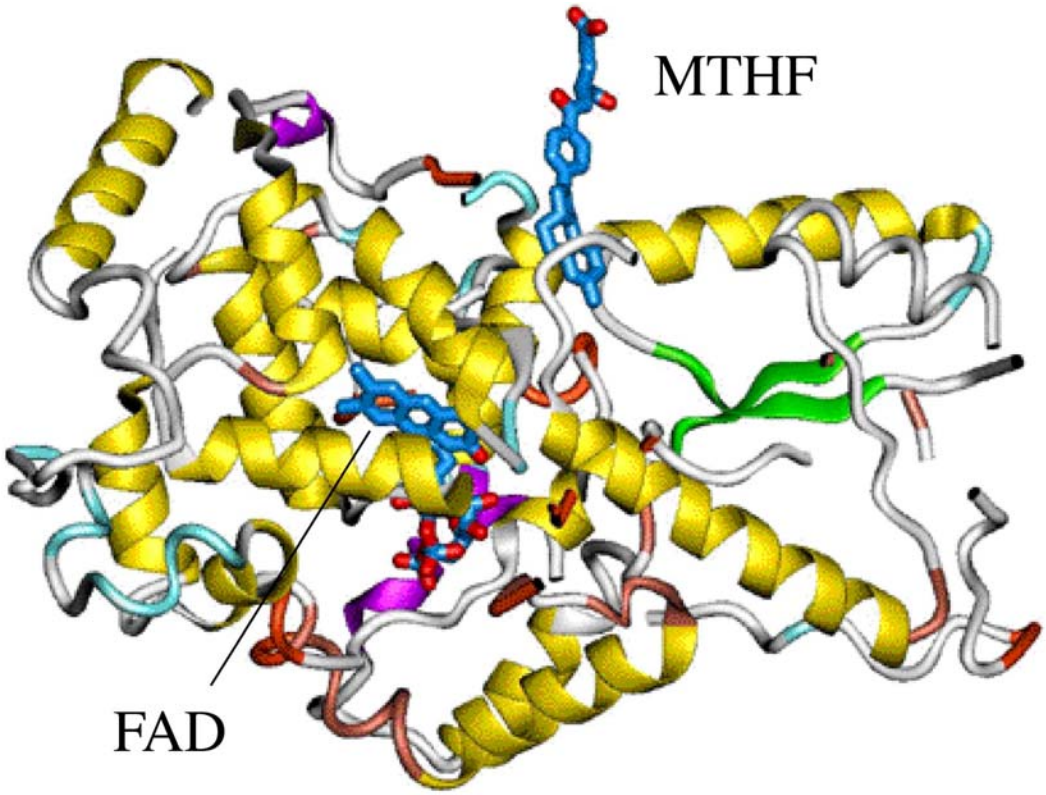


Figure 21 Continued. Bioinformatic and cladistic analysis of chicken CRY2 **B:** Ribbon diagram based upon homology modeling of gCRY2 showing the likely positions of the flavin chromophore (FAD) in the center of the molecule and the pterin co-factor (MTHF) on the surface.

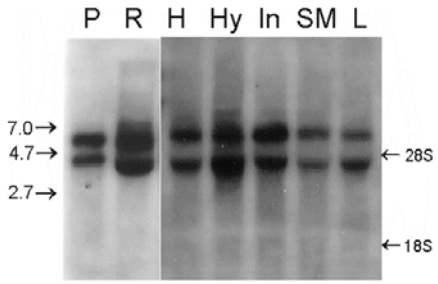
domain. The residues that form the FAD-binding pocket are located in the middle of the predicted protein and are significantly positively charged, including residues 233, 243, 257–261, 264–265, 296, 299, 301–302, 305, 361–365, 367–368, 371, 390, 394, 396, 401–403, and 405–406. The pterin-binding pocket is much smaller, since this cofactor is partially bound at the surface. The putative binding residues are 112–114, 326 and 399.

Because of the close similarity of *gCry2* to the prokaryote photolyases, it was possible to model gCRY2 in homologous regions and to construct a putative structure for the predicted protein, such as the ribbon diagram of the overall modelled chicken cryptochrome structure including MTHF and FAD cofactors (Figure 21B). The FAD-binding domain contains residues within 3 Å of the FAD chromophore, buried in the center of the protein. The MTHF (pterin)-binding domain contains residues within 5 Å of the flavin molecule. This cofactor is partially bound at the surface.

Tissue distribution of *gCry2* mRNA

Northern blot analysis at high stringency revealed that *gCry2* mRNA is expressed at high levels in the pineal gland and retina (Figure 22A). *gCry2* probes hybridized to two transcripts (approximately 4.2 and 5.2 kb). Multiple tissue northern analysis revealed that *gCry2* mRNA is widely expressed in the chicken, including the heart, liver, skeletal muscle, intestine and brain (Figure 22A). The existence of daily rhythms in *gCry2* mRNA was examined using Northern blot analysis in RNA prepared from pineal tissues (ZT 4 to ZT 24). The expression of *gCry2* mRNA oscillated on a 24 h basis in a LD cycle such that *gCry2* exhibited high levels at late night (Figure 22B). ISH of the chick

A.



B.

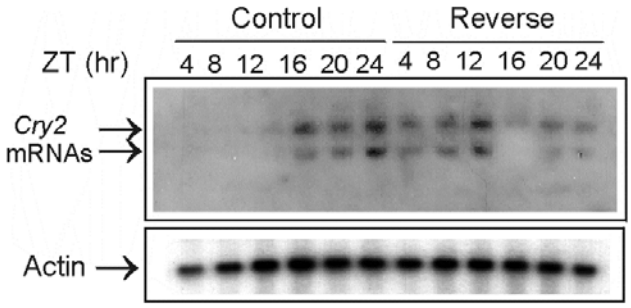


Figure 22. Northern blot analysis of gC*ry2* mRNA expression. A: Two transcripts (~4.2 and 5.2 kb) were present in all tissues examined. PolyA+ RNA (2 μg) from pineal gland and retina and total RNA (20 μg) from other tissues were loaded. All RNA samples were isolated from the indicated tissues dissected at ZT-20. The blots were repeated with similar results on independently obtained samples. P=pineal, R=retina, H=heart, Hy=hypothalamus, In=intestine, L=liver, SM=skeletal muscle. B: Rhythm in *gCry2* mRNA persists in LD in chicken pineal gland. Levels are high during late night and are entrainable to LD cycles, since reversal of the LD cycle in the birds reverses the phase of *gCry2* levels.

brain, using radioactive-labelled probes, revealed an expression of *gCry2* mRNA in areas associated with phototransduction and the visual system, including the visual SCN (vSCN), optic tectum (TeO), and lateral septum (LS) (Figure 23). Non-radioactive digoxigenin-labelled ISH confirmed *gCry2* mRNA expression in the pineal gland, LS, and also revealed expression in the chick retina. Retinal *gCry2* mRNA expression is observed primarily in the inner nuclear layer (INL), photoreceptor layer (PL), and to a lesser extent, in the ganglion cell layer (GCL) (Figure 24). *gCry2* mRNA is expressed in both photoreceptive pinealocytes (Pin) and interstitial cells of the pineal gland (Int), vSCN, and ventrolateral geniculate nucleus (GLv), stratum opticum (Sop), stratum griseum et fibrosum (SGF), and stratum griseum centrale (SGC) layers of the TeO (Figure 25A-C). However, it is important to point out that the level of expression in either the vSCN or medial SCN (mSCN), albeit present, is not particularly strong, when compared to either pineal or retinal expression (Figure 23, Figure 24 and Figure 25).

4. Discussion

We report here the isolation and initial characterization of *gCry2*. Analysis of the predicted amino acid sequence indicates that *gCry2* is a phylogenetically conserved ortholog of mammalian *Crys* (Figure 21A), complete with a flavin-binding site, a pterin-binding site and a DNA photolyase domain (Figure 21B). Northern blot analysis of *gCry*2 detected two transcripts in all tissues examined, which is similar to human *Cry2* mRNAs (Kobayashi et al., 2000) but not the mouse, where there appears to be only one transcript (Miyamoto and Sancar 1999). It is conceivable that the two *Cry2* transcripts in

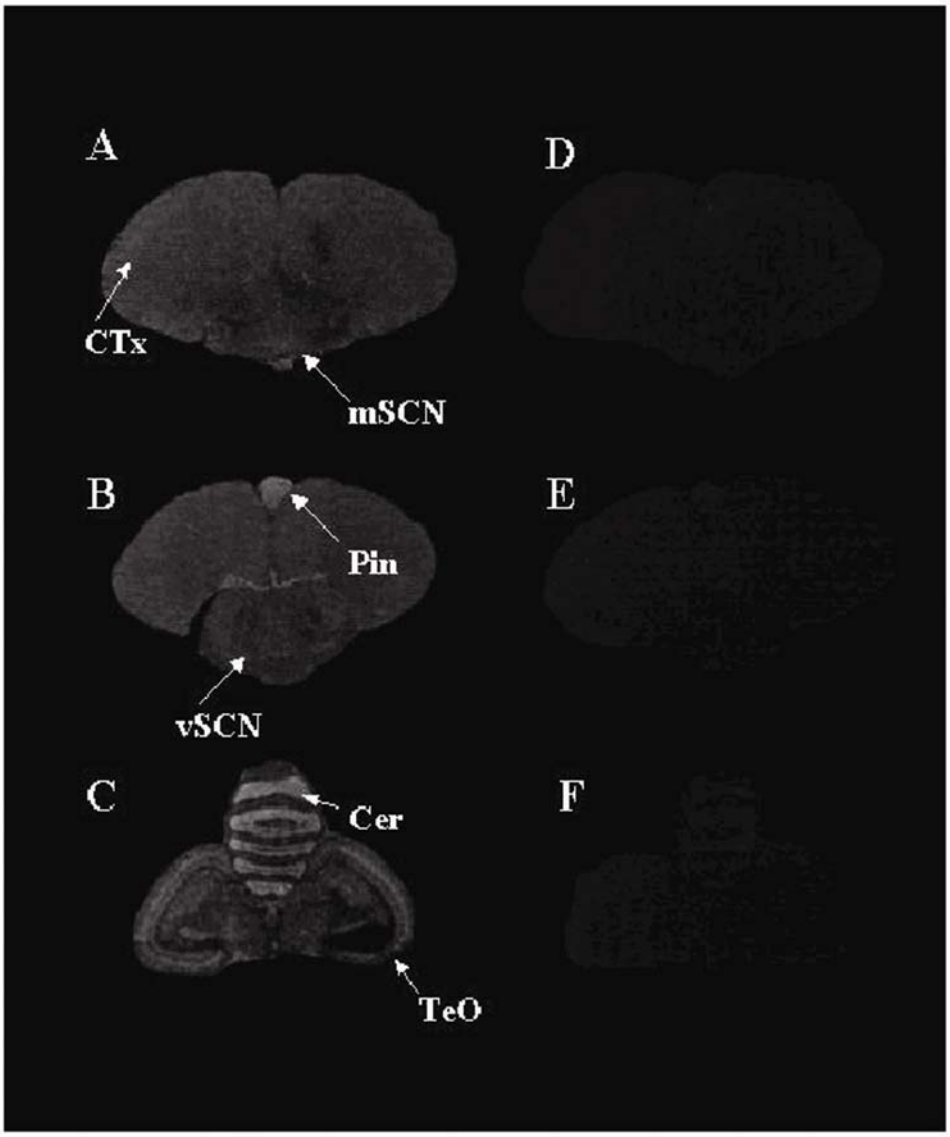


Figure 23. ISH analysis of *gCry2* mRNA expression in the chicken brain. These coronal sections are displayed in rostral (A), intermediate (B), and caudal (C) aspects of the brain. Note high expression in the TeO, Pin, and cerebellum (Cer). Relatively low levels of expression are found in the mSCN, vSCN and cortex (CTx). Corresponding sense controls (D, E, F) exhibit very little, if any, hybridization.

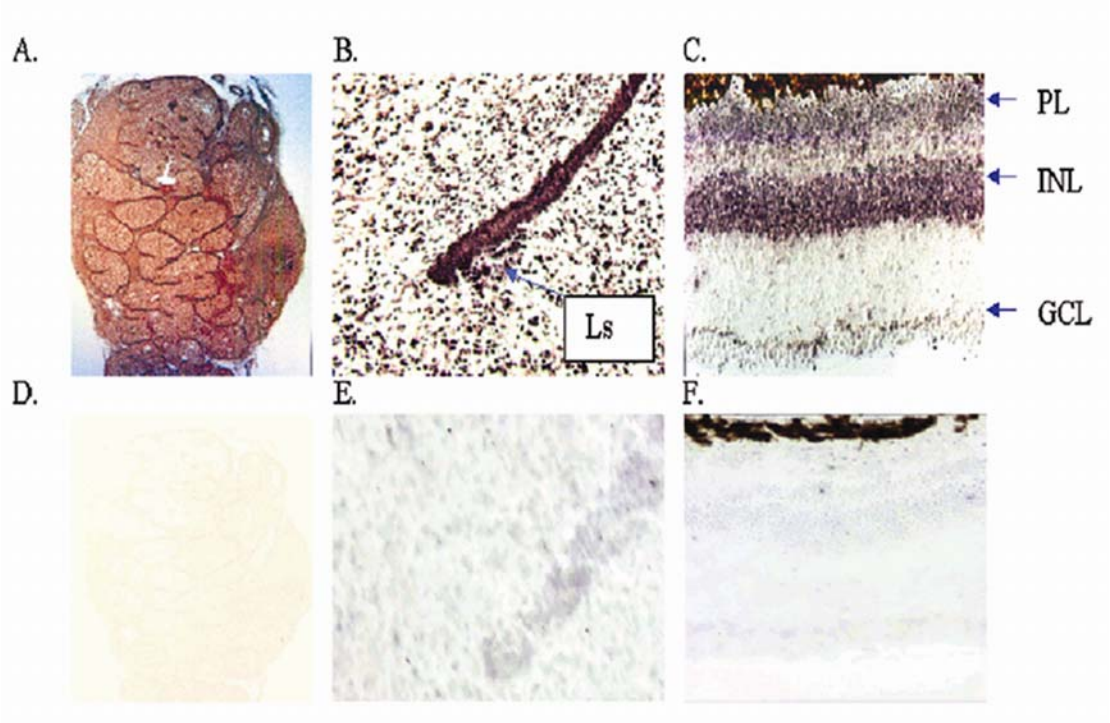


Figure 24. Digoxigenin ISH for *gCry2* mRNA in the pineal gland, lateral septum, and retina. These data show broadly distributed, but specific, expression in most of the pineal gland (A, D). In the lateral septum (Ls) (B, E), a concentration of *gCry2* cells were observed in ependymal regions, which have been shown to contain opsins. Expression in the INL, PL, and GCL layer of the retina (C).

chicken and human were due to alternative polyadenylation site usage as sequence analysis revealed a consensus polyadenylated tail at a premature location in the 3′-UTR, approximately 1 kb from the polyA+ tail for g*Cry*2. The wide distribution is similar to the profile seen in mammals (Sancar 2000; Miyamoto and Sancar 1999; Kobayashi et al., 2000).

There are, however, several important differences in the expression patterns among the mammalian cryptochromes and *gCry2*. First and foremost, *gCry2* is expressed by known photoreceptive cells in the retinae, the pineal gland and in the putative deep-brain photoreceptor region of the LS (Figure 23 and Figure 24), whereas, in mammals, the cryptochromes are not expressed by canonical photoreceptor cells (Sancar 2000). This expression pattern coincides with opsin and opsin-like immunohistochemical staining in these structures in a variety of non-mammalian vertebrate species (Zordan et al., 2001; Silver et al., 1988; Foster et al., 1993; Kojima et al., 2000; Okano et al., 2000; Zhu and Green 2001) and resembles the cryptochrome expression pattern in the zebrafish and *Xenopus* (Kobayashi et al., 2000; Zhu and Green 2001). In addition, ISH revealed *gCry2* mRNA in the retinal ganglion cell and INLs of the retina, also similar to the situation in *Xenopus* (Zhu and Green 2001) and in the mouse (Miyamoto and Sancar 1999). Further, we find broad *gCry2* expression in retinorecipient and integrative structures of the visual system (Figure 23), which is not the case in mammals (Sancar 2000). It is interesting to note that, while we observe strong hybridization in the photoreceptive elements of the circadian clock in retinal,

A. **B.** **C.**

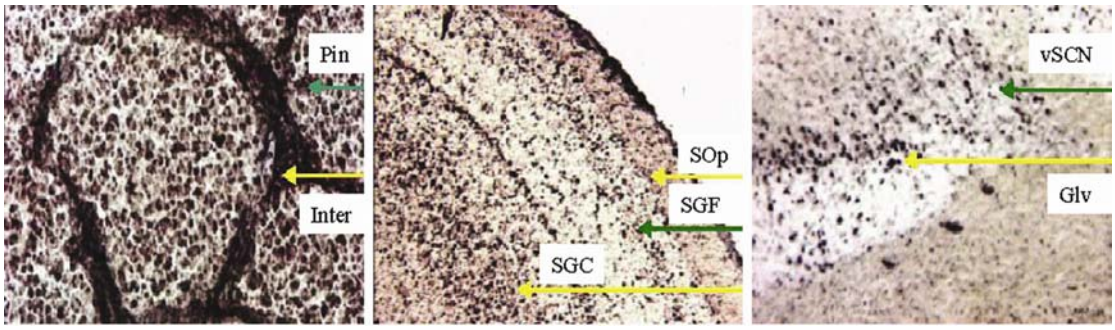


Figure 25. Digoxigenin ISH for *gCry2* mRNA in discrete brain regions

Expression in both Pin and Inter (A), Sop, SGF, and SGC layers of the TeO (B) and vSCN, and GLv (C).

pineal and brain photoreceptors, we see only moderate expression in the two candidates for the avian SCN, which is also the case in mammals (Miyamoto and Sancar 1998). This observation stands in sharp contrast to the situation for the *per* genes, which are expressed abundantly in the mSCN (Yoshimura et al., 2000; Brandstatter et al., 2001).

Light is a major environmental time cue in the entrainment of circadian rhythms (Pittendrigh et al., 1991). Visual phototransduction has been extensively characterized at the molecular level, although the identity of the photoreceptors mediating circadian photoentrainment in vertebrates is uncertain (Zordan et al., 2001). Conceivably, molecules that mediate circadian photoreception may include both opsin and non-opsin-based pigments (Miyamoto and Sancar 1998; Sancar 2000; Zordan et al., 2001). In addition to the better-known visual pigments, several novel non-visual opsins have been identified in vertebrates, including pinopsin (Okano et al., 1994; Max et al., 1995) melanopsin (Provencio et al., 1998) and parapinopsin in the pineal (Blackshaw and Snyder 1997), among many others (Sancar 2000; Zordan et al., 2001).

In non-mammalian vertebrates, the pineal gland is a directly photoreceptive structure on which light has three major effects: (1) the acute suppression of melatonin production, (2) resetting the phase of the endogenous circadian oscillator and (3) the prevention of damping of the output rhythm (Takahashi et al., 1989, Provencio et al., 2000; Zatz et a;., 2000). It is possible that some or all of these effects are mediated by opsin-based photopigments, which mediate phototransduction via a vitamin a-dependent retinaldehyde chromophore (Zordan et al., 2001). Certainly many of these photopigments are present in the avian pineal gland. However, it is important to point

out that, although the acute effects of light on chick pineal melatonin are reduced with vitamin A deprivation, the phase-shifting effects of light in cultured chick Pin are unaffected by >95% depletion of total and of protein bound retinaldehyde (Zatz 1994). This observation raises the possibility that a non-opsin-based photopigment may underlie circadian phase-shifting and entrainment in the chick pineal. There is a growing body of evidence in favor of this scenario in mammals. Using triple-mutant mice lacking rods and most cones (rd/rd) as well as both mCRY proteins, have recently reported that classical opsins and CRYs serve functionally redundant roles in circadian phototransduction (Selby et al., 2000). Further, examination of the circadian photoresponse in vitamin A-depleted retinol-binding protein (RBP) −/− mice as measured by acute *mper* gene induction in the SCN in response to light. These authors reported that ocular retinal is not required for light signalling to the murine circadian pacemaker (Thompson et al., 2001)

In spite of recent molecular breakthroughs and high sequence similarities to the mammalian clock genes, these genes role in the avian circadian system is undetermined. However, co-expression of the putative positive elements in COS-7 cells activates a chicken AANAT E-box luciferase reporter construct (Chong et al., 2000), suggesting elements of the proposed transcription/translation feedback model interact with a known circadian output. It is not clear at this point whether *gCry2* is involved in the phototransduction associated with entrainment and/or is a clock component itself.

CHAPTER VI

MELANOPSIN EXPRESSION IN THE CHICK RETINA AND PINEAL GLAND

1. Introduction

The vertebrate eye functions as the primary recipient and integrator of photic information from the surrounding environment. Visual photoreception occurs primarily within rod and cone photoreceptors of the retina. Retinal photosensitivity however, is not exclusive to these cell types. Several groups have discovered that ganglion, horizontal and amacrine cells respond to light in several vertebrate species (Soni et al., 1998; Berson 2003; Sekaran et al., 2003). In addition to visual processing, the retina performs several nonvisual functions. These include the entrainment of the circadian system by relaying photic information to the master oscillator in mammals (Freedman et al., 1999), the suprachiasmatic nucleus (SCN), the acute suppression of melatonin production (Lucas et al., 1999), and pupillary constriction (Lucas et al., 2003). In nonmammalian vertebrates entrainment of circadian rhythms and induction of photoperiodic responses survive enucleation (Menaker 1972), suggesting the presence of functional extraocular photoreceptors. Extraocular photosensitive structures have been identified in several different organisms, including the nonmammalian pineal gland, the parietal eye of reptiles, parapineal structures of anamniote vertebrates, and also deep tissues within the hypothalamus and septum. Within these structures and cell types several opsin photopigments have been discovered which can be categorized into several discrete classes. A photosensory class comprising the rod and cone opsins, a vertebrate

ancient opsin class (Soni and Foster 1997) and a pinopsin class (Okano et al., 1994). The other classes include exo-rhodopsin (Mano et al., 1999), parapinopsin (Blackshaw and Snyder 1997), tmt-opsin (Moutsaki et al., 2003), RGR-opsin (Jiang et al., 1993), encephalopsin (Blackshaw and Snyder 1999), peropsin (Sun et al., 1997), and melanopsin (Provencio et al., 1998). The precise functions of the non-visual opsins are not completely understood although several of these isolated in pineal tissue are implicated in photic regulation of the hormone melatonin by virtue of the fact that they are expressed in photoreceptive pinealocytes (Foster and Hankins 2002). Nonetheless, the precise photopigments that control retinal responses to light and the photoentrainment of the circadian system remain unknown.

A leading candidate for the circadian photopigment and the focus of intense research in recent years is melanopsin. Melanopsin was initially isolated in photosensitive dermal melanophores of *Xenopus laevis* (Provencio et al., 1998). In addition to melanophores, melanopsin mRNA is expressed in hypothalamic areas, and the inner nuclear layer of the retina. Since that time, melanopsin has been found in several organisms, including fish, birds, reptiles, rats, humans, and mice (Provencio et al., 2000; Gooley et al., 2001; Bellingham and Foster 2002; Drivenes et al., 2003). A major body of evidence regarding melanopsin in mice points to a role in circadian photoreception. First, retinal ganglion cells comprising the retinohypothalamic tract (RHT) express melanopsin and are photosensitive (Berson 2003; Gooley et al., 2001). Secondly, knockout studies indicate altered circadian photoentrainment and decreased pupillary constriction in response to pulses of light (Hattar et al., 2003; Lucas et al.,

2003; Panda et al., 2003). Third, biochemical data suggest that melanopsin is capable of forming a functional photopigment and of activating transducin (Newman et al., 2003). Taken together these data support the idea that melanopsin may be involved in the photoentrainment of the circadian system. Since the photopigment(s) required for photic entrainment in avian species are unknown, we have investigated melanopsin expression within two avian tissues harboring circadian oscillators, the pineal gland and retina of chicks.

2. Methods and Materials

White leghorn cockerels were obtained from Hy-Line International (Bryan, TX) and maintained for three weeks in a light:dark (LD) cycle of 12 h:12 h [lights on Zeitgeber time (ZT) 0-12] with food (Purina Starteena) and water ad libitum. Animals maintained in conditions of constant darkness were sacrificed using an IR viewer. Animals were treated in accordance with PHS guidelines, these procedures have been approved by the Texas A&M University Laboratory Animal Care Committee (AUP #2001-163). The cDNA encoding the full-length melanopsin clone was isolated from a chick pineal cDNA library, as part of our chick pineal EST sequencing project previously described (Bailey et al., 2003). Briefly, the cDNA library was constructed from pineal mRNA collected at ZT-18 using a Lambda Zap II cDNA Synthesis Kit (Stratagene). Sequences were compared to a full length melanopsin clone in the Genbank database (accession no. AY036061).

Total RNA was isolated from tissues using Trizol solution (Invitrogen, Carlsbad, CA) as described by the manufacturer. Northern blots were performed as previously described (Bailey et al., 2003). Unless otherwise stated, total (10ug each lane) was fractionated on 1.5% agarose/0.66 M formaldehyde gel, and probed for *opn4*. Probes were labeled with [32P] dATP by random priming (DECA Prime II kit, Ambion, Austin, TX). The final wash was at 42°C in 0.1 X SSC containing 0.1 % SDS for 30 min. Blots were exposed to X-ray film (Biomax MS, Kodak, Rochester, NY) for 2 days and their images scanned. Transcript sizes were estimated by comparison with ribosomal RNA bands. Data were normalized for variation in RNA loading and transfer efficiency by comparison to the 18S ribosomal band, which was obtained by digital photography of the blot after transfer.

Radioactive *in situ* hybridization was performed as follows. Animals were sacrificed by decapitation; brains and eyes were removed and rapidly frozen in isopentane at −40°C. Briefly, following fixation, deproteination, and acetylation, slides were hybridized with sense and antisense cRNA probes for *opn4*. Probes encoding the open reading frame were generated in the presence of [33P] dUTP, in vitro with T3 and T7 RNA polymerases for sense and antisense probes, respectively. Sections were incubated overnight at 53°C and then subsequently washed in SSC and then dehydrated in 100% ethanol. Sections were exposed to BioMax MS film (Kodak) for 36 h. Non-radioactive *in situ* hybridization techniques were carried out as stated above, however slides were hybridized with digoxigenin-labeled probes synthesized using a DIG RNA Transcription Kit (Roche). Following prehybridization, sections were incubated with the

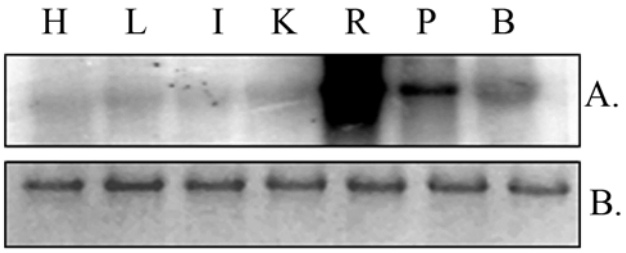


Figure 26. Northern blot analysis of multiple tissues for melanopsin mRNA. B=brain without pineal tissue, L=liver, I=intestine, H=heart, M=skeletal muscle, K=kidney, R=retina. P=Pineal Gland A: mRNA expression for *opn4* is widespread, with highest levels in retina and pineal tissue. B: 18S ribosomal RNA. All RNA samples were isolated from the indicated tissues dissected at ZT-14. Blots were repeated with similar results on independently obtained samples.

RNA probe (200 nmol/ml) in hybridization buffer at 45ºC for 16 hrs, and subsequently washed three times in 60% formamide/0.2X SSC. To visualize the hybridization a color reaction was then performed 8-10 hours.

3. Results

Northern blot analysis

Multiple tissue northern analysis at high stringency revealed that *opn4* mRNA is widely expressed in the chicken, with expression visualized in all tissues tested. The *opn4* probes revealed the highest levels of expression in the pineal gland and retina (Figure 26). Using RNA prepared from pineal gland tissues collected across one day of LD and one day of DD (ZT 2-ZT 22, CT 2-CT 22) a daily and circadian analysis was performed for the pineal gland and retina. In the pineal gland, expression of *opn4* mRNA oscillated on a 24 h basis in a LD and DD cycle such that *opn4* mRNA exhibited high levels during the night and reduced mRNA abundance in the daytime (Figure 27A). Additionally, northern blot analysis of retina also showed no apparent rhythm of *opn4* mRNA, in DD and a slight increase during the night in LD (Figure 27B).

In situ hybridization

ISH of the chick brain, using radioactive-labeled probes, revealed an expression of *opn4* mRNA in areas associated with phototransduction and the visual system, including the pineal gland (Pin), optic tectum (TeO), and cerebellum (Cer) (Figure 28). Non-radioactive digoxigenin in situ hybridization of *opn4* mRNA in the retina revealed

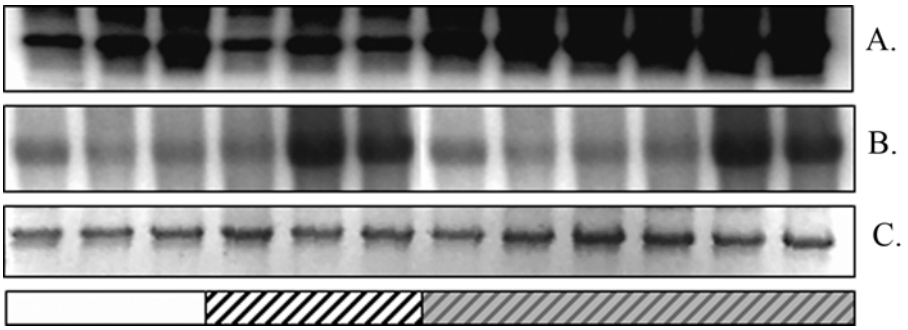


Figure 27. Circadian regulation of *opn4* in the pineal gland and retina. A. In the pineal gland levels are high during nighttime and decreased in the day. B. In the retina levels are slightly increased during nighttime in LD but not DD. C. The 18S ribosomal RNA. Blots were repeated with similar results on independently obtained samples.

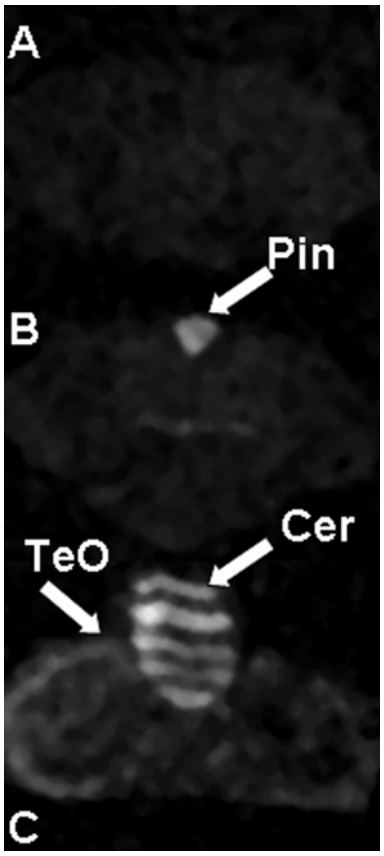


Figure 28. ISH analysis of *opn4* mRNA expression in the chicken brain. These coronal sections (ZT-14) are displayed in rostral (A), intermediate (B), and caudal (C) aspects of the brain. Note high expression in the optic tectum (TeO), pineal (Pin), and cerebellum (Cer). Corresponding sense controls were blank after hybridization (data not shown).

robust expression. Here, *opn4* mRNA predominated in the inner nuclear layer (INL), ganglion cell layer (GCL) and also in the photoreceptor layer (PL) (Figure 29A). Expression in the GCL appeared to reside within the retinal ganglion cells themselves, expression in the INL could be of either glial (Mueller cells) or neural (amacrine or bipolar) origin, expression in the photoreceptor layer appeared to be primarily in the inner segments. Little, if any expression was seen in the sense controls (Figure 29B). We could not exclude expression in the RPE, since digoxigenin precipitate would be obscured by the pigment.

4. Discussion

There are several important differences in the expression pattern of melanopsin among mammals and birds. In chicks, *Opn4* is expressed by known photoreceptive cells in the retinae, and the pineal gland whereas, in mammals, melanopsin has not been detected in the pineal gland nor rods and cones. The melanopsin expression pattern of chicks highly resembles that of *cry2* expression in the chicken (Bailey et al., 2002), wherein the mRNA of both genes are located within the same structures and cell types including the pineal, cerebellum, and optic tectum of the brain, and the ganglion cell layer GCL, inner nuclear layer (INL) and photoreceptors layer (PL) of the retina. This study also is the first report that melanopsin mRNA is regulated on a circadian basis, in the pineal gland, where increases *opn4* mRNA in late subjective night is found. This oscillation again closes resembles that of *cry2* in chicks, yielding the possibility that *opn4* and *cry2* function in accord to help integrate photic information (Van Gelder 2003).

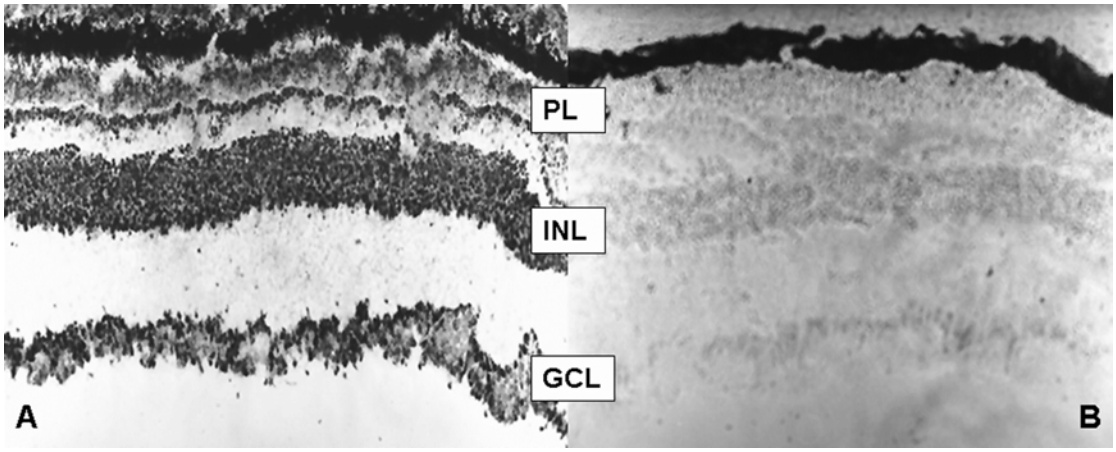


Figure 29. Non-radioactive digoxigenin in situ hybridization of *opn4* mRNA in the chick retina. A. Retinal *opn4* mRNA expression was widely expressed, with message predominating in the inner nuclear layer (INL), ganglion cell layer (GCL) and photoreceptor layer (PL). B. Little, if any expression is seen in the sense control.

In mammals the biological clock that controls behavioral, physiological and biochemical rhythms resides in the SCN. The localization of a master pacemaker to a single structure, as seen in mammals, varies depending on the species in question. For example, in birds it comprises a complex system of multiple oscillators coupled together. These oscillators are located in the retina, pineal and the SCN (Cassone, M. Menaker 1984; Gwinner and Brandstatter 2001; Underwood et al., 2001). Previous studies have shown that all three are critical components of the avian circadian system, equally important in regulating overt rhythmicity. The avian pineal gland and retina share several key processes: (1) both are directly photoreceptive, (2) both contain circadian oscillators, and (3) both rhythmically synthesize and release the indoleamine neurohormone melatonin. As such they represent two sets of circadian pacemakers residing in the same organism, which one would expect to employ identical molecular mechanisms to generate and regulate their rhythmic functions. Since birds possess intracranial photoreceptors that are sufficient for circadian entrainment and photoperiodic time measurement, we believe the presence of the photopigment melanopsin within pineal tissues and in INL, RGL, and PL of the ocular retina provides a candidate for such a function.

CHAPTER VII

CONCLUSIONS AND PROSPECTUS

The present studies sought to determine candidate molecular clock components in the avian species *Gallus domesticus*. The determination of candidate avian clock genes was accomplished by examination of two avian circadian oscillators, the pineal gland and ocular retinae. Given the fact the avian system is not readily amenable to genetic manipulation, an alternate experimental approach was devised. Utilizing DNA microarrays, we determined the transcriptional profile of the pineal gland and retina, unveiling the mRNA profiles of several thousand genes over the course of one day in LD and one day in DD. A close examination of the two datasets revealed common rhythmic transcripts in both oscillators. Through further northern and *in situ* analyses, we have produced several candidate avian clock components and now pose new questions as to the function and interactions of these two oscillators in the avian system.

The transcriptional profile of the pineal gland advances the circadian field on several fronts. First, it allowed the identification of avian orthologs of mammalian clock genes, including putative negative elements *per2, per3, cry1,* and *cry2,* and positive elements *clock, bmal1,* and *bmal2* as well as *ck1e, dbt* and *tim.* All attempts to uncover any ortholog of the *period 1* gene failed, raising the possibility that mammalian clock genes may not be ubiquitous among avian species. In addition to the discovery of the orthologs, the transcriptional profiling of both oscillators established the rhythmic mRNA profiles of avian clock genes and their phase relationships. Specifically, *bmal1*

and *bmal2* mRNA are expressed rhythmically in LD and DD such that mRNA expression peaks at early night (ZT14/CT14), and *clock* exhibits a rhythmic mRNA profile with peak mRNA levels in the late subjective day. The oscillations of mRNA abundance for these genes are at a decreased (1.5 fold) amplitude. The putative negative elements, *per3* and *cry1* are also expressed rhythmically; however these oscillations occur at robust levels in the pineal gland. Increased levels of *per3* mRNA abundance occur in the late night/early day (ZT22-2/CT22-2), while c*ry1* message reaches peak mRNA levels during the day in LD and DD. An interesting feature of these two putative negative element oscillations is the phasing. Wherein, *per3* and *cry1* reach peak values of mRNA abundance at approximately opposite times of day. This is entirely inconsistent with the mammalian mRNA profiles for these genes within the SCN. Further the putative positive elements all peak at roughly the same time of day; which is consistent with mammalian data. However the time at which the putative positive elements reach peak mRNA abundance is not in an antiphase relationship to that of the negative element *cry1*. *Cry2* mRNA oscillated with a low amplitude bimodal rhythm with peak message at midday and late night. *Per2* was determined to exhibit an oscillating mRNA profile via northern analysis obtaining peak values during the day, but did not appear rhythmic on the microarray data. Presumably this is due to a poor PCR spot on the array. None of the other avian clock gene orthologs appeared rhythmic in the pineal gland under our analysis.

Examination of the transcriptional profile for the retina revealed several important similarities and disparities in mRNA patterns to that of the pineal gland. Not

surprisingly, the avian retina expresses avian clock genes including putative negative elements *per2, per3, cry1,* and *cry2,* and positive elements *clock, bmal1,* and *bmal2.* However, the patterns of mRNA abundance for certain clock genes in the retina do differ from their respective pattern of mRNA accumulation in the pineal gland. Retinal *bmal1* and *bmal2* are expressed rhythmically in LD and DD such that expression peaks at early night (ZT14/CT14), while *clock* does not appear significantly rhythmic under our criteria. Of the putative negative elements in the retina, only *per3* and *cry1* are expressed rhythmically at sufficient levels in LD and DD, such that *per3* is expressed predominantly in the late night/early day (ZT22-2, CT22-2) while *cry1* is expressed throughout the day in LD and subjective day in DD. Like the pineal gland other avian orthologs of the chick clock genes including *tim*, *dbt* and *ck1e* were found to be arrhythmic.

The temporal distributions of mRNA abundance in the pineal and retina for these clock proteins are interesting in several respects. First, there is no strict antiphase relationship of positive and negative elements that has been observed in *Drosophila* and mammalian models (Hardin 2000; Shearman et al., 2000). In chicks the *bmal* genes are expressed at high levels coincidentally with *cry1*. Second the *clock* gene is rhythmic in pineal gland which is also not the case in mammals. Third, the putative negative elements *per3* and *cry1* oscillate in roughly opposite phases. The differences between the temporal distributions of these mRNA species in these chick clock tissues and those of mammals and flies suggests that these genes may not function in the avian circadian system as they do in mammals. These avian orthologs may also perform other non-clock

related functions altogether. It is important to note that we have not examined the localization or regulation of the proteins these genes encode. As a result we can not rule out the function and interaction of these genes similar to that of the mammalian system.

In addition to the avian orthologs of mammalian clock and their rhythmic mRNA profiles in the pineal and retina, several very intriguing clock regulated transcripts were identified. First, the rhythmic expression of immune function genes was not expected. However, it has been shown that the chick pineal gland is a lymphopoietic organ (Cogburn and Glick 1981; 1983), and it is likely that the local regulation of lymphoid activity translates into both daily and seasonal regulation of lymphatic and immune activity in this species. Several studies on rodent immune function have similarly pointed to daily and seasonal changes in B- and T-lymphocyte activity (Brock 1983; Planelles et al., 1994; Haldar and Singh 2001). These changes have been associated with melatonin's actions on these cells in the blood (Demas and Nelson 1998; Drazen et al., 2000; Drazen et al., 2001).

Second, in both the pineal gland and retina tissues, GAPDH and cytochrome c oxidase are expressed rhythmically. Several recent studies have pointed to rhythmic processes involving metabolic activity that are independent of clock gene function. First, induction of clock gene rhythms does not necessarily induce metabolic rhythmicity in cultured cells (Allen et al., 2001). Wherein the induction of robust clock gene rhythms in fibroblast lines by serum shock has no effect on glucose utilization in these cells, even though rhythms of glucose utilization are accompanied by clock gene expression rhythms in SCN cells. Secondly, induction of metabolic rhythms in cultured astrocytes

does not necessarily result in rhythmic clock gene expression, since glucose utilization rhythms can be induced in chick astrocytes with cycles of melatonin (Adachi et al., 2002) but clock genes are not responsive to melatonin *in vitro* (Peters et al., 2003). McKnight and others have shown that redox state in *in vitro* systems can determine the affinity of negative elements such as the cryptochromes for positive element activation of clock gene promoter regions (Rutter et al., 2002). Both GAPDH and cytochrome oxidase have the potential of altering redox state via the reduction of NAD+. Thus, it is possible that the rhythms of intermediary metabolic elements we observe here in the retina and in the pineal gland may underlie a semi-independent oscillatory mechanism.

Thirdly, several ribosomal proteins, and elongation factor 2 are expressed rhythmically in both tissues, suggesting protein synthesis and modification may play a role in regulating circadian function in these two tissues. Both 40S and 60S ribosomal proteins are regulated at the mRNA levels in LD and DD, as are several sequences associated with proteosomal proteolysis, suggesting a global regulation of processes by the clock via the regulation of translational machinery. Translational control of clock components and their outputs that are independent of transcriptional regulation have been described in many model systems, including vertebrates. For example, in the filamentous fungus *Neurospora crassa*, circadian regulation of the clock gene *frq* and its protein FRQ can be separated by entrainment to long and short photoperiods, suggesting independent regulation of these two components (Tan et al., 2004). In *Gonyaulax*, circadian regulation of GAPDH levels and activity, a common clock-regulated protein (Morre et al., 2002), appears to be independent of mRNA levels (Fagan et al., 1999),

and, while AANAT activity is rhythmically regulated in the sheep pineal gland, no rhythm in mRNA levels can be determined (Coon et al., 1999). This post-transcriptional regulation of key clock components and their outputs may be the result of circadian regulation of either rhythmic protein synthesis and/or proteolysis.

Finally, the oscillations of mRNA species encoding phototransductive elements and retinaldehyde transport are interesting. The vertebrate visual cycle process involves at least three different retinal cell-types, the photoreceptors, pigmented epithelial cells and Muller glia. Thus, the problem of transported retinaldehyde, and by-products from one compartment to another and from the periphery into the retina is likely solved by transport proteins. One of these is purpurin (Berman et al., 1987), whose mRNA is rhythmically expressed with a very large amplitude in the retina and pineal gland. Purpurin was expressed highly during the early day and late night in LD and DD in both the retina and the pineal.

A series of experiments in mammals lacking functional rods and cones indicates normal circadian entrainment in these animals and the existence of an opsin based circadian photopigment responsible for entrainment. Interestingly, our experimental analysis determined the rhythmic mRNA profiles of several opsin bases photopigments within the pineal gland and retina including the leading candidates for the circadian photopigment in mammals, melanopsin (Provencio et al., 1998) and cryptochrome 1 (Sancar 2000). Additionally, several opsin-like and binding proteins associated with visual cycle function, such as peropsin (*Rrh*) (Sun et al., 1997), Retinal G-protein coupled receptor opsin (RGR-Opsin) (Jiang et al. 1993), retinal pigmented epithelium 65

(*Rpe65*) (Hinterhuber et al., 2004) were expressed rhythmically under both LD and DD conditions in either the pineal gland or retina. The rhythmic regulation and distribution of these genes in avian oscillators and in cell types associated with circadian photoreception provides candidates for the circadian photopigment in birds.

To further narrow the list of avian clock candidates a more detailed examination of several candidate photoisomerase/photopigment genes was conducted. Expression and regulation of these genes within photoreceptive cells and areas associated circadian clock and/or melatonin biosynthesis would provide a greater chance of these genes functioning as clock components. The mRNA profiles of peropsin, (*Rrh*) and RGR-opsin (*Rgr*), melanopsin (*opn4*) and cryptochrome 2 (*cry2*) were further characterized. g*Rgr* and *gRrh* message exhibited increased transcript abundance occurring in the subjective day for both genes in both the pineal gland retina. *In situ* hybridization indicates g*Rgr* and *gRrh* message are located within pineal tissues and in retinal layers associated with biological clock function including the pineal parenchyma, where melatonin biosynthesis is known to occur, and in the inner nuclear layer (INL) and retinal ganglion cell layers (RGL) of the ocular retina, where circadian photoreception is present. Intriguingly, both of these putative photoisomerases are also expressed throughout the brain. Similar to most non-mammalian vertebrates, birds possess extraocular photoreceptors that are sufficient for circadian entrainment, suggesting that these genes may mediate regeneration of extraocular opsin involved in these processes.

Further, the expression patterns of the *opn4*, and *cry2* genes of birds, is also very interesting. As stated above, these two genes are widely believed to play a crucial role in

circadian photoreception in mammals. In chicks, *opn4* and *cry2* are expressed in retinal layers associated with biological clock function including the inner nuclear layer (INL) and retinal ganglion cell layers (RGL) of the ocular retina, where circadian photoreception is present, and also the photoreceptor layer where melatonin biosynthesis occurs. In addition, a striking overlap of mRNA distribution in the brain exists, wherein both genes are located within structures associated with the visual system including the pineal gland, cerebellum, and optic tectum. Lastly, melanopsin and cryptochrome 2 mRNA is regulated on a circadian basis in the pineal gland, *opn4* and *cry2* express a robust rhythm in mRNA abundance reaching peak values in late subjective night.

Together these data yield a significant insight into the mechanisms of the avian circadian system. This analysis has provided several candidate genes which may be responsible for clock function. These data also provide evidence that the avian pineal and retina are more diverse in function than previously appreciated. The regulation of several important aspects of avian physiology at the transcriptional level including immune and stress and metabolic function indicates such a role. In addition the data indicate a very tightly linked distribution and regulation of several clock regulated genes and pose the possibility that the candidate genes examined here function to help integrate photic information, and/or play a role in circadian rhythm generation. Naturally being a candidate clock component does not constitute a real clock gene. Therefore it is vital that these candidates presented here are further examined at the protein level and for functionality. A potential approach for this genetic manipulation is the knock-down or overexpression study of these genes protein products in an *in vitro* system of

pinealocytes. Experimental evidence indicates pinealocytes in culture continue to produce the hormone melatonin rhythmically for several cycles in constant conditions. This circadian output provides a direct marker of the fitness of the pinealocyte system *in vitro*. Therefore application of an RNAi or opposite approach of gene overexpression, to the pinealocyte cell culture system would provide an avenue to pursue protein function. One would expect that this system, once perfected, would allow the application of a construct to inhibit or induce a specific clock protein in the pineal cells. If the construct was effective at inhibiting or inducing the specific protein and that protein was functioning as an integral component of the pineal clock then the output rhythm of melatonin production would be altered. This type of manipulation would provide an invaluable tool to genetically dissect the avian circadian clock at a molecular level and to directly examine the candidates determined here.

In summary, the chick pineal gland and retina share many conserved rhythmic mRNA species. Among the common elements between the rhythmic transcriptomes in these tissues are key components of phototransduction pathways, intermediary metabolism and protein synthesis and degradation and orthologs of mammalian clock genes. However, the details of their regulation are both similar and different, raising the reasonable possibility that the "clocks" in each of these tissues are differentially regulated. These rhythmically regulated conserved genes of the pineal and retina should be considered as top candidates for clock regulation and further tested through *in vitro* manipulation of the pineal gland to solidify their function as clock components or merely clock controlled elements.

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APPENDIX

Dear Mr Bailey

FEBS Letters, Vol 513, No 2-3, 2002, Pages 169-174, Bailey et al, "Chickens' Cry2: molecular analysis"

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Texas A&M University, Graduate student, 1999-Present
Functional genomics of the chick circadian system.

PUBLICATIONS

1. Bailey MJ, Chong NW, Xiong J, Cassone VM. Chickens' Cry2: molecular analysis of an avian cryptochrome in retinal and pineal photoreceptors. FEBS Lett. 2002. 513, 169-74.

2. Bailey MJ, Beremand PD, Hammer R, Bell-Pedersen D, Thomas TL, Cassone VM. Transcriptional profiling of the chick pineal gland, a photoreceptive circadian oscillator and pacemaker. Mol Endocrinol. 2003. 17, 2084-95.

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