Screening RNAi Transformants Of Chlamydomonas For Reduced Expression Of The Photoreceptor Cryptochrome

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SCREENING RNAi TRANSFORMANTS OF *CHLAMYDOMONAS* FOR REDUCED EXPRESSION OF THE PHOTORECEPTOR CRYPTOCHROME

A Capstone Experience/Thesis Project

Presented in Partial Fulfillment of the Requirements for

the Degree Bachelor of Science with

Honors College Graduate Distinction at Western Kentucky University

By

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*****

Western Kentucky University
2011

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ABSTRACT

*Chlamydomonas reinhardtii* is a unicellular green alga. In *C. reinhardtii*, three types of photoreceptors are known to be present: rhodopsins, phototropins, and cryptochromes. The single cryptochrome is the most likely photoreceptor for adjusting the circadian clock to the daily light/dark cycles, because cryptochromes are involved in clock entrainment in higher plants and insects. In this segment of the research, *C. reinhardtii* strains, which were genetically modified through transformation with a RNA interference construct, were screened for reduction in cryptochrome compared to the control strain. After *C. reinhardtii* cultures were harvested in complete darkness, all soluble proteins were extracted from the cells. The proteins were subjected to Western blot (immunoblot) analysis with chemiluminescent detection. Quantification of the images obtained with a digital camera revealed cryptochrome reductions between 0 and 50 percent in the ten strains tested. Should the strains with 50 percent reduction show a reduced ability to entrain to light/dark cycles, such results would suggest that cryptochrome is the primary photoreceptor that controls circadian clock resetting in *C. reinhardtii*.

Keywords: Chlamydomonas, photoreceptor, cryptochrome, RNA interference, circadian clock, western blot analysis
Dedicated to my family and those who helped me along the way
ACKNOWLEDGMENTS

That I was able to perform research and write an honors thesis is a result of extreme generosity by my primary advisor, Dr. Sigrid Jacobshagen. I truly thank her for her willingness to take me on as a student researcher and for her continual patience, encouragement, and support. Her guidance throughout this process has been greatly appreciated.

I would also like to thank Dr. Lesa Dill for her gracious assistance in helping me revise my thesis from the beginning. Her teaching in class and on this project has made me a better writer, not to mention a better reader. Lastly, I extend thanks to Dr. Walter Collett for serving on my thesis committee and also to the Honors College for providing the framework for my thesis and providing me with indispensable resources and advice.

Finally I would like to honor my dad, mother, and sister, who have supported and encouraged me until the very end. Without them I would not have been in the position to even start a thesis. They know they are cherished beyond compare.

“But thanks be to God, who has given us victory through our Lord Jesus Christ.”

1 Corinthians 15:57
August 19, 1989……………………………….. Born – Bowling Green, Kentucky

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Second Major Field: Chemistry

Concentration: Pre-Medicine
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CHAPTER 1

INTRODUCTION

Almost every aspect of lives of organisms is controlled by an endogenous mechanism known as a circadian clock. Metabolic, physiological, and behavioral processes show daily rhythms because of this clock. The clock can be described by three parts: the input sector, the central oscillator, and the output sector (Fig. 1.1). Environmental changes such as light versus dark provide time cues which the input sector decodes and sends as information to the central oscillator for entrainment. The central oscillator creates cycles of about a day and sends timing messages to the output sector, producing rhythmic behavior such as larval eclosion in insects, sleep/wake cycle in mammals, and flowering in plants (4). However, any depiction of the circadian clock as a simple three-component system involving one-way flow belies the level of interaction between input and output pathways. Instead, the input and output pathways are subject to regulation through various feedback mechanisms involving the central oscillator and each other. For example, light signaling is specifically known to be adjusted by output pathways during the daytime.

For plants, this internal clock maximizes fitness by ensuring that photosynthetic
The circadian clock is composed of three main components: input sector, central oscillator, and output sector. The central oscillator maintains a roughly 24 hour cycle and produces output in the form of circadian rhythms in phototaxis and metabolic changes or in flowering times. The input sector senses changes in factors like temperature and light over time in the environment which lead to entrainment of the circadian rhythm.
components are ready for light-capturing at dawn, and it also regulates chemotaxis toward nutrients in algae so that the organism can locate and accumulate nutrients when solar energy is low or unavailable. In mammals, the circadian clock enables cycles of optimum readiness and temperature levels during the daytime while allowing a state of rest at night. The circadian clock has also been shown in the green alga Chlamydomonas reinhardtii to regulate cell division (2, 7). The timing of cellular division to night hours allows organisms as diverse as yeast, cultured mammalian cells, and plant cells to reduce DNA damage from UV light (11).

The approximately 24-hour cycling created by the circadian clock is in part a result of the change in concentration or reactivity of cellular components. The clock must, however, also have a means to synchronize itself with day/night cycles. In most situations, a prolonged period of light must be present in order for the organism to reset its circadian clock. Once set, the circadian clock can be maintained regardless of a lack of external cues. Previously entrained plants, which are placed under conditions of constant light, will still produce a rhythm. For example, a human who is placed inside a structure closed off from outside sunlight will continue to show signs of sleepiness in cycles of approximately twenty-four hours. Two environmental factors that are known to be able to entrain the circadian clock by acting as Zeitgebers (time givers) are light and temperature, the former considered to be the most influential. Under normal circumstances, cooler temperatures often mark the night, while warmer temperatures indicate the day. Increasing amounts of light prepare the organism for the day's activities. Conversely, decreases in light signal the end of day for the organism.
Because light is so important in development and throughout the life of plants, it is necessary that there be an effective structure to capture light or receive external cues from the environment to entrain the clock. Perception is accomplished through photoreceptors, which are proteins that are present in both plants and animals. These photoreceptors are responsible for both absorbing light and mediating light responses. Since the initial discovery of a blue-light photoreceptor in Arabidopsis (1), more photoreceptors have been discovered in other species. More commonly seen are the melanopsins (in mammals), rhodopsins, cryptochromes, phototropins, and phytochromes.

Cryptochromes are present in animals and plants (3). In fruit flies and higher plants cryptochromes have been implicated in the entrainment of the circadian clock by light (5). In mammals, including humans, they form part of the central oscillator mechanism instead. It is not known whether cryptochrome is part of the circadian clock in the green alga Chlamydomonas reinhardtii. Cryptochromes are blue/ultraviolet-A photoreceptor flavoproteins, which are subject to degradation in the presence of light (10). Additionally, absorption spectrum tests on cryptochrome have indicated a slight peak noticeable in the green light region, suggesting that the photoreceptor may produce responses to green light as well (13). The resemblance of N-termini between cryptochrome and the type II photolyase enzymes, which mediate DNA repair, indicate a homology. The photolyase and cryptochrome also share similar three-dimensional structures, including an α/β domain and a helical domain (9). This finding suggests that the photolyase, a flavoprotein as well, could be cryptochrome's evolutionary precursor (3). This relationship is thought to be the reason why cryptochromes, like photolyases,
may function by mediating a light-dependent redox reaction. Two forms of the *C. reinhardtii* cryptochrome exist with molecular masses of approximately 126 and 143 kD, which are thought to be a result of post-translational modification (12). Cryptochromes have been demonstrated to act as a requirement for phytochrome A signaling to the circadian clock in both blue and red light in plants. Although debatable, experiments show that cryptochromes do not comprise a part of the central oscillator in plants as is observed in mammals (4).

In this study, I propose to investigate the extent to which strains of *C. reinhardtii* with a RNA interference construct for cryptochrome show reduced expression of the cryptochrome photoreceptor. RNA interference is a method by which a particular messenger RNA (mRNA) is degraded in the cell so production of the protein encoded by the mRNA is reduced. Earlier studies involving the photoreceptor phototropin have shown that knock-down strains can be successfully isolated using the RNA interference method. The knock-down strains were defective in their blue-light-dependent sexual life cycle, indicating that phototropin is the photoreceptor that mediates this response (8). Further experimentation with the cryptochrome knock-down strains will test for possible impaired resetting of the circadian clock as well as production of abnormal circadian rhythms in these strains. Reduced resetting abilities will indicate cryptochrome’s potential role as primary mediator of photoentrainment in *C. reinhardtii*. 
Strain Maintenance

The RNA interference construct for cryptochrome was previously cloned and transformed into *C. reinhardtii* by Matthew Young. All transformants, numbering approximately 50, were individually transferred from a multi-strain HSM media plate to a single YA media plate, onto which they were grown underneath a plant light. Because these plates were heavily contaminated with bacteria, the antibiotic ampicillin to a final concentration of 100µg/ml and 200µg/ml was added to suppress bacterial growth. Streaking was performed weekly initially and biweekly thereafter.

Cell Harvesting

When the cultures were sufficiently free of bacteria, 50 milliliter liquid TAP medium in 125 milliliter Erlenmeyer flasks were inoculated and grown on a shaker to late log phase (2 to 3 x 10^6 cells/ milliliter). The flasks were placed into dark for approximately 15 hours by a wrapping of aluminum foil. In complete darkness, each sample flask was unwrapped and 10 milliliters were transferred to each of two 15 milliliter centrifuge tubes. These tubes were spun in a clinical centrifuge at the highest
setting for 1 minute and 30 seconds and the supernatant discarded. Four hundred microliter of previously prepared Laemmli Final Sample Buffer (62.5 mM Tris-HCl, pH 6.8; 2% SDS; 50 mM dithiothreitol (DTT); 10% glycerol; 0.001% bromophenolblue) was then added to each tube. These tubes were vortexed for 20 seconds before being frozen in liquid nitrogen and finally stored in the -80°C freezer.

Sample Preparation for Electrophoresis

Each sample was thawed and vortexed thoroughly to ensure re-suspension of the pellet. The sample was then treated with a Sonifier at 40% amplitude 5 times for 10 seconds with 20 seconds cooling interval. After sonication the samples were transferred to a microfuge tube with screw cap and boiled for 1 min, cooled on ice for 2 minutes, and spun for 2 minutes in a microcentrifuge at 17,000 x g. The supernatant was then transferred to a fresh microfuge tube. The absorbance of the supernatant was measured at 280 nanometers and 260 nanometers by combining 20 microliter of supernatant in 980 microliter of water in UV-plastic cuvets. Using these data and the formula for raw extracts the volumes to be loaded for electrophoresis were calculated (Protein conc. (mg/ml) = 1.55 A_{280nm} – 0.76 A_{260nm}).

SDS-Polyacrylamide Gel Electrophoresis

Three separate gels were prepared at one time: one, which was stained for proteins, and two, which were transferred to a membrane for antibody hybridization. For each a resolving gel was made (375 mM Tris-HCl, pH 8.8; 0.1% SDS; 7.5% acrylamide/
bisacrylamide; 0.5 mg/ml ammonium persulfate 0.03% TEMED), followed on top by a stacking gel (125 mM Tris-HCl, pH 6.8; 0.1% SDS; 3.9% acrylamide/bisacrylamide; 0.5 mg/ml ammonium persulfate 0.1% TEMED) with combs inserted, according to protocol. These gels were then placed into the electrophoresis setup inset while the inner chamber was filled with electrophoresis buffer (24.8 mM Tris; 134.2 mM glycine; 0.1% SDS). The outer chamber was also filled with electrophoresis buffer and the sample slots were rinsed with the buffer before loading. Ten micro-liters of pre-stained marker proteins were loaded followed by the remaining samples, including the CC48 control sample, per pre-calculated volumes so that equal amounts of total proteins were loaded in each lane. The electrophoresis was performed at 160 volts for 60 minutes. Once completed, gels were removed from the glass plates so that one could be incubated in Coomassie staining solution while the other two in transfer buffer. The gel stained with Coomassie blue was first incubated in 12% trichloroacetic acid for 10 minutes before it was rinsed with nanopure water and then incubated in Coomassie staining solution (20 ml 10% \((\text{NH}_4)_2\text{SO}_4\), 2% phosphoric acid; 5 ml methanol; 0.5 ml 5% (m/v) Coomassie Brilliant Blue G-250) overnight. The staining solution was then removed before the gel was briefly rinsed with methanol and stored in 7% acetic acid.

**Western Blotting**

Two membranes were cut and soaked in transfer buffer (25 mM Tris; 192 mM glycine; 20% methanol) along with Whatman filter paper, fiber pads, and the two gels
from the electrophoresis. After preparing two gel sandwiches from the above materials, the blotting setup was assembled and the proteins transferred from the gels to the membranes at 350 milliamperes, 100 volts for one hour. Afterwards the gels were stained with Coomassie, while the membranes were stained with Ponceau solution (0.2% Ponceau S; 1% acetic acid) to make all protein bands visible. Protein marker bands were marked with a pencil before the membranes were set to dry on filter paper in the dark.

Detection by Chemiluminescence

The membranes were soaked in PBS-TM (PBS-T with 3% dried milk) for 1 hour with gentle shaking to block nonspecific protein binding sites. In separate containers, a 1:5000 dilution of the primary anti-cryptochrome antibody in 50 milliliter of PBS-TM was made, while a 1:250,000 dilution in 50 milliliter of PBS-TM was made for the anti-β subunit of CF1 control antibody. The membranes were incubated in these solutions for 1 hour with gentle shaking. Next the membranes were rinsed twice and washed once for 15 minutes, and twice for 5 minutes with PBS-T (1 x PBS; 0.1% Tween-20). Then the secondary antibody was made at a 1:5000 dilution in 50 milliliter of PBS-TM and both membranes were incubated in the same container. Then the membranes were rinsed and washed again with PBS-T as before. Filter paper slightly larger than the membranes was cut and laid onto overhead foil to be used for each membrane. The filter papers were wetted individually with ECL solution, which consisted of 2 milliliter of ECL soln. 1 (100 mM Tris-HCl, pH 8.5; 2.5 mM luminol (aminophthalhydrazide); 0.4 mM p-coumaric acid) mixed with 2 milliliter of ECL soln. 2 (100 mM Tris-HCl, pH 8.5;
Air bubbles were rolled out using a disposable pipette. Then each membrane was placed on top of a filter paper and Saran™ Wrap was laid on top of it.

Using an Alpha-Innotech FluorChem HD2 Imaging System, chemiluminescence was detected for each membrane. After pushing the "Acquire" button for the camera in the computer program, opening the aperture fully, centering and zooming to fit the membrane onto the computer screen, and focusing the camera, the cabinet door was closed and the membranes were exposed. The empty filter setting was used, "Chemi Display" was selected and "High/Medium" was chosen as the setting for Sensitivity/Resolution. The Cryptochrome membrane was initially exposed for 30 minutes and, if necessary, exposure was repeated with different times for optimal results. The anti-β subunit of CF1 control membrane was initially exposed for 5 minutes.

Quantification of Cryptochrome Expression

Using the option “band analysis” under “analysis tools” of the imaging program, a box of equal area was drawn around each cryptochrome band, the control band for each lane, as well as background regions. An integrated density value (IDV) was automatically calculated for each box drawn. The average IDV for the background was subtracted from each cryptochrome band and each control band to eliminate the background from the area under each band. In order to account for any unevenness in sample loading, cryptochrome data were normalized to each lane’s control band. In the end, the data were calculated as percentage of cryptochrome amount in each transformant as compared to the wild-type.
Statistical Analysis

Two independent experiments were performed. Statistical analysis of the difference values between the mean values for wild-type CC48 control and RNAi strains was accomplished by performing a Student’s t-test for correlated samples. Statistical significance was accepted at $p \leq 0.05$. Statistical calculations were made using a statistical calculator programmed by Vassarstats.
Wild-type and strains transformed with the RNA interference construct were grown until late log phase, at which point the cultures were put into dark for an overnight period (12-15 hrs). Cell harvesting was performed in the dark to prevent light degradation of cryptochrome. Gel electrophoresis separated all proteins well, as shown for the example gel in figure 3.1. Although not clearly visible, the cryptochrome protein appears at approximately 160 kDa in size. It is clear from the protein gel that all protein samples were loaded in relatively equal concentrations, with the exception of strain #28. Protein gels for the second of the two independent experiments with these particular strains as well as that for wild-type CC48 and strains #36, #37, #40, #41, and #43 were comparable to figure 3.1.

Blotting of two membranes for treatment with anti-CF1 control antibody and anti-cryptochrome antibody resulted in even transfers, as shown in figure 3.2. The blotted membranes revealed a lightness of color in lane 5 for strain #28, which is consistent with lane 5 in figure 3.1. Staining of the protein gels after transfer indicated only small amounts of residual proteins that did not transfer (not shown). Cryptochrome and control membranes for wild-type CC48 and strains #36, #37, #40, #41, and #43 are shown in
**Figure 3.1.** SDS-PAGE of proteins of *C. reinhardtii* transformants. Separated proteins were stained with Coomassie Brilliant Blue. Lanes – 1: Marker proteins; 2: wild-type CC48 3: #20; 4: #21; 5: #28; 6: #31; 7: #33. Each lane was loaded with 228.3 microgram of total protein. This gel demonstrates that proteins are equally loaded in terms of concentration per lane with the exception of lane 5.
Figure 3.2. Blotting of proteins onto nitrocellulose membranes. Transferred proteins were stained with Ponceau S. Lanes 2-7 show transferred proteins for CC48, #20, #21, #28, #31, #33, respectively, as well as marker proteins (lane 1). A, Membrane used to detect β-subunit of chloroplast ATP synthase with the anti-CF1 control antibody. B, Membrane used to detect cryptochrome protein with the anti-cryptochrome antibody.
Clear separation between bands allows for differentiating strains. The strains do not show apparent signs of major reduction in their cryoprobe amount (figure 3.3 A). Roughly equal intensities of control bands can be detected (figure 3.3 B) suggesting equal loading of total protein in each lane. The cryoprobe bands were quantitated and normalized to the control bands using their integrated density values. The results of this analysis are shown in figure 3.4. The integrated density value of wild-type CC48 was set as 100%, which allowed for representing the integrated density values of the other strains as a percentage of the wild-type strain. All strains except #36 displayed over 100% expression of the cryoprobe protein, with #36 showing over 75% expression of the protein.

The exposed membranes of the experiment testing wild-type CC48 and strains #20, #21, #28, #31, and #33 are shown in figure 3.5. One band, #31, shows particularly low amounts as a visible sign of possible reduction (figure 3.5 A). Despite the slight smear visible between lanes 2-3 of the control membrane (figure 3.5 B), the control bands appear roughly equal, which was confirmed in the quantitative analysis. A second independent experiment was conducted testing the same strains, which is shown in figure 3.6. Again, distinctive separation and roughly equal appearance of control bands (figure 3.6 B) suggest equal loading of total protein in each lane. The average of both independent experiments after quantitation, together with the standard deviation, is illustrated in figure 3.7. With wild-type CC48 set as 100% expression of cryoprobe protein, strains #20 and #31 show reduction of about 50%. The differences in expression
between strains #20 and #31 and the wild-type were determined as statistically significant (p = 0.04 for RNAi strain #20 and p = 0.05 for RNAi strain #31, Student’s t-test for correlated samples, one tail). This indicates that cryptochrome expression by strains #20 and #31 was significantly lower than the control strain.
Figure 3.3. Western Blot analysis of cryptochrome amounts: Strain set 1. Detection was accomplished by chemiluminescence of wild-type CC48 (lane 1) and strains #36, #37, #40, #41, #43 (lanes 2, 3, 4, 5, and 6, respectively). A, Membrane incubated with anti-cryptochrome antibody. B, Membrane incubated with anti-β subunit of chloroplast ATP synthase as control antibody.
Figure 3.4. Quantitation of cryptochrome amounts: Strain set 1. Percentage of cryptochrome expressed by strains #36, #37, #40, #41, and #43 relative to wild-type CC48 control (100% expression). Data were calculated by normalizing the integrated density value (IDV) for the cryptochrome band of each strain against the CF1 control antibody band for each strain.
Figure 3.5. Western Blot analysis of cryptochrome amounts: Strain set 2. Detection was accomplished by chemiluminescence of wild-type CC48 (lane 1) and strains #20, #21, #28, #31, #33 (lanes 2, 3, 4, 5, and 6, respectively). A, Membrane incubated with anti-cryptochrome antibody. B, Membrane incubated with anti-β subunit of chloroplast ATP synthase as control antibody.
Figure 3.6. Independent repeat experiment of Western Blot analysis of cryptochrome amounts: Strain set 2. Detection was accomplished by chemiluminescence of wild-type CC48 (lane 1) and strains #20, #21, #28, #31, #33 (lanes 2, 3, 4, 5, and 6, respectively). A, Membrane incubated with anti-cryptochrome antibody. B, Membrane incubated with anti-β subunit of chloroplast ATP synthase as control antibody.
Figure 3.7. Quantitation of cryptochrome amounts: Strain set 2. Percentage of cryptochrome expressed by strains #20, #21, #31, and #33 relative to wild-type CC48 control (100% expression). Data were calculated by normalizing the integrated density value (IDV) for the cryptochrome band of each strain against the CFI control antibody band for each strain. The data represent the average of two independent experiments. Standard deviation bars are included to show range of difference between the two independent experiments.
CHAPTER 4

DISCUSSION

It is not yet known what specific function the protein cryptochrome has in *Chlamydomonas reinhardtii* (11). It stands as a likely candidate for the primary photoreceptor that mediates photoentrainment. Identification of the photoreceptor responsible for photoentrainment will lead to a greater understanding of the other components of the organism’s circadian system as well as the interactions between them. Screening of the RNAi transformants in this experiment provides strains for future testing that show significant decreases in cryptochrome protein expression. Possession of these strains allows for the testing of any abnormalities in the resetting of their circadian clock in response to blue light. An abnormality will suggest that cryptochrome plays a primary role as mediator of photoentrainment.

Analysis of RNAi strains #20 and #31 reveals a moderate reduction of about 50% (figure 3.7). Additionally, the relatively low standard deviation associated with the results from two independent experiments suggests a remarkably low variation between them. Moreover, the t-test performed demonstrates that the difference in expression between the RNAi strains and the wild-type CC48 control strain was significant. Percent
reductions were determined by interpreting the integrated density values calculated by the imaging program for the RNAi strains as a percentage of that of the CC48 control. In order to determine this, background areas around the bands had to be eliminated. Most of the strains tested display only low levels of reduction, if at all. This can be explained by considering the original transformation of the RNAi strains, whereby the gene in the RNAi construct has been silenced, which is a common occurrence in *C. reinhardtii*. As a result, some strains express less cryptochrome than others.

Normal amounts of variation between experiments cannot practically be avoided, though serious efforts were taken to ensure continuity between them. Liquid cultures of RNAi transformants were grown overnight in both independent experiments, and the number of hours in which the cultures were in complete darkness varied by no more than 2-3 hours. It is possible, though not known, that a difference in a few hours in darkness could affect protein accumulation in the samples. Research is currently being conducted in this research lab to determine whether a significant difference exists in protein expression for samples harvested at various times in darkness.

Subsequent research will focus on strains such as #20 and #31 to see if they produce abnormal rhythms in response to blue light pulses. This research will use a phototaxis machine, which measures rhythms of phototaxis of small algal samples in an automated fashion (6). Rhythms that are not properly reset in response to light pulses might indicate an altered or impaired circadian clock and could provide data which support the hypothesis that cryptochrome is the primary photoreceptor involved in photoentrainment in *C. reinhardtii*. 


