Cloning of "Animal Cryptochrome" cDNA from the Model Organism CHLAMYDOMONAS REINHARDTI for Functional Analysis of Its Protein Product

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CLONING OF “ANIMAL CRYPTOCHROME” cDNA FROM THE MODEL ORGANISM *CHLAMYDOMONAS REINHARDTII* FOR FUNCTIONAL ANALYSIS OF ITS PROTEIN PRODUCT

A Thesis
Presented to
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In Partial Fulfillment
Of the Requirements for the Degree
Master of Science

By
Shobha Lavanya Silparasetty
December 2009
CLONING OF “ANIMAL CRYPTOCHROME” cDNA FROM THE MODEL ORGANISM CHLAMYDOMONAS REINHARDTII FOR FUNCTIONAL ANALYSIS OF ITS PROTEIN PRODUCT

Date Recommended __September 21st, 2009__

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Dean, Graduate Studies and Research Date
I would like to express my gratitude to my thesis advisor, Dr. Sigrid Jacobshagen, who helped me understand the theme of the thesis. I am so grateful to have studied under her. She always encouraged me when things went bad and boosted my spirits, when I was low. Her patience with language is also appreciated. I would also like to thank my other graduate committee members, Dr. Claire Rinehart and Dr. Cheryl Davis. I would also like to thank the Dean of the Graduate Studies, Dr. Richard Bowker, and all the departmental staff for their support.

I would like to thank Dr. John Andersland for his ideas and suggestions on my presentations throughout my graduate studies. I thank Mr. Rick Fowler for his help and invaluable suggestions on PCR, allowing me to use his facilities at the Water’s Lab. My special thanks to our lab technician and a good friend of mine, Mrs. Jennifer Forbes-Stovall. Last but not the least, I would like to thank Ali Wright, Kishore Polireddy and all other graduate students for their suggestions on working with the thermocycler.

Finally, I would like to thank my parents in India for their love and constant support.
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CLONING OF “ANIMAL CRYPTOCHROME” cDNA FROM THE MODEL ORGANISM CHLAMYDOMONAS REINHARDTI FOR FUNCTIONAL ANALYSIS OF ITS PROTEIN PRODUCT

Chlamydomonas reinhardtii, a unicellular green alga, is a model organism to study the circadian clock. Cryptochromes are the blue light photoreceptors that entrain the clock in some organisms. The CPH1 protein of C. reinhardtii resembles the cryptochromes of the plant model Arabidopsis, but it is not yet known if CPH1 entrains the circadian clock in C. reinhardtii. Recent reports have suggested the existence of one more cryptochrome in C. reinhardtii, which resemble the cryptochromes of animals. However, the amino acid sequence of this protein shows even higher sequence similarity with the 6-4 DNA photolyase of Arabidopsis. DNA photolyases are involved in the repair of UV light-induced DNA damage using the energy of blue light. In order to determine if the “animal cryptochrome” gene of C. reinhardtii actually encodes a 6-4 DNA photolyase rather than a photoreceptor, an experimental design was developed to test whether the protein product is able to rescue an E. coli mutant defective in its DNA photolyase gene. The proposed experimental design is as follows: In a first step, the coding region of the “animal cryptochrome” cDNA is cloned. In a second step, the cDNA is inserted in-frame into an E. coli expression vector. In a third step, the construct is
transformed into an *E. coli* photolyase mutant (*phr*<sup>−</sup> *uvr*<sup>−</sup> *recA*), its expression induced, and the strain tested for better survival after UV light exposure.

In the present study, to accomplish the first step of cloning the “animal cryptochrome cDNA, total RNA was successfully extracted from *C. reinhardtii* 4 hrs into the light phase of a 12 h light/12 h dark cycle and reverse transcribed into cDNA using oligo(dT) primers. After several initial unsuccessful attempts at amplifying animal cryptochrome from cDNA or genomic template with a variety of primers and conditions, a short fragment with the expected size of 186 bp was successfully amplified with both templates. However, even this fragment was not reliably obtained in every PCR assay. Because of this difficulty, real-time PCR was finally performed in the presence of DMSO (dimethylsulfoxide) and betaine. These two adjuvants have been reported to improve amplifications particularly for GC-rich templates. *C. reinhardtii* DNA is especially GC-rich with an average of 64% Gs and Cs. The improved conditions allowed for the reliable amplification of the 186 bp fragment from genomic template. It also enabled the amplification of a larger fragment of 528 bp from the same template. These results suggest that a combination of 5% DMSO and 1M betaine is optimal for the amplification of *C. reinhardtii* DNA and thus can serve as the basis for successful amplification of the entire 1788 bp coding region of the animal cryptochrome cDNA.
CHAPTER I
INTRODUCTION

*Chlamydomonas reinhardtii* is a unicellular green alga with a cell wall, two anterior flagella that are involved in motility and reproduction, and a chloroplast that is required for photosynthesis (Merchant *et al.*, 2007; Shrager *et al.*, 2003; Rochaix, 1995). It represents a model organism for research on eukaryotic cellular processes (Harris, 1989) because it has a completely sequenced, haploid genome (Shrager *et al.*, 2003; Merchant *et al.*, 2007) available at the NCBI website (Grossman *et al.*, 2003) and responds to a large number of environmental changes (Mittag and Wagner, 2003; Shrager *et al.*, 2003; Mittag *et al.*, 2005). One major research area for which *C. reinhardtii* serves as model organism is the study of the circadian clock (Mittag *et al.*, 2005; Breton and Kay, 2006).

The circadian clock is an endogenous timer with an approximately 24 hr period that is found in most eukaryotes and in some photosynthetic bacteria (Devlin, 2002). It allows organisms to adjust to the daily environmental changes and to the seasonal variations (Takahashi, 1993). Conceptually, the circadian clock is made up of three components (Figure 1): the core central oscillator, the input pathways, and the output pathways (Devlin, 2002; Aronson *et al.*, 1994). The central oscillator consists of interconnected transcriptional-translational feedback loops resulting in rhythmic release of transcripts and production of clock proteins. There is some evidence that a single cell may contain more than one central oscillator. In the organism *Gonyaulax*, at least two oscillators have been reported that are not coupled under certain experimental
Figure 1: Model of the circadian clock (Devlin, 2002). The three components of the clock model are: the central oscillator responsible for the generation of the rhythms in a cell, the input pathway that entrains the oscillator to the daily environmental time cues, and the output pathway that transfers the signals from the oscillator to different biological processes that can be measured. In addition, the output pathway might also regulate the input pathway, for example by gating the expression of input photoreceptors to only certain times of a circadian cycle.
conditions (Merrow et al., 2005). The input pathways serve to entrain the central oscillator by perceiving the daily changes in environmental stimuli to reset the circadian clock. Light and temperature are the most important environmental stimuli for the entrainment process. Output pathways couple timing information from the oscillator with downstream genes causing cellular processes to exhibit a circadian rhythm. In mammals and insects, the master clock is located in a group of neurons in the brain while peripheral clocks are located in other parts of the body (Bell-Pedersen et al., 2005). These peripheral clocks can function autonomously although there is some coupling to the master clock. The peripheral clocks in insects are also directly entrained by light, which is not the case in mammals (Bell-Pedersen et al., 2005).

The circadian clock gives rise to circadian rhythms of different biological processes like amino acid uptake, nitrogen metabolism, photosynthesis, carbohydrate metabolism, respiration, etc. (Johnson, 2004). These rhythms are widespread and involve functions as diverse as the human sleep-wake cycle and cyanobacterial nitrogen fixation. In the plant model Arabidopsis, a number of physiological processes like leaf and petal movements, opening of stomata, elongation of hypocotyls, and induction of flowering are rhythmically controlled by the circadian clock (Green et al., 2002). In C. reinhardtii, circadian rhythms can be observed in various physiological behaviors like phototaxis, chemotaxis, cell division, UV sensitivity, and adherence to glass (Jacobshagen et al., 2001).

Since circadian rhythms are regulated by the circadian clock, they show three basic characteristics (Johnson and Hastings, 1986). First, they are self-sustaining oscillations whose period length under constant conditions is about 24 hr. Secondly; they
show temperature compensation of their period. Temperature compensation is a mechanism of maintaining the same rate of a rhythm even at different environmental temperatures. This is an important requirement in order for the circadian clock to be truly adaptive. The third characteristic of a circadian rhythm is that environmental signals reset the phase and thereby entrain the rhythm to the daily environmental changes. These rhythms can persist without external time cues, which imply that they are driven by an internal clock.

If light acts as an environmental stimulus to reset the circadian clock, it is first perceived by particular photoreceptors. In the fruitfly *Drosophila*, clock entrainment is mediated by a cryptochrome-type photoreceptor encoded by a single gene (Stanewsky *et al.*, 1998). Cryptochromes are flavin-containing photoreceptors (Cashmore, 2003; Devlin, 2002) that absorb in the blue and UV-A region of the light spectrum (Immeln, *et al.*, 2007). In the model plant *Arabidopsis*, cryptochrome- as well as phytochrome-type photoreceptors were reported to reset the circadian clock (Thompson and Sancar, 2002). Phytochromes are unique to plants and algae. They absorb light in the red and blue region. *Arabidopsis* contains two different cryptochromes and five different phytochromes. Nearly all of these photoreceptors are reported to reset the circadian clock (Devlin, 2002). The only exception is phytochrome C, whose involvement has not been tested. The two cryptochromes of mammals were also found to be involved in the circadian clock, but surprisingly they function as part of the central oscillator rather than the input pathway (Cashmore, 2003). The photoreceptor that resets the circadian clock in mammals is a rhodopsin-type photoreceptor called melanopsin (Hattar *et al.*, 2003).
Rhodopsins are photoreceptors that absorb light in the blue-green region (Fuhrmann et al., 2001).

The kind of photoreceptor that resets the circadian clock in *C. reinhardtii* is not known. It could be any of the three types of photoreceptors experimentally demonstrated in the organism or even an unknown type. The photoreceptors reported for *C. reinhardtii* are of the cryptochrome, rhodopsin, and phototropin type (Huang and Beck, 2003; Sineshchekov et al., 2002; Fuhrmann et al., 2001). Phototropins are a class of photoreceptors unique to plants and algae that absorb light in the blue and UV-A region (Devlin, 2002). In *Arabidopsis*, there is some evidence that they are not involved in resetting the circadian clock. The most likely candidate for circadian clock entrainment in *C. reinhardtii* is probably a cryptochrome-type photoreceptor. Cryptochromes are of particular interest in this alga, because it was recently reported that *C. reinhardtii* might contain a second cryptochrome with similarity to animal cryptochromes (Mittag et al., 2005) in addition to the well-characterized cryptochrome with similarity to plant cryptochromes (Small et al., 1995). However, the “animal cryptochrome” might actually function as a DNA photolyase rather than a photoreceptor.

There is a close evolutionary relationship between cryptochromes and DNA photolyases (Thompson and Sancar, 2002). DNA photolyases are enzymes involved in the repair of UV-induced DNA damage by photoreactivation using the energy of near UV/blue light (Nakajima et al., 1998; Merrow et al., 2005; Thompson and Sancar, 2002). Cryptochromes and DNA photolyases together form a single protein family. Based on sequence comparisons, plant and animal cryptochromes are thought to have evolved from DNA photolyases independently (Figure 2) (Devlin, 2002; Merrow et al., 2005). Plant
cryptochromes show a higher similarity to type II photolyase whereas animal cryptochromes show a higher similarity to 6-4 photolyases (Cashmore, 2003). An organism with both types of cryptochromes has not been reported.

The cryptochrome of *C. reinhardtii* characterized by Small and co-workers (Small *et al.*, 1995; Reisdorph and Small, 2004) clusters with the cryptochromes from plants (Figure 2). When searching the *C. reinhardtii* genome for similar proteins to the ones involved in the circadian clock of other model organisms, Mittag and coworkers (2005) made a surprise discovery of a gene (C_430042 in the second version of the genome), whose protein product shows high similarity to the single cryptochrome of *Drosophila* and to the two cryptochromes of mice. They concluded that this gene most likely encodes a functional cryptochrome of the animal type. However, this gene is now annotated as UVR3 in the fourth version of the *C. reinhardtii* genome, because it shows an even higher sequence similarity to the UVR3 gene from *Arabidopsis*. UVR3 was identified as the defective gene in an *Arabidopsis* mutant with increased sensitivity to UV light (Jiang *et al.*, 1997; Nakajima *et al.*, 1998). The gene was found to encode a 6-4 photolyase (Nakajima *et al.*, 1998). Since animal cryptochromes and the 6-4 photolyases of *Arabidopsis* and *Drosophila* are so similar in sequence that they form a single group within the cryptochrome/photolyase phylogenetic tree (Figure 2), it is likely that the gene of *C. reinhardtii* now annotated as UVR3 encodes a 6-4 photolyase although with high similarity to animal cryptochromes.

An investigation into whether or not the protein shows 6-4 photolyase activity is important in two ways. On the one hand, it is important to establish the function of the
Figure 2: Phylogenic tree of the cryptochrome/DNA photolyase protein family. (Cashmore et al., 1999). Cryptochrome 1 of *Chlamydomonas reinhardtii* (Cr CRY1, blue box) clusters with the plant cryptochromes, which form a sister group to the type II photolyases. Animal cryptochromes and 6-4 photolyases (red box) cluster together in a single group (green box), which is more distantly related to the plant cryptochromes and type II photolyases. At: *Arabidopsis thaliana*, Dm: *Drosophila melanogaster*, Mm: *Mus musculus*, Hs: *Homo sapiens.*
gene. On the other hand, it allows for a more precise interpretation of results from functional analyses of the plant cryptochrome in this organism. If it can be established that the “animal cryptochrome” gene encodes a 6-4 photolyase, it means that C. reinhardtii contains only a single cryptochrome and therefore that possible overlapping functions from a second cryptochrome do not need to be considered. If the UVR3 gene does not encode a 6-4 photolyase, it probably encodes a cryptochrome and research into the need for two cryptochromes in C. reinhardtii will be especially interesting.

The first goal of this project was to develop an experimental design so that the “animal cryptochrome” of C. reinhardtii can be tested for possible 6-4 photolyase activity in the future. The second goal was to clone the cDNA of this gene, which represents the first step in the proposed experimental strategy.
CHAPTER II
MATERIAL AND METHODS

II. 1. Strain and Growth Conditions

*Chlamydomonas reinhardtii* strain CC124 was obtained from the *Chlamydomonas* Culture Collection at Duke University. Liquid stock cultures were obtained by inoculating 50 mL of 0.3 high-salt medium (0.3 HSM) (Sueoka, 1960) in 125 mL Erlenmeyer flasks from slants and growing them photoautotrophically on an orbital shaker (Innova 2100 Platform shaker; New Brunswick Scientific, NJ). Stock cultures were maintained at room temperature with continuous shaking at 157 rpm under alternate 12hr light and 12hr dark cycles.

For RNA extractions, cells were inoculated from the liquid stock into 1 L bottles of 0.3 HSM at a concentration of $10^4$ cells/mL. The experimental cultures were grown photoautotrophically in an incubator programmed to a constant temperature of 20°C and aeration by an aquarium pump. Two 20W Gro-lux fluorescent light tubes on either side illuminated the culture bottles with a light intensity of 46 µE/m$^2$s from each side during the light phase of 12hr light / 12 hr dark cycles. Cells were subjected to at least three light/dark cycles to synchronize them prior to harvesting.

II. 2. Determination of Cell Density

To determine the cell density of a culture, a 1 mL sample was treated with a drop of tincture of iodine (0.25 g iodine in 100 mL 95% ethanol) to immobilize the cells (Harris, 1989) and the number of cells was counted using a hemacytometer.
II. 3. Harvesting and RNA extraction

*C. reinhardtii* was grown as described (see growth conditions). Cells were harvested in late logarithmic phase of growth at $2 \times 10^6$ cells/mL 4 h after the beginning of the light phase.

Ice (60g) was placed into a 250 mL centrifuge bottle and 167 mL *C. reinhardtii* culture was added immediately to the ice to keep the cells in their original physiological state. Samples were centrifuged at 4900×g for 5 min at 4°C. Cells were resuspended in a small volume of original supernatant and transferred to a 50 mL centrifuge tube (Corning Inc., Corning, NY). Cells were collected by centrifugation at 1900×g for 10 min at 4°C, frozen in liquid nitrogen, and stored at -80°C.

Total RNA was isolated using TRIzol reagent (Life Technology). Isolation was performed as suggested by the manufacturer’s instructions except that 8 mL Trizol reagent was used for $4 \times 10^8$ *C. reinhardtii* cells. At the end of the procedure, dried RNA pellets were dissolved in 100 µL diethylpyrocarbonate (DEPC)-treated water, vortexed, and incubated in a water bath at 55-60°C for 10 min. RNA samples were stored at -80°C.

II. 4. Spectrophotometry

Concentration and purity of the extracted RNA was determined by measuring the absorbance of the sample at 260 and 280 nm in a spectrophotometer (Ultrospec 3000; Amersham Pharmacia Biotech, Uppsala, Sweden). The concentration of an available genomic DNA sample was also determined using a spectrophotometer and confirmed to be 550 µg/mL.
II. 5. First Strand Synthesis

The cDNA was prepared using RETROscript (RetroScript Kit; Ambion, Corp., Austin, TX) according to the manufacturer’s instructions. The one step procedure of this kit involves the addition of all components for reverse transcription (RT) and polymerase chain reaction (PCR) at the same time, and is generally used for the amplification of relatively abundant targets. However, the two step procedure was selected for this project because it was more efficient and sensitive for rare targets. The two step procedure involves as a first step the synthesis of cDNA by reverse transcription (RT step) followed by the amplification of cDNA using gene specific primers (PCR step).

Total RNA contains all cellular RNA including mRNA, rRNA, and tRNA. Total RNA was reverse-transcribed to cDNA according to the RETROscript protocol using initial heat denaturation and oligo dT primers provided with the Retroscript kit. The initial heat denaturation was performed at 85°C for 3 min due to the high GC content of *C. reinhardtii* RNA. Oligo dT primers were used because they only hybridize to poly (A)+ RNA, which represents the vast majority of mRNA.

The two buffers 10X RT and 10X PCR provided with the RETROscript kit was used for different purposes. The 10X RT buffer was used at a concentration of 1X (50mM Tris-HCl, pH 8.3; 75mM KCl; 3mM MgCl$_2$; 5mM DTT) during the RT step, because it is reported to give a higher yield of cDNA. The 10X PCR buffer was used at a concentration of 1X (10mM Tris-HCl, pH 8.3; 50mM KCl; 1.5mM MgCl$_2$) for some PCR amplifications. The human template mRNA provided with the kit was used as a positive control for efficiency of cDNA synthesis.
II. 6. Primer design

Genomic (accession number NW_001843783) and mRNA (XM_001698002) sequences of the “animal cryptochrome” gene were downloaded from the NCBI website and used as basis for the primer design. The gene is now annotated as UVR3. Table 1 lists all of the primers that were designed and tested in a PCR assay. Primers for “animal cryptochrome” were obtained from IDT Technologies and primers for CABII (Table 2) were obtained from Genosys, whereas the retroscript control primers came with the RETROscript cDNA synthesis kit. Vector NTI, Beacon 2.1, and Primer Express softwares were used to design the oligonucleotide primers.

II. 7. Bioanalyzer

RNA quality was assessed using a Bioanalyzer (Agilent Technologies) according to the manufacturer’s instructions. The Bioanalyzer is able to measure both the purity and the intactness of a nucleic acid sample using very small volumes. Each kit for the Bioanalyzer is provided with a specific dye concentrate and polymer gel matrix. The gel matrix is designed to have many sets of interconnected microchannels that act like a sieve during electrophoresis and allow the separation of RNA or DNA based on size. Smaller fragments migrate faster than larger ones. The fluorescent dye intercalates into the RNA or DNA and is then detected by its laser-induced fluorescence. The results are interpreted in the form of electropherograms and false gel images. The RNA 6000 Nano Kit (Agilent) was used with RNA nanochips for the detection of RNA and the DNA 7500 and 1000 Kits were used with DNA nanochips for DNA fragments amplified from
Table 1: Primers used in the amplification of “animal cryptochrome” by PCR

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
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<tr>
<td>DRJUVR3REVERSE2</td>
<td>CACTCCCGGAACACCCCG</td>
</tr>
<tr>
<td>DRJUVR3BamH1Forward</td>
<td>AGCAGGATCCCGGAGGAGTGAAGAAGACGCATC</td>
</tr>
<tr>
<td>DRJUVR3BamH1Reverse</td>
<td>TTGTGGATCCATCACATCCCTCGGAGG</td>
</tr>
<tr>
<td>ShobhaBAMH1Forward</td>
<td>ATTGGATCCAAATGGCAGGAGTGAACAGGC</td>
</tr>
<tr>
<td>ShobhaBAMH1Reverse</td>
<td>TATGGATCCCGGTCCAGAGCTCGTAGATGT</td>
</tr>
<tr>
<td>ShobhaBamH1FP-001</td>
<td>CATTAGGATCCATATGGTTCCCGCAAGG</td>
</tr>
<tr>
<td>ShobhaBamH1RP-001</td>
<td>CATTAGGATCCGAGAAGAAGGCGAGG</td>
</tr>
<tr>
<td>ShobhaBamH1FP-002</td>
<td>CATTAGGATCCGCATCGATATGGTTCCCGCAAGG</td>
</tr>
<tr>
<td>ShobhaBamH1RP-002</td>
<td>CATTAGGATCCGACAGAAGATTGCCGTAAGGC</td>
</tr>
<tr>
<td>ShobhaBamH1FP-005</td>
<td>ATTATGGATCCATCGACGACCGCGACTACC</td>
</tr>
<tr>
<td>ShobhaBamH1RP-005</td>
<td>AGTATGGATCCGAAACTTGGCGATGAGC</td>
</tr>
<tr>
<td>ShobhaBamH1UVR-FP-006</td>
<td>AGTCTGGATCCCATGGCAGGAGTGAAGAAGC</td>
</tr>
<tr>
<td>ShobhaBamH1UVR-RP-006</td>
<td>TATAGGATCCGGCGCTCGTAGATGTACTTG</td>
</tr>
<tr>
<td>ShobhaBamH1UVR-FP-008</td>
<td>AATTCGGATCCGGCAACCDGGGAGGTGTGTTC</td>
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<tr>
<td>ShobhaBamH1UVR-RP-008</td>
<td>AATTTGGATCCCCGGAAGTACTGGGAGAAGA</td>
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<tr>
<td>Shobha- 009-FP</td>
<td>CTTGCACTAGAATTGCCCTGAGCA</td>
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<tr>
<td>Shobha- 009-RP</td>
<td>TTGCCAGCCAGATGTACACGC</td>
</tr>
<tr>
<td>DR.J- 009-FP</td>
<td>TGTGCTGCGACGTGGCG</td>
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<tr>
<td>DR.J- 009-RP</td>
<td>AGAATCATGTCGGCGCATAGACAGCC</td>
</tr>
<tr>
<td>Shobha-FP- 010</td>
<td>TGTCGCCCTACCTCAAGTTC</td>
</tr>
<tr>
<td>Shobha-RP- 010</td>
<td>GGTGTGCTGCCAGTGGACAA</td>
</tr>
<tr>
<td>Shobha-FP- 011</td>
<td>GGTGTCAAGGACATGCCAGCC</td>
</tr>
<tr>
<td>Shobha-RP- 011</td>
<td>GCAAATGCCAGTGGCACCCTCGC</td>
</tr>
<tr>
<td>Primer name</td>
<td>Sequence</td>
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<td>------------------------------------</td>
</tr>
<tr>
<td>CABII-Sense</td>
<td>CCTGGACTACCTGGGCAACC</td>
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<tr>
<td>CABII-Antisense</td>
<td>CGCGGTAGCCCTCGATCAG</td>
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<tr>
<td>Retroscript control Forward</td>
<td>TTCCGCAAGTTACCTACC</td>
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<tr>
<td>Retroscript control Reverse</td>
<td>CGGGCCGCGCATGCTTTACG</td>
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</table>
genomic DNA and cDNA samples. The ladder provided with each kit by the Agilent company was used as standard.

II. 8. Polymerase Chain Reaction (PCR)

For results III. 3.

PCR was performed in a PTC200 thermocycler (Peltier thermal cycler, MJ Research). Retroscript control RNA was evaluated by amplification with the Retroscript control primers and \( C. \) *reinhardtii* RNA with the CABII control primers (Table 2). These amplification results allowed us to the efficiency of the first strand cDNA synthesis. PCR was carried out in a total volume of 25 µl containing 1X Retrocript PCR buffer (10 mM Tris-HCl, pH8.3; 50 mM KCl; 1.5 mM MgCl\(_2\)), 125 µM of each dNTP (Invitrogen), 1 U of Supertaq polymerase (Ambion), 200 nM of each primer, and 2.5 µl of cDNA synthesis solution. Amplification conditions were as follows: initial denaturation at 94°C for 4 min; 30 cycles of 94°C for 30 sec, 44°C (Retroscript control primers) or 55°C (CABII control primers) for 30 sec, 72°C for 1 min; final extension of 72°C for 5 min. When the CABII primers were included as controls in any of the following PCR reactions, 55°C was used as the annealing temperature unless otherwise specified.

For results III.4.

When primer pairs #1 (DRJUVR3REVERSE2 and DRJUVR3BamH1Forward), #2 (DRJUVR3BamH1Forward and DRJUVR3BamH1Reverse), or #3 (ShobhaBAMH1Forward and ShobhaBAMH1Reverse) (Table 1) were used with genomic DNA as template, amplification was carried out in a total reaction volume of 25 µl containing 1X master mix (5 PRIME) (30 mM Tris-HCl buffer pH 8.3, 1.5 mM
MgCl$_2$, 200 µM of each dNTP, 50 mM KCl, 0.625 U Taq DNA Polymerase), 200 nM of each primer, and genomic DNA template in the amount of 50 ng, 100 ng and 200 ng in three individual assays. For magnesium optimizations, additional MgCl$_2$ was added from a 25 mM stock solution provided with the 5 PRIME kit and 200 ng of genomic DNA was used as template. The PCR conditions used for amplification were: initial denaturation at 94°C for 4 min; 35 cycles of 94°C for 30 sec, annealing temperature at 5°C below the melting temperature of the primers (64°C for #1, 55°C for #2, and 60°C for #3) for 30 sec, 72°C for 5 min; final extension at 72°C for 6 min. Additional experiments were carried out with an annealing temperature gradient of 5°C above and below the primer melting temperatures for each of the three primer pairs using 200 ng of genomic DNA as template. All other conditions were the same as in the fixed annealing temperature assays. The three primer pairs were tested with cDNA as template. The total volume, amounts in the reaction mix as well as amplification conditions used were exactly the same as with the genomic DNA as template except that genomic DNA was replaced with 2.5 µl of cDNA solution.

For results III. 5.

For the amplification of smaller fragments from genomic DNA with primer pairs ShobhaBamH1FP-001 and ShobhaBamH1RP-001, ShobhaBamH1FP-002 and ShobhaBamH1RP-002, ShobhaBamH1FP-005 and ShobhaBamH1RP-005, ShobhaBamH1UVR-FP-006 and ShobhaBamH1UVR-RP-006, as well as ShobhaBamH1UVR-FP-008 and ShobhaBamH1UVR-RP-008 (Table 1), PCR was carried out in a total volume of 50 µl containing 1X Retrocript PCR buffer, 125 µM of each dNTP, 1 U of PlatinumTaq polymerase (Invitrogen), 200 nM of each primer, and
200 ng genomic DNA. Amplification conditions were as follows: initial denaturation at 94°C for 4 min; 30 cycles of 94°C for 30 sec, annealing temperature of 5°C below the melting temperature of the primers (41.7°C for 001, 53°C for 002, 51°C for 005, 57.8°C for 006, and 55°C for 008) for 30 sec, 72°C for 1 min; final extension at 72°C for 5 min. Primer pair ShobhaBamH1UVR-FP-008 and ShobhaBamH1UVR-RP-008 was also tested with an annealing temperature gradient of 50°C to 60°C under otherwise identical conditions as for the 55°C annealing temperature assay.

For results III.6.

For the amplification of small fragments from the 5’ or 3’ untranslated region (UTR) of the genomic DNA with primer pairs Shobha- 009-FP / Shobha- 009-RP and DR.J- 009-FP / DR.J- 009-RP, respectively, PCR was carried out in a total volume of 50 µl containing 1X Invitrogen PCR buffer (20 mM Tris- HCl pH 8.4, 50 mM KCl), MgCl₂ in the amount of 1.5 mM, 2 mM, 2.5 mM, 3 mM or 3.5 mM, 125 µM of each dNTP, 1 U PlatinumTaq polymerase (Invitrogen), 200 nM of each primer, and 200 ng genomic DNA. Amplification conditions were as follows: initial denaturation at 97°C for 4 min; 30 cycles of 94°C for 50 sec, annealing temperature of 5°C below the melting temperature of the primers (54.5°C for Shobha-009, 60°C for DR.J-009) for 40 sec, 72°C for 45 sec; final extension at 72°C for 5 min. Hot start PCR was applied, where the polymerase was only added after the initial denaturation step of the PCR reaction. Primer pair Shobha- 009-FP / Shobha- 009-RP was also tested with an annealing temperature gradient of 56.6°C to 70°C with 3 mM MgCl₂ under otherwise identical conditions as described above. When the Shobha- 009-FP / Shobha- 009-RP primer pair was used with
cDNA as template, 3 mM MgCl₂ and an annealing temperature of 54.6°C was applied with all other conditions identical as for the experiments with genomic DNA as template.

For results III.7.

To decrease the possibility of nonspecific primer binding, touch down PCR (Korbie & Mattick, 2008) was also performed with the Shobha-009-FP / Shobha-009-RP primer pair and cDNA from batch1 as template according to the following protocol: hot start with initial denaturation at 97°C for 5 min; followed by a total of 35 cycles at 94°C for 50 sec denaturation, 70°C to 54°C for 30 sec annealing with decrement of 2°C for every two cycles but 15 cycles at the lowest temperature, 72°C extension for 45 sec; and final extension at 72°C for 5 min. A 2.5 µl sample of cDNA was added in place of the genomic DNA, otherwise the reaction volume and components were identical to those described for the primer pair above for 3 mM MgCl₂. When the experiment was repeated with cDNA from batch1 and batch2, conditions were identical to the batch1 assay, except that 37 cycles were performed with 4 cycles at the highest annealing temperature and 20 cycles at the lowest annealing temperature and the volume of cDNA mix added as template was reduced to 1 µl.

II. 9. Real-Time PCR

Real-time PCR (iCycler, Version 3.0, BIO-RAD) was performed for the amplification of genomic DNA in the presence of DMSO (dimethylsulfoxide) and betaine (N,N,N,-Trimethylglycine). The 50µL reaction mixture was composed of 25µL 2X SYBR Green Supermix (100 mM KCl, 40 mM Tris-HCl (pH 8.4), 0.4 mM of each
dNTP, 50 U/ml iTaq DNA polymerase, 6 mM MgCl₂, SYBR Green I and 20 nM fluorescein dye), 250 ng of genomic template, and 250 nM reverse and forward primers. The quantification of the double stranded DNA was carried out using SYBR green dye that binds to double-stranded DNA, absorbs blue light, and emits green light. The real-time PCR amplification was carried out with genomic template and primer sets Shobha-008-FP / Shobha-008-RP, Shobha-009-FP / Shobha-009-RP, DrJ-009-FP/ DrJ-009-RP, Shobha-010-FP / Shobha-010-RP, Shobha-011-FP / Shobha-011-RP (Table 1) and CAB-II control primers (Table 2) with initial denaturation at 94°C for 5 min followed by 40 cycles of 94°C for 50 sec denaturation, 56°C-58°C for 30 sec annealing, 72°C for 45 sec extension; and final extension at 72°C for 6-10 min. To reduce the inhibitory affect of DMSO on DNA Taq Polymerase, a combination of DMSO and betaine (5% DMSO + 1M betaine) was also used for uniform amplification of DNA with varying GC content under otherwise the same conditions.

II. 10. DNA electrophoresis

PCR amplification products were analyzed by agarose gel electrophoresis followed by ethidium bromide staining to determine their presence and size. Generally 0.7% gels were used with the genomic template and electrophoresis was performed either in 1xTAE (40 mM Tris-acetate, pH 8.18; 1mM EDTA) or 0.5xTBE (45 mM Tris-borate, pH 8.0; 1 mM EDTA). The amplified products of touch down PCR from cDNA as template and from real-time PCR were observed on 1.5% agarose gels using 0.5X TBE buffer. 1xTAE buffer was initially used but later replaced by 0.5xTBE to improve the resolution of the gel. After electrophoresis, gels were stained in the respective
electrophoresis buffer containing 1µM ethidium bromide for 30 min, followed by destaining in electrophoresis buffer for 20 min. Computerized images of the destained gels visible on a UV transilluminator were captured with a digital camera (DC40, Kodak Scientific Imaging System or Fluorchem HD2 Alfa Innotech Gel Imaging System, Version 1.2.1.0).

II. 11. cDNA Library Screening

The construction and screening of cDNA libraries is a common technique in the analysis of mRNA transcripts. The “core” library available from the *Chlamydomonas* Center provides the advantage that it contains cDNA derived from cultures under four different conditions. The “core” library is provided by the *Chlamydomonas* Center as four separate libraries that were generated from strain 21 gr (CC-1690) grown either (a) in Tris-acetate-phosphate (TAP) medium under moderate light (approximately 75 μE/m²s), (b) TAP medium in the dark, (c) high-salt minimal medium (HS) at ambient levels of CO₂ or (d) HS medium in air supplemented with 5% (v/v) CO₂ (Grossman, 2003). These cDNA libraries had been constructed using a λZAP II vector system inserting the cDNAs into the pBluescript phagemid.

In the present study, a control oligonucleotide probe for the pBluescript portion of the phagemid (5’GGTATCTTTATAGTCCTGTCGGGTTT3’) and a respective probe for animal cryptochrome cDNA (5’TGCTGGTGCTGCGTGGCAAACCCGAGGTGTTT3’) was designed using Vector NTI software (IDT technologies). Chemiluminiscence (Alkaline phosphatase direct labeling; Amersham, GE Health Care Life Sciences) was used for detection according to the manufacturer’s instructions. The oligonucleotide
probe labeled with the enzyme alkaline phosphatase binds to the target DNA. The CDP-star substrate reacts with the enzyme and generates signals, which are then visualized using HD2 Alfa Innotech Gel Imaging System, Version 1.2.1.0.
III.1. Proposed Experimental design to test for possible 6-4 photolyase activity

In order to determine if the “animal cryptochrome” gene of *C. reinhardtii* actually encodes a 6-4 DNA photolyase rather than a photoreceptor, an experimental design was developed to test whether the protein product is able to rescue an *E. coli* mutant defective in its DNA photolyase gene. The proposed experimental design is as follows: In a first step, the coding region of the “animal cryptochrome” gene will be cloned. This can be accomplished by isolating total RNA from *C. reinhardtii*, reverse transcribing all polyA+ RNA into cDNA, amplifying the coding region of the gene-specific cDNA by PCR using appropriate primers, and cloning the amplicon into the vector pGEM-T (Promega), which has been specifically designed for easy cloning of PCR amplification products. It will then be necessary to confirm the correct sequence through DNA sequencing. The second step will be to clone the coding region in-frame into an *E. coli* expression vector such as pET-15b (Novagen). The pET-15b vector carries an N-terminal His•Tag sequence followed by a thrombin site and three unique restriction sites for cloning. The His•Tag of the fusion product can be used for the purification of the protein. The thrombin site is a protease cleavage site that allows for the removal of the His•Tag after purification. Expression of the inserted gene in *E. coli* can be induced by addition of isopropyl-β-D-1-thiogalactopyranoside (IPTG) to the culture. In order to achieve the correct in-frame insertion of the animal cryptochrome cDNA into the pET-15b vector, a properly
positioned \textit{BamHI} restriction site will be added to the end of each primer used for cDNA amplification in the first step. Since there is no \textit{BamHI} site in the coding region of the animal cryptochrome cDNA, the entire fragment can be excised from pGEM-T using \textit{BamHI} and inserted into the \textit{BamHI} site of the expression vector. The correct orientation of the fragment as well as the correct sequence of the fusion regions will have to be confirmed by DNA sequencing. The final step will be to transform the construct into a strain of \textit{E. coli} such as NKJ3002 (Nakajima \textit{et al.} 1998) that is defective in its photolyase gene, nucleotide excision repair, and RecA gene and test for better survival after UV light exposure when the construct is expressed and blue light is provided. This test will also enable to distinguish whether UV-induced pyrimidine [6-4] pyrimidone photoproducts or cyclobutane pyrimidine dimers (CPDs) are repaired. These two products constitute 20-30\% and 70-80\% of the total UV products, respectively (Nakajima \textit{et al.} 1998), and are repaired by different photolyases. The defective \textit{E. coli} strain can be transformed with an expression plasmid carrying the \textit{E. coli} CBP photolyase gene like pKY137 (Nakajima \textit{et al.} 1998) and tested whether survival after UV exposure is highest, when the strain is expressing both plasmids.

\textbf{III.2. Analysis of quantity and integrity of the extracted RNA}

The amount of total RNA extracted from \textit{C. reinhardtii} was determined spectrophotometrically. It was found to be 824 µg/mL. An indication on the purity of an RNA sample can also be gained spectrophotometrically through its $A_{260}/A_{280}$ ratio. However, this ratio also depends on the pH, because the absorbance at 280 nm decreases with increasing pH while the absorbance at 260 nm is unaffected (Wilfinger \textit{et al.}, 1997).
A pure RNA in water will have an $A_{260}/A_{280}$ ratio between 1.7 and 1.8. The $A_{260}/A_{280}$ ratio of the total RNA sample extracted and diluted with pure water was 1.8, which implies that there was little or no DNA or protein contamination.

The integrity of the RNA sample was analyzed with a Bioanalyzer, which separates the RNA by size and plots it as an electropherogram. Figure 3 shows a comparison of two electropherograms. The one for total RNA extracted from *C. reinhardtii* using the TRIzol method is compared with a typical electropherogram provided by the Bioanalyzer company (2100 Agilent) for total mammalian RNA extracted with TRIzol. The TRIzol method does not remove small RNAs, which form a small peak at the beginning of the electropherogram. In both electropherograms, the peaks representing the cytosolic 18S and 28S ribosomal RNA are indicated with arrows. The 28S/18S ratio of peak height should be close to 2. Any discrepancy in this ratio might be due to enzymatic degradation or mechanical shearing of the RNA during the isolation procedure. Figure 3 shows that the ratio of heights are very similar in the two electropherograms indicating that the RNA extracted from *C. reinhardtii* shows integrity that is acceptable. *C. reinhardtii* also contains large amounts of chloroplastic rRNA. In its electropherogram, the 16S rRNA of the chloroplast shows up as a separate peak, whereas the respective 25S rRNA is not separated from the 28S rRNA peak. This is also typical when RNA is separated by gel electrophoresis.
Figure 3: Integrity of the isolated RNA as determined with the Bioanalyzer.

(A) A typical electropherogram image provided by Agilent (2100 Agilent) for total RNA of mammals extracted using TRIzol. (B) Electropherogram of total RNA extracted from *C. reinhardtii* using Trizol.
III.3. Efficiency of cDNA first strand synthesis

Successful synthesis of cDNA was tested using PCR followed by separation with the Bioanalyzer or gel electrophoresis. Two positive and four negative controls were prepared. One positive control made use of the human control RNA and control primers provided by the Agilent kit. The other positive control included the *C. reinhardtii* RNA together with the CABII control primers (Table 2). Negative controls were prepared, where RNA was directly used as template during the PCR reaction without prior transcription into cDNA. It allows for detection of possible DNA contamination in the RNA samples. Another set of negative controls included the PCR reaction without any template. It tests for possible DNA contamination in any of the PCR solutions besides the one containing the template.

Figure 4 shows the electropherograms of the two positive controls used for testing the efficiency of cDNA synthesis. The fragment amplified based on the Retroscript control RNA and control primers shows the expected size of 361 bp (Figure 4A), whereas the fragment amplified based on the *C. reinhardtii* RNA and the CABII control primers shows the expected size of 110 bp (Figure 4C). No such fragments are detectable with the negative controls when RNA was used directly in the PCR assay (Figure 4B and D) or when template was omitted altogether (not shown). The results were further confirmed through separation by gel electrophoresis instead of the Bioanalyzer. They indicate that cDNA synthesis was successful and that no contaminating DNA was contained in the RNA extracted from *C. reinhardtii*. 
Figure 4: Analysis of cDNA first strand synthesis by PCR and the Bioanalyzer. 

The first and last peak in each electropherogram represents the lower and upper marker, respectively. (A) Electropherogram of the positive control provided with the cDNA kit. Retrospect control RNA was reverse transcribed into cDNA followed by PCR amplification using the Retrospect control primers. Peak 4 shows the expected size of 361 bp. (B) Electropherogram of a negative control, because the sample was treated as in (A) except that RNA was directly used as template for PCR amplification. (C) Electropherogram of the *C. reinhardtii* positive control. *C. reinhardtii* total RNA was reverse transcribed into cDNA followed by PCR amplification using the CABII control primers. A peak at the expected size of 110 bp is detected. (D) Electropherogram of a negative control, because the sample was treated as in (C) except that RNA was directly used as template for PCR amplification.
III.4. Amplification of the entire coding region from genomic DNA

In order to determine whether the primers designed to amplify animal cryptochrome cDNA were able to do so, they were first tested using genomic DNA as a template. Genomic DNA has the advantage that the presence of animal cryptochrome template can be relied upon, exactly once per genome, whereas the cDNA may or may not contain the specific template. The disadvantage of genomic DNA is that amplicon sizes may be much larger due to inclusion of introns.

PCR was used for the amplification of genomic DNA with two sets of primers designed to amplify the entire coding region of the animal cryptochrome gene (Table 1): DRJUVR3BamH1Forward and DRJUVR3BamH1Reverse as well as ShobhaBAMH1Forward and ShobhaBAMH1Reverse. The expected amplicon size was 6237 bp and 5499 bp, respectively. In addition, the DRJUVR3BamH1Forward primer was used with DRJUVR3REVERSE2 for amplification of only part of the coding region to give a smaller amplicon of 1141 bp. Gradients of annealing temperature and magnesium ion concentration were employed to optimize the amplification conditions. Negative controls were prepared that included all PCR components except the DNA template. No amplification could be detected with any of the primer pairs under any of the tested conditions (data not shown).

Failure to amplify the entire coding region of animal cryptochrome from genomic DNA could be due to the large amplicon size in combination with the high GC content of *C. reinhardtii* DNA. An attempt was therefore made to amplify directly from cDNA using these primer pairs, as this would result in smaller amplicons of about 1700 bp. However, no fragments could be detected after amplification with cDNA template.
Amplification of smaller regions of genomic DNA

Due to the difficulties in amplifying the unusually large coding region of genomic DNA, new primers were designed that would amplify only a small region of genomic DNA. If successful, the primers would provide the tool to test whether animal cryptochrome cDNA was present in the cDNA sample obtained from the extracted total RNA.

Several new primer pairs (ShobhaBamH1-001, ShobhaBamH1-002, ShobhaBamH1-005, and ShobhaBamH1UVR-006 in Table 1) were designed to give amplicon sizes of 1202 bp, 165 bp, 143 bp, and 1347 bp, respectively. No amplicons were obtained with any of these primer sets. Another primer pair (Shobha- BamH1UVR-008), which was designed with the program Beacon 2.1 instead of Vector NTI to give an amplicon size of 997 bp, also did not yield an amplicon despite attempts at optimization of annealing temperature and magnesium ion concentration. Only primer dimers were observed (data not shown).

In a fresh attempt, primers were designed to amplify either the 3’ untranslated region (UTR) (DrJ-009-FP and -RP) or the 5’ UTR (Shobha-009-FP and -RP). The expected amplicon size was particularly short with 186 bp for both primer sets.

Using the primer pair Shobha-009, a fragment of the expected size of 186 bp could be amplified. Figure 5 shows an example gel for the optimization of the magnesium ion concentration. A gradient of 1.5 to 3.5 mM final concentration was used. Amplification was detected with each concentration but 3.0 mM was found to be optimal.
Figure 5: Gel electrophoresis of PCR products amplified from genomic DNA with Shobha-009 (lane 4-9) and Dr.J-009 primers (lane 11-16): optimization of magnesium ion concentration. Lane 1: CABII control primers. Lane 2: 100 bp Marker. Lane 4: No template and 1.5 mM Mg^{2+}. Lane 5: 1.5 mM Mg^{2+}. Lane 6: 2.0 mM Mg^{2+}. Lane 7: 2.5 mM Mg^{2+}. Lane 8: 3.0 mM Mg^{2+}. Lane 9: 3.5 mM Mg^{2+}. Lane 11: No template and 1.5 mM Mg^{2+}. Lane 12: 1.5 mM Mg^{2+}. Lane 13: 2.0 mM Mg^{2+}. Lane 14: 2.5 mM Mg^{2+}. Lane 15: 3.0 mM Mg^{2+}. Lane 16: 3.5 mM Mg^{2+}.

**PCR conditions:** Hot start PCR with initial denaturation at 97°C for 4 min; 30 cycles of 94°C for 50 sec, annealing temperature of 5°C below the melting temperature of the primers (54.5°C for Shobha-009, 60°C for DR.J-009) for 40 sec, 72°C for 45 sec; final extension at 72°C for 5 min.
Optimization of the annealing temperature was also carried out. At lower temperatures, there is a greater possibility for non-specific binding of the primers. With the increase in temperature the stringency of binding increases but the amount of product also decreases. Hence an optimal temperature is required for optimal amplification. Figure 6 shows the electrophoresis gel of an annealing temperature gradient optimization from 54°C to 70°C. Although amplification can be detected with each temperature, 55.4°C was found to be optimal for maximum amplification. This temperature is 5 °C below the melting temperature for the Shobha-009 primer pair.

The primer pair DrJ-009 also resulted in an amplicon of the expected size with genomic DNA as template (Figure 5). Since a few faint, non-specific bands were also observed with this primer pair, the Shobha-009 primer pair was chosen for further experimentation.

III.6. Amplification of smaller fragments from cDNA

Since the Shobha-009 primer pair yielded the expected amplicon size with genomic DNA as template, the next step was to test whether amplification from cDNA could be achieved using the same primer pair.

Figure 7 shows that amplification with the Shobha-009 primers at the optimized conditions was successful with genomic DNA as template (lane 11) but not with cDNA (lane 9). There are two possible explanations for these results. Either the amount of mRNA specific for animal-like cryptochrome in the total RNA extracted was too low or the preparation of cDNA from the RNA was inefficient. Although the CABII control primers had yielded the expected amplicon from the cDNA (see Figure 4), the gene
Figure 6: Gel electrophoresis of PCR products amplified from genomic DNA with Shobha-009 primers: optimization of annealing temperature. Lane 1: 100 bp Marker. Lane 2: No template, 55.4°C. Lane 3: 55.4°C. Lane 4: No template, 56.6°C. Lane 5: 56.6°C. Lane 6: No template, 58.5°C. Lane 7: 58.5°C. Lane 8: No template, 60.8°C. Lane 9: 60.8°C. Lane 10: No template, 63.5°C. Lane 11: 63.5°C. Lane 12: No template, 65.8°C. Lane 13: 65.8°C. Lane 14: No template, 67.5°C. Lane 15: 67.5°C. Lane 16: No template, 68.8°C. Lane 17: 68.8°C.

PCR conditions: Hot start PCR with initial denaturation at 97°C for 4 min; 30 cycles of 94°C for 50 sec, annealing temperature gradient from 54°C to 70°C for 40 sec, 72°C for 45 sec; final extension at 72°C for 5 min.
Figure 7: Gel electrophoresis of PCR products amplified from cDNA with Shobha-009 primers. Lane 1: 100 bp Marker. Lane 2: CABII primers without template. Lane 3: CABII primers and genomic DNA as template. Lane 4: CABII primers without template. Lane 5: CABII primers without template. Lane 6: CABII primers and genomic DNA as template. Lane 7: CABII primers without template. Lane 8: Shobha-009 primers without template. Lane 9: Shobha-009 primers with cDNA as template. Lane 10: Shobha-009 primers without template. Lane 11: Shobha-009 primers with genomic DNA as template.

PCR conditions: Hot start PCR with initial denaturation at 97°C for 4 min; 30 cycles of 94°C for 50 sec, annealing temperature 54.5°C for 40 sec, 72°C for 45 sec; final extension at 72°C for 5 min.
family amplified by these primers is highly expressed in *C. reinhardtii* (Jacobshagen *et al.*, 2001) and therefore enough target might even be present in less efficient cDNA preparations.

To test whether the efficiency of the cDNA preparation could be improved, two fresh batches of cDNA were prepared and used as template for amplification with the Shobha-009 primers. Figure 8 show that amplification with the CABII control primers gave an amplicon with the expected size of 110 bp for batch1 (lane 3). When genomic DNA was used as control template with the Shobha-009 primers, a band with the expected size of 186 bp could also be detected (lane 8). However, only a non-specific band at approximately 1100 bp instead of the expected 186 bp was observed for the Shobha-009 primers with either of the two cDNA preparations (lane 5 and 7). This result suggests that the stringency of primer binding to the cDNA template needed to be improved. The non-specific band could be due to another gene with low affinity for the primers that is highly expressed and therefore present in large copy numbers in the cDNA preparations to serve as template.

III.7. Touch down PCR for improved specificity of amplification

A derived method of PCR known as Touch down PCR was used to increase the specificity of amplification by reducing the incident of non-specific primer binding. In this technique, PCR cycles are set such that the earlier cycles start at a higher annealing temperature followed by lower annealing temperatures for each subsequent set of cycles. Higher annealing temperatures increase the specificity of primer binding but also reduce the efficiency of binding. Lowering the annealing temperature in a step-wise fashion thus
Figure 8: Gel electrophoresis of PCR products from two fresh cDNA batches with Shobha-009 primers. Lane 1: 100 bp Marker. Lane 2: CABII primers without template. Lane 3: CABII primers and cDNA as template (batch1). Lane 4: Shobha-009 primers without template. Lane 5: Shobha-009 primers with cDNA as template (batch1). Lane 6: Shobha-009 primers without template. Lane 7: Shobha-009 primers with cDNA as template (batch2). Lane 8: Shobha-009 primers with genomic DNA as template. Lane 9: Shobha-009 primers without template.

**PCR conditions:** Hot start with initial denaturation at 97°C for 5 min; 30 cycles of 94°C for 50 sec denaturation, 55°C for 40 sec annealing, 72°C extension for 45 sec; and final extension at 72°C for 5 min.
increases the likelihood of sequence-specific amplicons during the first cycles that can serve as additional templates during the cycles with lower annealing temperatures.

In a first attempt, an amplicon of the correct size could be observed with genomic DNA as control template for both the CABII and Shobha-009 primers, but neither primer pair gave rise to an amplicon when batch1 of the freshly prepared cDNA was used as template (data not shown). Therefore, the experiment was repeated including this time also batch2 of the freshly prepared cDNA. As shown in Figure 9, the results of this experiment are not entirely clear-cut. For example, the CABII control primers when used with no template as control (lane 2, 6, and 10) already gave a weak band of a size (~110 bp) only expected with cDNA as template. It suggests some contaminating cDNA in these samples. Possible cDNA contamination can also be observed in some of the no template controls for the Shobha-009 primers (lane 4, 8, and 12), although the band indicating the contamination (~186 bp) is extremely weak. In addition, when the CABII control primers were used with cDNA from batch1 as template (lane 3), an additional band is detectable that has a size (~347 bp) only expected when genomic DNA is used as template. It suggests contaminating genomic DNA in the batch1 cDNA preparation. However, this band cannot be observed with the batch2 cDNA (lane 7). Instead, the band at the expected size of 110 bp is greatly increased in density compared to the no template control. It suggests that batch2 contains sufficient CABII-specific cDNA to serve as template for the control primers and that it does not contain contaminating genomic DNA. When the same cDNA preparation (batch2) is used with the Shobha-009 primers (lane 9), the band of the expected size (186 bp) is also greatly increased in density.
Figure 9: Gel electrophoresis of Touch down PCR products with Shobha-009 primers. Lane 1: 100 bp Marker. Lane 2: CABII primers without template. Lane 3: CABII primers and cDNA template (batch1). Lane 4: Shobha-009 primers without template. Lane 5: Shobha-009 primers and cDNA as template (batch1). Lane 6: CABII primers without template. Lane 7: CABII primers and cDNA as template (batch2). Lane 8: Shobha-009 primers without template. Lane 9: Shobha-009 primers and cDNA as template (batch2). Lane 10: CABII primers without template. Lane 11: CABII primers and genomic DNA as template. Lane 12: Shobha-009 primers without template. Lane 13: Shobha-009 primers with genomic DNA as template.

PCR conditions: Hot start with initial denaturation at 97°C for 5 min; followed by 35 cycles of 94°C for 50 sec denaturation, 70°C to 54°C for 30 sec annealing with decrement of 2°C for every two cycles but 15 cycles at the lowest temperature, 72°C extension for 45 sec; and final extension at 72°C for 5 min.
compared to the no template control suggesting that next to CABII-specific cDNA this batch also contains sufficient animal cryptochrome-specific cDNA.

The experiment shown in Figure 9 was not repeatable. In fact, for some unknown reason the Shobha-009 primers even failed to give rise to the correct amplicon with the genomic control template, despite many attempts that also included fresh preparations of every single solution. There is some indication now that the problems were most likely due to a defective thermocycler.

Since it was not possible to draw any firm conclusion yet on the feasibility of amplifying the entire coding region of animal cryptochrome from cDNA despite the many optimizations, the reasonableness of continuing with further optimizations was questioned. An alternative technique for the cloning of a particular cDNA is the screening of an already available cDNA library. Although this technique also involves the design of a proper oligonucleotide probe for specific hybridization with the target cDNA, it does not involve amplifications by PCR. It therefore seemed more sensible to switch to this technique. However, when this technique was used with a cDNA library obtained from the *Chlamydomonas* Center, it did not work as efficiently as necessary for two reasons. The pBluescript control probe initially designed to study the efficiency of the method is expected to generate a signal with every single phage plaque. Yet a proper signal was only detected with about 56 to 68% of the plaques (data not shown). False positives, where the signal could not be assigned to an existing plaque, were also detected with a rate of 15 out of 102 total signals. In addition, the titer of the library turned out to be about $10^3$ times lower than suggested, which was later confirmed by the *Chlamydomonas* Center from which the library was purchased and which is probably due to its old age.
Since cDNA library screening was not possible with the required efficiency, another option of using real-time PCR with improved PCR conditions specifically for the amplification of GC-rich templates was chosen. The temperature optimization carried out suggested 56°C to be optimal for the amplification of genomic template with Shobha-009 and CAB II primers (data not shown). Figure 10 shows the results of PCR reactions, for which the genomic template was varied between 100 ng and 250 ng, the primer concentration between 100 nM and 250 nM, and the DMSO concentration between 5% and 8%. Amplification for the CABII control primers is observed under any condition, but it was optimal at 250 ng of genomic template, 250 nM primer concentration, and 5% (v/v) DMSO (Lane 7). The gel shows that 5% DMSO yields a much higher amount of amplicon than 8% DMSO (compare Lane 7 with Lane 8). However, the Shobha-009 primers did not yield any amplicon (expected: 186 bp, Lanes 11-18), and the Shobha-011 primers showed only non-specific amplification of about 325 bp instead of the expected amplicon of 750 bp (Lanes 19-26).

The experiment was modified by adding a combination of 5% DMSO and 1 M betaine to each amplification assay, by investigating the effect of an increased amount of polymerase, and by testing a greater variety of primers for the animal cryptochrome gene. Figure 11 shows that three primer pairs gave an amplicon of the expected size – the control CAB II primers (347 bp, Lane 3 and 4), the DrJ-009 primers (186 bp, Lane 12 and 13), and the Shobha-010 primers (528 bp, Lane 15 and 16). Three other primer pairs did not yield an amplicon of the expected size – the Shobha-008 primers (997 bp, Lane 6 and 7), the Shobha-009 primers (186 bp, Lane 12 and 13), and the Shobha-010 primers (750 bp, Lane 18 and 19). A higher amount of polymerase was tested, since the use of
**Figure 10:** Gel electrophoresis of real time-PCR products with genomic DNA as template. Order of conditions listed for each lane: primer conc., template amount, % DMSO. Lane 1: 100 bp Marker. Lane 2: No template. Lane 3: 250nM, 250ng, 0%. Lane 4: 250nM, 100ng, 0%. Lane 5: 250nM, 100ng, 5%. Lane 6: 250nM, 100ng, 8%. Lane 7: 250nM, 250ng, 5%. Lane 8: 250nM, 250ng, 8%. Lane 9: 100nM, 250ng, 5%. Lane 10: 100nM, 250ng, 8%. Lane 11: No template. Lane 12: 250nM, 100ng, 0%. Lane 13: 250nM, 100ng, 5%. Lane 14: 250nM, 100ng, 8%. Lane 15: 250nM, 250ng, 5%. Lane 16: 250nM, 250ng, 8%. Lane 17: 100nM, 250ng, 5%. Lane 18: 100nM, 250ng, 8%. Lane 19: No template. Lane 20: 250nM, 100ng, 0%. Lane 21: 250nM, 100ng, 5%. Lane 22: 250nM, 100ng, 8%. Lane 23: 250nM, 250ng, 5%. Lane 24: 250nM, 250ng, 8%. Lane 25: 100nM, 250ng, 5%. Lane 26: 100nM, 250ng, 8%.

**PCR conditions:** Initial denaturation at 94°C for 5 min followed by 40 cycles of 94°C for 50 sec denaturation, 56°C for 40 sec annealing, 72°C for 45 sec extension; and final extension at 72°C for 6 min.
Figure 11: Gel electrophoresis of real time-PCR products with genomic template and a mixture of 5% DMSO and 1 M betaine. DMSO and betaine were always added, primer conc. was always 250nM, and template amount always 250ng. Lane 1: 100 bp Marker. Lane 2: CABII primers without template. Lane 3: CABII primers. Lane 4: CABII primers, additional polymerase. Lane 5: Shobha- 008 primers without template. Lane 6: Shobha- 008 primers. Lane 7: Shobha- 008 primers, additional polymerase. Lane 8: Shobha- 009 primers without template. Lane 9: Shobha- 009 primers. Lane 10: Shobha- 009 primers, additional polymerase. Lane 11: DrJ-009 primers without template. Lane 12: DrJ-009 primers. Lane 13: DrJ-009 primers, additional polymerase. Lane 14: Shobha- 010 primers without template. Lane 15: Shobha- 010 primers. Lane 16: Shobha- 010 primers, additional polymerase. Lane 17: Shobha- 011 primers without template. Lane 18: Shobha- 011 primers. Lane 19: Shobha- 011 primers, additional polymerase.

**PCR conditions:** Initial denaturation at 94°C for 5 min followed by 40 cycles of 94°C for 50 sec denaturation, 56°C for 30 sec annealing, 72°C for 45 sec extension; and final extension at 72°C for 10 min.
DMSO was reported to reduce the efficiency of Taq Polymerase (Diefenbach and Dveksler, 2003). Interestingly, a greater smear can be observed in the samples with the additional Taq Polymerase (Lane 4, 13, and 19 as compared to Lane 3, 12, and 18). The experiment was performed again to test its repeatability. An attempt was also made to study the use of only betaine instead of betaine in combination with DMSO. Figure 12 shows gel electrophoresis results of the real-time PCR reaction with genomic template in the presence of either 5% DMSO or 1M betaine or 2M betaine with no DMSO. First, the results from the previous experiment are repeatable. When using 5% DMSO and 1 M betaine in the amplification assay, an amplicon of the expected size can again be observed for the CABII control primers (347 bp, Lane 4 and 8), the DrJ-009 primers (186 bp, Lane 16), and the Shobha-010 primers (528 bp, Lane 20). The Shobha-011 primers (750 bp, Lane 24) again did not yield an amplicon of the expected size. However, this time the Shobha-009 primers (186 bp, Lane 12) did give the expected amplicon.

Secondly, the same results were generally obtained with 2M betaine as with the combination of 5% DMSO and 1 M betaine (Lane 6, 10, 14, 18, 22, and 26 as compared to Lane 4, 8, 12, 16, 20, and 24). It indicates that betaine alone is equally effective in promoting specific amplification from the GC-rich template as the combination of DMSO and betaine. Generally, the experiments depicted in Figure 11 and 12 show that amplification of a larger fragment (528 bp with the Shobha-010 primers) is possible through the use of DMSO and/or betaine. However, in order to conclusively prove that the correct fragment was amplified with the Shobha-010 primers, the 528 bp amplicon has to be sequenced.
Figure 12: Gel electrophoresis of real time-PCR products from genomic template with either 5% DMSO and 1M betaine or 2M betaine alone. Primer conc. was always 250nM, and template amount always 250ng. Lane 1: 50 bp Marker. Lane 2: 100 bp Marker. Lane 3: No template, DMSO+betaine. Lane 4: DMSO+betaine. Lane 5: No template, betaine. Lane 6: betaine. Lane 7: No template, DMSO+betaine. Lane 8: DMSO+betaine. Lane 9: No template, betaine. Lane 10: betaine. Lane 11: No template, DMSO+betaine. Lane 12: DMSO+betaine. Lane 13: No template, betaine. Lane 14: betaine. Lane 15: No template, DMSO+betaine. Lane 16: DMSO+betaine. Lane 17: No template, betaine. Lane 18: betaine. Lane 19: No template, DMSO+betaine. Lane 20: DMSO+betaine. Lane 21: No template, betaine. Lane 22: betaine. Lane 23: No template, DMSO+betaine. Lane 24: DMSO+betaine. Lane 25: No template, betaine. Lane 26: betaine.

**PCR conditions:** Initial denaturation at 94°C for 5 min followed by 40 cycles of 94°C for 50 sec denaturation, 56°C for 30 sec annealing, 72°C for 45 sec extension; and final extension at 72°C for 10 min.
CHAPTER IV
DISCUSSION

IV.1 Development of an experimental design

Since the gene of *C. reinhardtii* previously identified as encoding a possible animal-like cryptochrome photoreceptor (Thompson and Sancar, 2002; Mittag et al., 2005) shows greater sequence similarity to the 6-4 DNA photolyase of *Arabidopsis*, an experimental strategy was developed to test the protein for possible 6-4 DNA photolyase activity.

DNA photolyases are enzymes involved in the repair mechanism of UV induced DNA damage by photoreactivation (Thompson and Sancar, 2002; Nakajima et al., 1998; Waterworth et al., 2002). Photoreactivation is a process where these enzymes use the energy of blue light (360-420nm) to repair the lesions in DNA (Waterworth et al., 2002; Peterson and Small, 2001). The two enzymes that repair pyrimidine dimers are CPD (Cyclobutane pyrimidine dimer) photolyases and 6-4 DNA photolyases. They act specifically on cyclobutane pyrimidine dimers and pyrimidine (6-4) pyrimidone products, respectively (Hirayama et al., 2003; Merrow et al., 2005; Cashmore, 2003).

In *E. coli*, UV-induced DNA damage is repaired by three mechanisms – 1) photoreactivation (*phr*), 2) nucleotide excision repair (*uvr*) and 3) post replication-recombination repair (*rec*). Post replication-recombination repair does not involve any enzymatic machinery, photoreactivation and nucleotide excision repair are mediated by DNA photolyase and *uvr*ABC excision nuclease, respectively (Sancar et al., 1984). Interestingly, *E. coli* contains a single DNA photolyase that is only able to repair cyclobutane pyrimidine dimers (Brash et al., 1985).
The strategy developed is similar to the strategies previously employed for testing other eukaryotic 6-4 photolyases (Nakajima et al., 1998; Waterworth et al., 2002). For example, Nakajima et al., (1998) studied and characterized the UVR3 gene of Arabidopsis, which when defective will render the organism more sensitive to UV light. The researchers were able to demonstrate 6-4 photolyase activity of the UVR3 protein by expressing the cDNA in a mutant of E. coli defective in photoreactivation, nucleotide excision repair, and recombination. After extraction of total RNA, poly (A+) RNA was separated from total RNA using oligo (dT) latex beads (Nakajima et al., 1998) and used for cDNA synthesis. The cDNA was amplified with primers derived from the conserved sequence of CPD and 6-4 DNA photolyases. 5’ and 3’ RACE was performed to acquire the entire translated region. The amplified DNA fragment was gel purified, inserted into a GST fusion vector, and transformed into XL1-blue E. coli cells. The expression of fused UVR3 and GST gene gave a fusion product. The expression of this fusion protein product resulted in an increased resistance to UV in mutant E. coli defective in its DNA repair mechanism. This reversal of DNA damage occurred only when blue light was supplied. The (6-4)-specific photolyase activity was demonstrated in vivo through the survival rates of the E. coli cells transformed with the photolyase gene. In addition, the authors induced expression of the gene in XL1-blue cells with IPTG, purified the GST-fusion protein by affinity chromatography on a glutathione-Sepharose column, and used it in an in vitro assay. The in vitro assay was based on a 49 bp DNA substrate containing the 6-4 photoproduct within a Msel restriction site (5’TTAA3’) that was initially resistant to digestion with Msel. The substrate became sensitive to Msel digestion after incubation
with the fusion protein and fluorescent light indicating that the protein has 6-4 photolyase activity.

An *in vitro* assay for 6-4 photolyase activity has also been used to identify the enzyme in *Xenopus laevis*, *Crotalus atrox*, and *Drosophila* (Todo *et al.*, 1997; Kim *et al.*, 1994). The assay was either carried out based on a *Msel* restriction site in the substrate as described above for *Arabidopsis* or based on a *Hpal* restriction site (5’GTTAAC3’).

An *E. coli in vivo* assay has been used to identify the 6-4 photolyase from the hexactinellid sponge, *Aphrocallistes vastus* (Schroder *et al.*, 2003). The photolyase cDNA from *A. vastus* was isolated and transfected into a mutant strain of *E. coli* SY2, defective in photoreversal activity. The expression of the photolyase gene caused the resistance of bacteria against the UV light. *E. coli* cells were harvested after growing them for 6hr at 26°C. The petri dish containing the cells suspended in saline was irradiated with UV-B lamp, followed by illumination with daylight fluorescent lamps for 1 hr at 25°C. Samples were plated on LB agar and incubated overnight at 26°C to score the surviving colonies. The survival rate was calculated as a percentage of the number of colonies formed with non-irradiated control.

Successful expression of recombinant *C. reinhardtii* genes in *E. coli* has been described before. For example, Keshkiaho and coworkers (2007) purified two different active prolyl 4-hydroxylases from *E. coli*. The enzymes play a major role in proper cell wall formation in *C. reinhardtii*. The strategy the authors employed included the amplification of gene specific cDNAs with flanking *Ndel* and *BglII* sites introduced through the primers. The products were cloned into the *Ndel-BamHI* digested bacterial vector pET15b in frame with an N-terminal His tag and transformed into the *E. coli*
(DE3) strain. The gene expression was induced with IPTG, samples were harvested, lYZed, and the protein purified with nickel chelating Sepharose affinity chromatography.

However, it might be difficult to express the cDNA for animal cryptochrome from *C. reinhardtii* in *E. coli*. *C. reinhardtii* generally shows a high GC content of about 64% in its nuclear genome (Merchant *et al.*, 2007; Immeln, *et al.*, 2007). This high GC content is partly due to a strong codon bias, where G and C in the third position of a codon is greatly overrepresented (Harris, 1989). The differences in codon usage between the *E. coli* expression system and *C. reinhardtii* impede translation of the cryptochrome protein due to less amounts of specific tRNA. This could be overcome by adding an extra gene for the most problematic tRNA into the *E. coli* strain so that this tRNA is present in appropriate amounts (Harris, 1989; Franklin *et al.*, 2002).

**IV.2. Cloning of the animal cryptochrome cDNA**

The first step in the experimental strategy developed for this project is the cloning of the animal cryptochrome cDNA by PCR. To accomplish this step, total RNA was successfully extracted (Figure 3) and reverse transcribed into cDNA as confirmed by *C. reinhardtii*-specific control primers (Figure 4). However, the efficiency of the two different primer pairs designed to amplify the entire coding region from the animal cryptochrome cDNA could not be confirmed using genomic DNA as control template, despite attempts at optimizing the annealing temperature and the magnesium ion concentration. This is perhaps not too surprising, because amplification of long GC rich templates is generally difficult. The high GC content of the template might prevent its complete denaturation (Bhaskaran *et al.*, 1996). Amplification of GC rich templates may
also lead to shorter fragments that lack the GC portion of the sequence (Diefenbach and Dveksler, 2003).

When using cDNA as template, the two primer pairs also did not yield the expected fragments for the entire coding region, at least in a basic PCR assay, despite the smaller size of this region (1788 bp). It therefore became necessary to confirm that enough animal cryptochrome cDNA was present in the cDNA mix to efficiently serve as template before further attempts at optimizing the PCR conditions were justified. Accordingly, four more primer pairs were designed to amplify various smaller regions of the genomic control DNA. If amplification could be achieved with at least one primer pair, this pair would provide a reliable probe for the presence of animal cryptochrome cDNA in the cDNA mix. However, none of these primer pairs yielded the expected amplicon size with the genomic control DNA. There are at least three possible reasons for this. (1) The conditions for amplification had not been fully optimized yet. Only the basic protocol of annealing temperature 5°C below the melting temperature of the primers and a magnesium ion concentration of 1.5 mM was tested. (2) The primer design program, which was Vector NTI, did not succeed in designing optimal primers. (3) There might be some differences in sequence for the animal cryptochrome gene from different strains of *C. reinhardtii*. The sequence used for the primer design is derived from strain CC-503 (cw92 mt+). This strain was chosen for sequencing the entire nuclear genome of *C. reinhardtii*, because it is a cell wall-less mutant and therefore extraction of genomic DNA is particularly easy. The strain used to extract genomic DNA for this project was CC125, which has a wild-type cell wall. There were some indications to suspect that the second reason was true. The primer design program seemed flawed, because surprisingly,
When aligning the primers with the sequence they were designed from, mismatches were detected.

Because of the unexpected mistake in the Vector NTI primer design function, new primer pairs were designed based on different software. A primer pair that would amplify a 997 bp fragment (Shobha-008) was designed using the Beacon 2.1 software but it did not yield the expected amplicon size despite attempts at annealing temperature and magnesium ion concentration optimizations. Two new primer pairs were finally designed to amplify particularly short fragments of 186 bp each. One of these primer pairs was designed with the Beacon software and the other with the Primer Express program. Both of these primer pairs yielded an amplicon of the expected size with genomic control DNA as template. This implies that the size of the amplicon might be a particularly important aspect when performing PCR with C. reinhardtii DNA, possibly because of its high GC content. For one of these pairs (Shobha-009, Figure 5 and 6), optimization of magnesium ion concentration and annealing temperature was carried out in order to be certain of the best conditions for amplification. Interestingly, the primer pair gave rise to an amplicon of the expected size under any of the magnesium ion concentrations and annealing temperatures tested. It seemed to suggest that the pair has a sequence particularly optimal. However, when more experiments were performed, there were problems with the repeatability and consistency of the results despite using exactly the same solutions and PCR amplification conditions (data not shown).

To improve the likelihood of successfully cloning the cDNA for animal cryptochrome, different options were considered. One option was to improve the amplification conditions particularly for GC rich templates using real-time PCR. Real-
time PCR allows for the visualization of the amplification reaction after every single cycle and therefore conclusions on the amplification efficiency. It can also measure melting curves for the final amplicons and therefore provide an indication of the number of different fragments present. The second option was to abandon the PCR technique altogether by screening a cDNA library with an oligonucleotide probe.

Due to problems with the PCR technique in terms of its reliability and consistency, the second option, i.e. the screening of an available cDNA library using a Dot Blot technique was initially chosen but then abandoned due to the low titer of the library and the insufficient reliability of the signal. The better option was to use real-time PCR and optimize the amplification of high GC templates. This choice finally allowed not only the reliable amplification of short sequences but even the amplification of a longer fragment of 528 bp. It was possible based on the addition of DMSO or betaine, two compounds that were reported to improve amplification from GC-rich templates (Henke et al., 1997). The results are only a first step on the path to truly clone the cDNA of animal cryptochrome from *C. reinhardtii*. But they indicate the strategy by which it might be achieved. Amplification of GC-rich DNA may also be improved by using adjuvants like BSA (bovine serum albumin), sulfonal, formamide, and glycerol to increase efficiency (higher amount of product) and specificity (lack of non-specific products) (Kitade et al., 2003; Henegariu et al., 1997). Other possibilities include the use of nucleotide analogs like 7-deaza dGTP and dITP or DNA denaturation by NaOH. A combination of DMSO, betaine and 7-deaza dGTP may be the most promising combination to further improve the specificity of amplification and succeed in amplifying the entire coding region from cDNA (Kitade et al., 2003). According to the
Boynton/Gillham laboratory (see the *Chlamydomonas* Center web page), 7-deaza-dGTP is required for the amplification of an exon of *C. reinhardtii* nuclear DNA with a G+C content of 73%. The analog is optimally used in a PCR assay at a concentration of 150µM in combination with 50µM dGTP while the other three dNTPs are used at the usual concentration of 200µM.

When considering improving the cDNA template for future experiments, there are also some choices. One option is to test extracted RNA from different times during the 12 h light/12 h dark cycle. Some photoreceptors were reported to show a diurnal or circadian rhythm of expression. RNA for the experiments reported here had been extracted 4 h into the light phase of a 12 h light/12 h dark cycle. Since animal cryptochrome is expected to function either as a protective enzyme against UV light damage or as a photoreceptor, expression of its gene could be at a higher level earlier in the light phase. A second option is to isolate polyA+ RNA from total RNA in an effort to improve the efficiency of cDNA synthesis. Yet a third option is to use the cDNA library that previously served in the screening attempt. Since the cDNA library is packaged into a phage, it can only be used after the DNA is released from the phage capsid. Hence, the cDNA library needs to be processed before it can be used as template. According to Dr. Silflow (personal communication), to prepare a crude phage DNA as template for PCR, a 25 microliter aliquot of the phage stock is freeze-thawed twice in liquid nitrogen. The sample is then boiled for 5 minutes and one or two microliters of the lysate is used in a 25 microliter PCR reaction. In order to reduce the amplification of unwanted fragments, Dr. Silflow’s lab is usually using nested primers at one end of the sequence. This is accomplished by first performing PCR with two primers, one of which hybridizes further away yielding a
longer amplicon. This reaction is then diluted 50-fold and one microliter of the dilution used as template in the second 25 microliter PCR reaction with the nested primer.

In summary, this project succeeded in developing an experimental approach to determine whether the “animal cryptochrome” of *C. reinhardtii* shows 6-4 DNA photolyase activity. It also succeeded in developing a protocol for PCR amplification of longer fragments from the GC-rich genome of *C. reinhardtii*. The results may be used in the future to clone the entire coding region of *C. reinhardtii* animal cryptochrome from cDNA as a first step in enabling the protein’s functional analysis.


