

## The Circadian Clock in *Chlamydomonas reinhardtii*. What Is It For? What Is It Similar To?<sup>1</sup>

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The physiology of the circadian (daily) clock has been well studied in the unicellular eukaryote *Chlamydomonas reinhardtii*. Circadian rhythms of phototaxis, chemotaxis, cell division, UV sensitivity, and adherence to glass have been characterized in this green alga. Circadian phototaxis was even shown to operate in outer space! The related phenomenon of photoperiodic time measurement of germination has been demonstrated. The *C. reinhardtii* system now offers genetic and proteomic opportunities that make it an excellent unicellular eukaryotic model organism to study the circadian clock at all levels of organization. Several clock-controlled genes have been identified as well as a clock-controlled RNA-binding protein that acts on circadian output. A computer-based search in *C. reinhardtii* for components of the circadian system that are similar to those from other model species has shown that some phototransduction components and especially kinases and phosphatases are well conserved in this green alga, while their target proteins appear to be different. The first functional proteomic approaches have discovered novel components of the circadian system, including a protein disulfide isomerase and a tetratricopeptide repeat protein.

### CIRCADIAN PROGRAMS IN *C. REINHARDTII* AND THEIR SIGNIFICANCE

Circadian (daily) rhythms are endogenous biological programs that control metabolic, physiological, and/or behavioral events to occur at optimal phases of the daily cycle. In addition to exhibiting a self-sustained oscillation in constant conditions that can be entrained to environmental cycles, circadian rhythms run at essentially the same rate at different ambient temperatures (i.e. they are "temperature compensated"). *C. reinhardtii* has long been a preferred model system for the analysis of circadian rhythms because of the

genetic and molecular techniques that have been developed for *C. reinhardtii* and are described elsewhere in this issue (Harris, 1989, 2001; Johnson et al., 1992; Fuhrmann, 2002). The availability of its complete nuclear, chloroplast, and mitochondrial genome sequences and of more than 200,000 expressed sequence tags (ESTs) that have been assembled into approximately 10,000 unique cDNAs (Grossman et al., 2003) render it a highly attractive model system.

The circadian clock in *C. reinhardtii* is known to modulate a number of processes. As is the case for other flagellates, *C. reinhardtii* is able to orientate itself toward the light, a process known as photoaccumulation or phototaxis. This process is known to be rhythmically modulated in some species; Victor Bruce demonstrated circadian rhythms of photoaccumulation in *C. reinhardtii* more than 30 years ago (Bruce, 1970). The algae swim toward a supplied light source with maximum accumulation during the day phase. Computerized apparatuses can automatically monitor this rhythm (Mergenhagen, 1984; Kondo et al., 1991; Johnson et al., 1992). Mergenhagen and Mergenhagen (1987) used an automated apparatus aboard a spacecraft to confirm that the photoaccumulation rhythm persisted in outer space when the cells orbited the earth every 90 min! The rhythms continued for at least 6 d in microgravity without any terrestrial cue of the time of day. Therefore, these studies of *C. reinhardtii* showed conclusively that the circadian clock can run independently from daily cycles of gravity, magnetism, cosmic ray irradiation, and so on.

There are also circadian rhythms in *C. reinhardtii* that peak during the night phase, for example, chemotaxis to ammonium (Byrne et al., 1992). The cells swim maximally toward this nitrogen source during the middle of the night phase, even though the light-dependent uptake of ammonium does not occur until dawn. The uptake of nitrite also peaks at dawn, as does the activity of nitrite reductase (Pajuelo et al., 1995). Chemotaxis versus phototaxis (as well as the uptake and further metabolism of N sources) is an example of temporal separation of processes in algae. In other words, the daily biological clock organizes a temporal program that triggers biological events to occur at specific phases throughout the daily environmental cycle; phototaxis during daytime optimizes photosynthesis, and chemotaxis (to ammonium) in the nighttime

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allows *C. reinhardtii* to find and store nutrients when solar energy is not available.

Another circadian rhythm in *C. reinhardtii* that peaks during the night is the ability of the cells to adhere to a glass surface (Straley and Bruce, 1979). Yet another important rhythmic property is the temporal control of cell division, as first studied by Bruce (1970) and later by Goto and Johnson (1995). Several clock mutants that have an altered period were isolated by Victor Bruce using the phototaxis rhythm as a screen (Bruce, 1972; Bruce and Bruce, 1978). These mutants were called *period* (*per*), a name that was also given to a famous clock gene in *Drosophila* (but they are almost certainly unrelated since there are no putative homologs of the *Drosophila per* gene in the *C. reinhardtii* genome; see below). Victor Bruce demonstrated with one of the long-period mutants (*per4*) that three independent rhythms (phototaxis, adhesion to glass, and cell division) were affected by the mutation, implying that a component of the central oscillator may be defective in the *per4* mutant (Straley and Bruce, 1979).

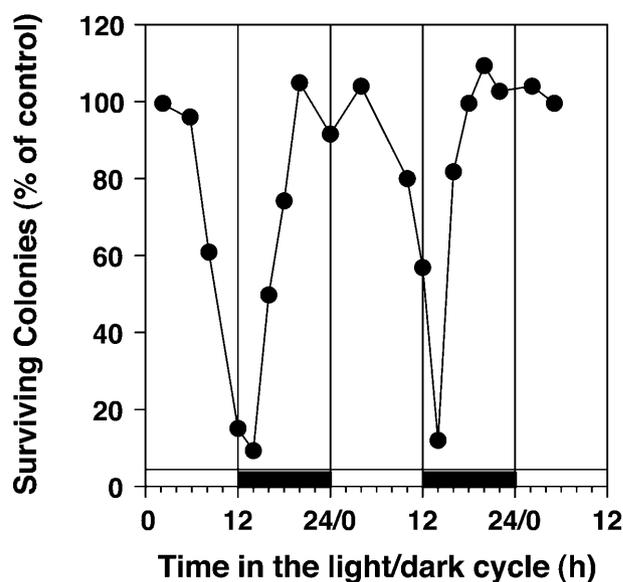
### Evolution of Circadian Timers

The question of why organisms have endogenous temporal programs is closely linked with identifying the selective forces that encouraged the original evolution of these timers. Perhaps an initial driving force for the early evolution of circadian clocks could have been to phase cellular events that are inhibited by sunlight to occur in the night. This idea has been called the "escape from light" hypothesis (Pittendrigh, 1993). That speculation seems plausible when one considers the numerous examples of microorganisms with 24-h cell division cycles in which DNA replication and cell division occur during the night (Edmunds, 1988). Some of the events of the cell division cycle in these microorganisms might be sensitive to sunlight, e.g. replication during S phase.

A prediction of this hypothesis would be that present-day organisms retain temporal regulation of light-sensitive processes to the night. Because the most generally deleterious wavelengths of sunlight are in the UV range, a daily rhythm of sensitivity to UV light in *C. reinhardtii* was tested (Nikaido and Johnson, 2000). As shown in Figure 1, these algae are more sensitive to UV light near sunset and into the early night. The rhythmic sensitivity persists in constant conditions. These data indicate that the circadian system in *C. reinhardtii* has programmed UV-sensitive processes to occur at phases of the daily cycle when UV levels will be low or absent in a manner that is consistent with the "escape from light" hypothesis (Nikaido and Johnson, 2000).

### Photoperiodism and Seasonal Responses

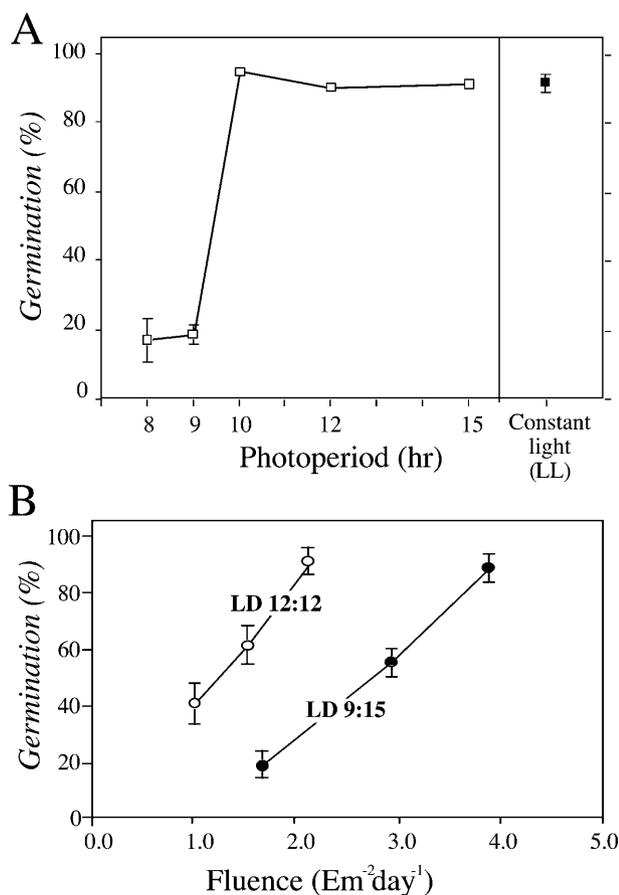
Plants and animals sense the season of the year by measuring the duration of the day and/or night in the natural environment and respond appropriately so as



**Figure 1.** Survival of *Chlamydomonas* cells after irradiation by UV light as a function of the time in a light/dark cycle. *Chlamydomonas* cultures were plated onto agar medium and treated with equal amounts of UV light at different phases of a 12-h-light/12-h-dark cycle. Survival was measured as the colony-forming ability of cells following treatment as compared to that of cells that were not irradiated with UV light (modified from Nikaido and Johnson, 2000).

to adapt to seasonal changes in their environment (Thomas and Vince-Prue, 1997). This phenomenon is called photoperiodic time measurement (PTM). PTM is of fundamental importance to the biological adaptations of organisms to their environment, especially in the cases of reproduction and development. The model for PTM that has become generally accepted is that a circadian (daily) clock is the timer that somehow measures the length of the night and triggers the developmental events controlled by photoperiodism, as first proposed by Bünning (1936) and now supported by multiple lines of evidence (Thomas and Vince-Prue, 1997).

PTM is well documented in multicellular eukaryotes, but few examples exist for unicellular organisms. It seems logical that unicellular organisms might also benefit from being able to anticipate and respond to seasonal changes in their environment. One of the few reports of a photoperiodic response in unicells is a study of PTM in *C. reinhardtii* (Suzuki and Johnson, 2002). In the life cycle of *Chlamydomonas*, nonoptimal conditions (e.g. nitrogen deprivation) provoke the differentiation of haploid vegetative cells into gametes, which mate and form diploid zygospores. Zygospores are dormant cells that cannot divide until after they undergo meiosis and germinate into four haploid vegetative cells. Zygotes resist stressful environmental conditions, such as freezing, darkness, desiccation, and starvation, better than vegetative or gametic cells (Harris, 1989; Suzuki and Johnson, 2002). As shown in Figure 2A, the germination efficiency of *Chlamydomonas* zygospores is enhanced (approximately 90%



**Figure 2.** Germination in different photoperiods in *Chlamydomonas*. A, Photoperiodic response curve. The germination efficiencies 10 d after mating are shown as a function of the light duration in light/dark cycles of 24 h total duration (white squares). Constant light (LL, filled square) represents cells continuously exposed to light for 24 h a day. B, Germination efficiencies in two different photoperiods (LD 12:12 versus LD 9:15) at different light fluences. Data are plotted as the total light fluence in a day, calculated as irradiance (light intensity,  $\mu\text{E m}^{-2} \text{s}^{-1}$ )  $\times$  (duration of light treatment in seconds). A and B, Germinated cells were counted at 10 d after mating, all data are shown with SEM (modified from Suzuki and Johnson, 2002).

germination) in long-day light/dark cycles, i.e. for photoperiods longer than 10 h, but is suppressed (<25% germination) in short-day light/dark cycles, i.e. for photoperiods of 8 to 9 h (Suzuki and Johnson, 2002). Comparisons of photoperiod versus fluence demonstrated that *Chlamydomonas* germination was a bona fide photoperiodic response and not merely a response to fluence (Fig. 2B). Thus, it is likely that the suppression of germination by short days is an adaptive response for the overwintering of *Chlamydomonas*.

#### MOLECULAR "OUTPUTS" OF THE CIRCADIAN CLOCK

So far, most of the studies on the molecular basis of the circadian clock in *C. reinhardtii* have focused on clock-controlled genes. In particular, circadian control

of transcriptional rate has been found for a number of genes, resulting in rhythms of RNA abundance over the circadian cycle. These data have been described in a recent review (Mittag and Wagner, 2003) and are summarized in Table I. Many of these clock-controlled genes encode proteins that are involved in photosynthesis and chloroplast metabolism, and most of these genes are expressed at a high rate during the subjective day. A few other genes, e.g. *Arf1*, *GAS-3*, *HSP70B*, and *cytochrome c*, are maximally expressed in the night. Most of these genes are encoded in the nuclear genome, while a few are encoded by chloroplast DNA (e.g. *Tuf A*, *chl rRNA*; Hwang et al., 1996). A possibly related observation is that the supercoiling status of chloroplast DNA itself oscillates over the daily cycle (Salvador et al., 1998). Because the expression of many promoters is sensitive to supercoiling status, gene expression within the chloroplast might be globally modulated by circadian changes of the topology of the chloroplast chromosome.

Other molecular studies concern the circadian binding activity of an RNA-binding protein called CHLAMY 1 (Mittag, 1996). This protein is an analog of CCTR (clock controlled translational regulator) from the dinoflagellate alga *Lingulodinium polyedra* (for review, see Mittag, 2003). Its binding activity increases at the end of the subjective day and stays at a high level until the middle of the subjective night. CHLAMY 1 interacts with mRNAs that have an UG repeat of at least seven units (Waltenberger et al., 2001), and we assume that it represses translation (Mittag, 2003). The UG-containing mRNAs encode mainly proteins of the nitrogen metabolic pathways (NRT2;3, a nitrite/nitrate transporter; nitrite reductase; Gln synthetase; argininosuccinate lyase) and carbon metabolic pathways (small subunit of Rubisco; LIP36-G1, a CO<sub>2</sub> shuffling protein in the outer chloroplast membrane; CCM, a CO<sub>2</sub> key regulator). CHLAMY 1 represents a novel type of

**Table I.** Circadian changes in mRNA abundance and/or transcription rate in *C. reinhardtii*

References: Jacobshagen and Johnson (1994); Memon et al. (1995); Fujiwara et al. (1996); Hwang et al. (1996); Jacobshagen et al. (1996, 2001); Savard et al. (1996); and Lemaire et al. (1999).

Time of Maximal Abundance (Day vs. Night) of mRNAs Encoding the Following Proteins	
Day Phase	
$\beta$ -Subunit of ATPase	$\beta$ -Tubulin
Carbonic anhydrase 1	D1 of PSII (Psb A)
Elongation factor tu (Tuf A)	Ferredoxin
Ferredoxin-NADP reductase	Fru-biphosphate aldolase
LI818, new type of Lhcp	Light-harvesting complex protein (LhcppII)
Thioredoxin h	Thioredoxin m
Phosphoribulokinase	PSA A of PSI
Night Phase	
ADP-ribosylation factor (Arf 1)	Cytochrome c
GAS-3, gamete-specific protein	HSP70B, chloroplastic heat shock protein

heteromeric RNA-binding protein that consists of subunits comprising three RNA-recognition motifs and three Lys-homology motifs (Zhao et al., 2004). CHLAMY 1's circadian binding activity appears to be controlled at the posttranslational level by time-dependent formation of protein complexes. One of the subunits of CHLAMY 1, named C3, is conserved in mammals, where it appears to be involved in myotonic dystrophy (Zhao et al., 2004). Interestingly, patients suffering from myotonic dystrophy have disturbances in their circadian system (Okumura et al., 2002).

#### EXPLORING THE *C. REINHARDTII* GENOME WITH REGARD TO COMPONENTS OF THE CIRCADIAN SYSTEM

The entire nuclear genome (version 2) of *C. reinhardtii* has been sequenced by the U.S. Department of Energy, and the information is available at the Joint Genome Institute (JGI) Web site (<http://genome.jgi-psf.org/chlre2/chlre2.home.html>). We searched the *C. reinhardtii* nuclear genome for potential homologs to genes that are known to encode components of the circadian system in other organisms (Table II). These include the clock model systems of the prokaryotic cyanobacterium *Synechococcus elongatus*, the fungus *Neurospora crassa*, the angiosperm *Arabidopsis thaliana*, the fruit fly *Drosophila melanogaster*, and the mammals *Mus musculus* and human (*Homo sapiens*). We scanned for amino acid sequences (National Center for Biotechnology Information [NCBI] protein search) in *C. reinhardtii* that show any extended similarity to protein sequences that are involved in the circadian system of the aforementioned model organisms by using the JGI BLAST page (<http://genome.jgi-psf.org/cgi-bin/runBlast?db=chlre2>) in combination with tBLASTn (protein versus translated nucleotides). The results were presented in the form of scaffolds. The depicted sequences within these scaffolds were then used to search within ESTs of *C. reinhardtii* ([http://www.biology.duke.edu/chlmy\\_genome/blast/blast\\_form.html](http://www.biology.duke.edu/chlmy_genome/blast/blast_form.html)) in parallel with the genome browser site of the JGI BLAST page that shows predicted gene models. The proteins that were used for screening JGI were also analyzed within the NCBI conserved domain search (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). All domains are listed in Table II. If a domain of a protein appears to have been conserved in the *C. reinhardtii* sequence, it is in bold. Percent similarity was calculated by browsing the protein sequence from *C. reinhardtii* against an NCBI protein BLAST. Regions of similarity (amino acid range) as well as their e-values are indicated in Table II, and the percent similarity is given with respect to these regions. In some cases, only small regions of similarity are shown in Table II, so the significance of the findings must be tempered by the comparison of the extent of a potentially homologous region of the *C. reinhardtii* protein to the total number of amino acids in the candidate protein.

In addition, we have searched the *C. reinhardtii* chloroplast and mitochondrial genomes for potential homologs to the clock-related genes of *S. elongatus* mentioned in Table II. For this purpose, the chloroplast and mitochondrial sequences of *C. reinhardtii* were translated in all six open reading frames and compared by the BLAST 2 Sequence Tool (<http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html>) with each single *S. elongatus* protein. However, no significant similarities were found to any of the clock-related proteins of *S. elongatus*.

#### The Significance of Potential Homologs with Regard to Their Circadian Function

In all circadian model systems studied so far, positive and negative feedback loops have been proposed to be key features (for review, see Dunlap, 1999; Harmer et al., 2001; Panda et al., 2002; Reppert and Weaver, 2002; Johnson, 2004). Within these loops, some molecular components are conserved among certain organisms with regard to their protein domain structure and their function. Thus, CLOCK and CYCLE act as transcription factors of clock components in flies and mammals. Casein kinases phosphorylate components of the endogenous oscillatory system in *N. crassa*, *Arabidopsis*, flies, and mammals, although the target proteins are often different. In some cases the protein domain architecture is conserved within a broad range of eukaryotes, but its function is different, as in the case of CRYPTOCHROME (CRY). CRY acts as blue-light photoreceptor in *Arabidopsis* and flies but is a component of the oscillatory loop in mammals. The similarity search can therefore suggest a clock-related *C. reinhardtii* component, but its function within the circadian system will have to be proven experimentally for each case.

#### Clock-Related Kinases and Phosphatases Are Well Conserved in *C. reinhardtii*

Progressive temporal phosphorylation of central oscillator proteins is thought to be crucial to the 24-h timing mechanism. In all eukaryotic examples (*N. crassa*, *Arabidopsis*, *D. melanogaster*, and mammals), casein kinases (CK1 and CK2) belonging to the Ser/Thr family of kinases are involved (Gorl et al., 2001; Panda et al., 2002; Yang et al., 2003; Daniel et al., 2004; Nawathean and Rosbash, 2004). While the phosphorylated target components are different depending on the organism (e.g. FREQUENCY [FRQ] in *N. crassa*, CCA1 in *Arabidopsis*, PERIOD [PER] in *D. melanogaster* and mammals, etc.), their temporal phosphorylation appears to be relevant for maintaining intact circadian rhythmicity. In *C. reinhardtii*, CK1 and CK2 (both subunits) are conserved (Table II). Furthermore, the Ser/Thr kinase SHAGGY, which has been shown to phosphorylate the interaction partners of PER and TIMELESS (TIM) in *D. melanogaster*, is also conserved in *C. reinhardtii*.

**Table II.** Clock-related proteins from model organisms that are conserved in *C. reinhardtii*

Sequence analysis was carried out as explained in the first paragraph of the section “Exploring the *C. reinhardtii* Genome with Regard to Components of the Circadian System.” Briefly, the JGI BLAST page was used in combination with tBLASTn in addition to a NCBI conserved domain search and NCBI protein BLAST. Clock proteins that do not show any significant similarity (labeled as “no hits” in the output file of the JGI blast) to proteins of *C. reinhardtii* are: *S. elongatus* KAI A, KAI B, KAI C, LDPA, PEX, RPOD2, and SAS A; *N. crassa* FRQ; Arabidopsis ELF3, GI, PHYB, PHYE, PIF3, and TEJ; *D. melanogaster* CLK, CYC, LARK, PDP1, PER, TIM, and VRI; and mammal BMAL1, BMAL2, m-CLOCK, CREB, DBP, DEC1, DEC2, NPAS2, mPER1, mPER2, mPER3, REV-ERB, and mTIM. Other proteins that show only a very limited similarity to proteins of *C. reinhardtii* are: *N. crassa* VVD, WC1, and WC2; and Arabidopsis ADO1, ADO2 (LKP2), ADO3 (FKF1), APRR1, APRR3, APRR5, APRR7, APRR9, CCA1, CO1, and ZTL. “Very limited similarity” was defined as those cases when the percentage of identical amino acids (AA; including functional identical ones) was below 16% over the total length of the protein. %, Similarity in percent within the positives (AA range); AA Range, x/y = number of (functional) identical amino acids (x) from the amino acid area of similarity (y); e-value, expect value is a parameter that describes the number of hits one can expect to see just by chance when searching a database of a particular size (the lower the e-value, or the closer it is to 0, the more significant is the match); Total No. AA, total number of amino acids of the protein from the organism used for the similarity search.

Organism/Protein	Domains <sup>a</sup> and Characteristics	NCBI No.	Gene Model/ESTs	%	AA Range	Total No. AA	e-value
Cyanobacteria ( <i>S. elongatus</i> )							
CIKA	Circadian input kinase	<b>His kinase A, HATPase, REC</b> and GAF domain; input pathway	AAF82192	C_50007/5.114.1.5	57	127/220	754 (1e–35)
CPMA	Circadian phase modifier	Similar to <b>NCAIR mutase (PurE)-related protein</b> ; output pathway	AAD29318	C_250166/No EST	48	112/230	260 (2e–21)
RPOD	RNA-polymerase sigma factor	<b>Sigma70 regions 2, 3, and 4, FlIA, RpoD and E domains</b>	2208419B	C_30171/3.55.1.51	62	194/308	391 (1e–54)
Fungi ( <i>N. crassa</i> )							
CKA	Casein kinase II catalytic subunit $\alpha$	<b>Ser/Thr kinase catalytic domain</b> ; component of central oscillator	AAM14624	C_70148 <sup>b</sup> /No EST	58	144/245	336 (7e–58)
CKB1	Casein kinase II regulatory subunit $\beta$ 1	<b>Casein kinase II regulatory subunit</b> ; component of central oscillator	Q8TG12	C_280152/28.27.1.0	59	121/20	333 (2e–41)
CKB2	Casein kinase II regulatory subunit $\beta$ 2	<b>Casein kinase II regulatory subunit</b> ; component of central oscillator	Q8TG11	C_280152/28.27.1.0	65	132/203	285 (6e–54)
HHP1	Casein kinase I homolog	<b>Ser/Thr kinase catalytic domain</b> ; component of central oscillator	EAA36589	C_70149/7.12.2.11	90	256/283	334 (1e–138)
PP1	Protein phosphatase 1, catalytic subunit	<b>PP2A catalytic, Metallphos and ApaH domains</b> ; component of central oscillator	Q9UW86	C_2260011/226.1.2.11	92	278/300	308 (1e–155)
PP2A	Protein phosphatase 2A, regulatory subunit	<b>B56 PP2A regulatory B subunit</b> ; component of central oscillator	CAC28812	C_1270036/127.5.2.11	63	264/418	642 (1e–107)
Higher plants (Arabidopsis and <i>Mesembryanthemum crystallinum</i> in one case)							
AtGRP7 (CCR2)	Arabidopsis Gly-rich protein (=cold circadian rhythm and RNA binding)	Gly rich, <b>RNA-recognition motif</b> ; output pathway	AAM62447	C_10096/1.211.1.5	67	53/79	175 (3e–11)
CCA1 <sup>c</sup>	Circadian clock associated 1	<b>Myb-like DNA-binding domain</b> ; component of the central oscillator	NP_850460	C_1060059/No EST	84	63/75	608 (2e–22)
CKI	Casein kinase 1	<b>Ser/Thr kinase</b> ; component of the central oscillator	BAB10977	C_70149/7.12.2.11	93	277/297	476 (1e–131)
CKIIA1	Casein kinase 2, $\alpha$ -chain 1	<b>Ser/Thr kinase</b> ; component of the central oscillator	NP_201539	C_970006 <sup>b</sup> /97.66.3.52, 97.66.1.51	74	146/196	409 (3e–67)
CKIIA2	Casein kinase 2, $\alpha$ -chain 2	<b>Ser/Thr kinase</b> ; component of the central oscillator	AAN41288	C_970006 <sup>b</sup> /97.66.3.52, 97.66.1.51	63	207/326	403 (2e–86)

(Table continues on following page.)

**Table II.** (Continued from previous page.)

	Organism/Protein	Domains <sup>a</sup> and Characteristics	NCBI No.	Gene Model/ESTs	%	AA Range	Total No. AA
						<i>e-value</i>	
CKIIB1	Casein kinase 2, $\beta$ -chain 1	<b>CKII regulatory subunit;</b> component of the central oscillator	S47967	C_280152/28.27.1.0	86	160/186 (4e-81)	287
CKIIB2	Casein kinase 2, $\beta$ -chain 2	<b>CKII regulatory subunit, DRP domain;</b> component of the central oscillator	CAB78767	C_280152/28.27.1.0	86	161/186 (3e-81)	282
COP1	Constitutive photomorphogenic 1	<b>WD40 repeats,</b> RING-finger, E3 ubiquitin ligase; input pathway	T01112	C_1310019/131.28.1.5	64	196/305 (1e-70)	675
CRY1 (HY4)	Cryptochrome 1 apoprotein (=Flavin-type blue-light receptor)	<b>FAD-binding domain 7, DNA-photolyase similar to PhrB-photolyases;</b> input pathway	NP_567341	C_190114 <sup>b</sup> /19.29.1.51, 19.29.2.52	62	299/478 (1e-120)	681
CRY2 (PHH1)	Cryptochrome 2 apoprotein (=blue-light photoreceptor)	<b>FAD-binding domain 7, DNA-photolyase similar to PhrB-photolyases;</b> input pathway	AAL16377	C_190114 <sup>b</sup> /19.29.1.51, 19.29.2.52	61	288/469 (1e-120)	612
ELF4	Early flowering 4 ( <i>M. crystallinum</i> )	<b>DUF1313 domain;</b> function unclear	AAQ73526	C_280056/28.99.2.51	72	39/54 (2e-7)	139
EPR1	Early phytochrome responsive 1	<b>Myb-like DNA-binding domain;</b> component of central oscillator	BAC98462	C_1060059/No EST	85	58/68 (8e-21)	346
LHY <sup>c</sup>	Late elongated hypocotyl	<b>MYB-type DNA-binding domain;</b> component of central oscillator	CAA07004	C_1060059/No EST	95	59/62 (2e-21)	645
NPH1	Nonphototropic hypocotyl protein 1 (Phototropin)	<b>PAS/PAC, Ser/Thr kinase domains;</b> input pathway (?)	NP_190164	C_120056 <sup>b</sup> /12.1.4.12, 12.1.2.51	63	337/532 (1e-130)	996
PHYA <sup>c</sup>	Phytochrome A	<i>PAS, HisKA, HATPase_c,</i> phytochrome, and GAF domains; input pathway	NP_172428	C_210006 <sup>b</sup> /No EST	37	43/116 (0.056)	1122
PHYC <sup>c</sup>	Phytochrome C	<i>PAS, HATPase_c,</i> phytochrome, and GAF domains; input pathway	NP_198433	C_210006 <sup>b</sup> /No EST	41	52/129 (1.2)	1111
PHYD <sup>c</sup>	Phytochrome D	<i>PAS, HisKA, HATPase_c,</i> phytochrome, and GAF domains; input pathway	NP_193360	C_210006 <sup>b</sup> /No EST	39	44/111 (0.006)	1164
Fruit fly ( <i>D. melanogaster</i> )							
CKII $\alpha$	Casein kinase 2, $\alpha$ -chain	<b>CKII catalytic subunit;</b> component of the central oscillator	AAA28429	C_970006 <sup>b</sup> /97.66.3.52, 97.66.1.51	70	140/199 (2e-58)	336
CKII $\beta$	Casein kinase 2, $\beta$ -chain	<b>CKII regulatory subunit;</b> component of the central oscillator	AAA28430	C_280152/28.27.1.0	75	143/190 (2e-64)	215
CRY	Cryptochrome	<b>DNA photolyase, FAD-binding 7 and PhrB-photolyase domain;</b> input pathway	AAC83828	C_430042/43.52.2.51	59	148/249 (9e-51)	542
DBT	Doubletime casein kinase (protein zeste-white 3)	<b>Ser/Thr kinase;</b> component of the central oscillator	AAD27857	C_70149/7.12.2.11	86	255/294 (1e-131)	440
PP2A <sub>tws</sub>	Protein phosphatase 2A, subunit B (twin)	<b>WD40 repeats and Ser/Thr-phosphatase 2A regulatory subunit;</b> component of central oscillator	NP_849681	C_180129/18.24.2.11	70	346/488 (1e-158)	500
PP2A <sub>wdb</sub>	Protein phosphatase 2A, subunit C (widerborst)	<b>B56 protein phosphatase 2A regulatory B subunit;</b> component of central oscillator	NP_651569	C_1270036/127.5.2.11	67	289/431 (1e-119)	524

(Table continues on following page.)

**Table II.** (Continued from previous page.)

Organism/Protein		Domains <sup>a</sup> and Characteristics	NCBI No.	Gene Model/ESTs	%	AA Range	Total No. AA
SGG	Protein kinase shaggy	<b>Ser/Thr kinase</b> ; component of the central oscillator	CAA37419	C_490046/49.49.6.11	79	270/340 <i>e-value</i> (1e–128)	514
Mammals (Human, <i>M. musculus</i> )							
CK1 epsilon	Casein kinase 1epsilon, ortholog to dDBT	<b>Ser/Thr kinase</b> ; component of the central oscillator	NP_689407	C_70149/7.12.2.11	91	268/294 (1e–144)	416
mCRY1	Mus cryptochrome 1	<b>DNA-photolyase, FAD binding 7</b> and <b>PhrB-photolyase domain</b> ; input pathway	NP_031797	C_430042/43.52.2.51	63	311/489 (1e–134)	606
mCRY2	Mus cryptochrome 2	<b>DNA-photolyase, FAD binding 7</b> and <b>PhrB-photolyase domain</b> ; input pathway	NP_034093	C_430042/43.52.2.51	64	315/489 (1e–133)	592
Hnat5	Human N-acetyl-transferase 5	GNAT family acetyltransferase 1 domain (1st step of melatonin biosynthesis); output pathway	NP_057184	C_70196 <sup>b</sup> /No EST	81	45/55 (5e–18)	178
h_HIOMT (ASMT)	Human hydroxy-indole-O-methyltransferase (=Acetyl-serotonin-O-methyltransferase)	<b>O-Methyltransferase 2 domain</b> (2nd step of melatonin biosynthesis); output pathway	AAA75289	C_1870014/187.14.2.31	61	71/116 (5e–22)	298

<sup>a</sup>Functional domains are written in (1) bold font if the domain is also present in the putative homolog in the *C. reinhardtii* genome and is situated within the depicted region of similarity, and (2) italic font if the domain is also present in the putative homolog in the *C. reinhardtii* genome but is situated outside of the depicted region of similarity. <sup>b</sup>Gene model still contains extended regions of “N” (nondetermined nucleotides).

<sup>c</sup>Proteins that show only very limited similarity to proteins of *C. reinhardtii* but have been listed due to functional implications.

Recently, it has been demonstrated that the protein phosphatase 2A (PP2A) regulatory subunits “twin” and “widerborst” are also part of the oscillatory loop in *D. melanogaster* by virtue of the observation that they dephosphorylate PER in a circadian manner (Sathyanarayanan et al., 2004). Both subunits are well conserved in *C. reinhardtii*. Furthermore, distinct roles for the catalytic subunit of protein phosphatase 1 (PP1) and for a regulatory subunit of PP2A have been found in the *Neurospora* circadian clock (Yang et al., 2004). Both can act on the central clock protein FRQ. PP1 is also highly conserved in *C. reinhardtii* (Table II). PP1 and PP2A have been localized to specific regions within the *Chlamydomonas* flagellar axoneme: PP1 is part of the central pair mechanism that controls flagellar motility, and PP2A appears to be anchored to the doublet microtubules (Yang et al., 2000). Both PP1 and PP2A are promising candidates for clock regulation of circadian taxes in *C. reinhardtii*.

Therefore, kinases and phosphatases that could be involved in circadian systems are present within the *C. reinhardtii* genome. In fact, there are potential homologs in the *C. reinhardtii* nuclear genome to all known eukaryotic clock-related kinases and phosphatases. On the other hand, the situation is different with regard to the clock components that are targets of these kinases/phosphatases; there were no significant similarities

found in the *C. reinhardtii* genome to the famous clock proteins FRQ, PER, or TIM.

#### Similarities of *C. reinhardtii* Genes to Clock Genes from Other Photosynthetic Organisms

It might be imagined that the likelihood of finding clock-homologous genes/proteins for *C. reinhardtii* would be greatest among its photosynthetic “sisters,” i.e. cyanobacteria and plants, represented by *S. elongatus* and Arabidopsis. However, if one excludes photo-receptor genes from the comparison, there are few central clock components that spring from the comparison. For example, there are no putative homologs in *C. reinhardtii* to the central cyanobacterial clock genes *kaiA*, *kaiB*, or *kaiC* of *S. elongatus*. The possible homologs that do appear are to *cikA*, *cpmA*, and *rpoD*. The *cikA* gene is a phytochrome-like gene that has a His-kinase domain that is similar to that of C\_50007. The function of *cpmA* is unknown, and therefore its similarity to C\_250166 is difficult to evaluate (also, no EST sequence exists). Finally, *rpoD* is a sigma factor for RNA polymerase and is therefore unlikely to be a clock-specific gene (and knockout of *rpoD* in cyanobacteria does not have a strong clock phenotype anyway).

Comparisons with plant clock genes (specifically those of Arabidopsis) likewise implicate similarities in

phototransduction and kinase pathways, but no other specific clock insights. Potential kinase homologs were discussed above, and potential homologs to phototransduction genes will be discussed in the next section. The similarities to putative central clock components of Arabidopsis are limited. No putative homologs exist in the *C. reinhardtii* genome for the Arabidopsis clock components *gi*, *elf3*, or *tej* (Eriksson and Millar, 2003). Only limited similarity can be found to *ztl*, *fkf1*, *lkp2*, or the APRR quintet *aprr1/toc1*, *aprr3*, *aprr5*, *aprr7*, and *aprr9*. There are also very short sections of similarity to the central clock genes *lhy*, *cca1*, *epr1*, and *elf4* (Table II), but these are probably just similarities to general-function domains found within these proteins (i.e. *myb*-like DNA binding domains in *lhy*, *cca1*, and *epr1* or DUF1313 domains in *elf4*) rather than to the parts of these clock genes that confer the clock-specific functions. In addition, there is a short section of similarity between the RNA recognition motif of the output gene *AtGRP7/ccr2* of Arabidopsis (Heintzen et al., 1997) and C\_10096. Therefore, despite the close evolutionary relationship between green algae like *Chlamydomonas* and plants like Arabidopsis, the major correspondences between putative clock genes are in photoreceptor, kinase, and phosphatase genes but not in the genes that are considered to be central components of the clockwork.

#### Potential Photoreceptors within the Circadian Input Pathway of *C. reinhardtii*

Several years ago, action spectra from light-pulse-treated cells revealed that blue as well as red light could reset the phase of the circadian clock in *C. reinhardtii* (Johnson et al., 1991; Kondo et al., 1991). Relevant wavelengths were at 620 nm and 650 to 670 nm in the red and at 520 nm and 450 to 480 nm in the blue. Phytochrome was excluded as a red-light photoreceptor since red/far-red reversibility was not observed. The experimental data available at that time suggested that photosynthetic electron transport could be involved.

A potential blue-light photoreceptor(s) has not been identified. The *C. reinhardtii* genome suggests some candidates, however. Two classes of blue-light photoreceptors that have been characterized in other organisms were found in the *C. reinhardtii* genome. One class is the CRYs (Small et al., 1995), which can act as circadian photoreceptors (e.g. in *Drosophila* or Arabidopsis) or as a component of the circadian clockwork (e.g. in mammals; Harmer et al., 2001), as mentioned above. The similarity search identified two genes that encode potential CRYs in *C. reinhardtii*. When Arabidopsis CRYs (1 or 2) were used for the search, one gene model was obtained (C\_190114; Table II). On the other hand, when either *D. melanogaster* or mammalian CRYs (1 or 2) were used for the search, a different gene model was the result (C\_430042; Table II). For both gene models, proteins are encoded that bear all the characteristic domains of CRY. Thus, we conclude that *C. reinhardtii* has two CRYs, one of which is more

closely related to Arabidopsis CRY and the other to animal CRY. Small and co-workers have sequenced a 7-kb region that includes the CRY protein according to their examinations (Small et al., 1995; Reisdorph and Small, 2004). If this sequence is taken and BLASTed against the *C. reinhardtii* genome, an extended region of similarity is found around gene model C\_190114. But the predicted protein of this gene model is not fully identical with the protein sequence depicted by Small et al. (1995), resulting in some discrepancies between the sequences, presumably within the C\_190114-bearing scaffold 19. Importantly, the second potential CRY deriving from gene model C\_430042 has not been described and investigated up to now. C\_430042 surely needs to be considered in experiments where CRY genes will be silenced since these CRY candidates could overlap functionally.

The other blue-light photoreceptor is phototropin (NPH1), an essential photoreceptor of the phototropic reaction of higher plants. NPH1 was discovered in Arabidopsis (Briggs and Christie, 2002; Kasahara et al., 2002) and was subsequently identified in *C. reinhardtii* (Huang et al., 2002). In *C. reinhardtii*, NPH1 controls multiple steps in its sexual life cycle, including changes in chemotaxis (Huang and Beck, 2003; Ermilova et al., 2004). Consistent with those effects, NPH1 can be found in the flagella of *C. reinhardtii* (Huang et al., 2004). The circadian clock in Arabidopsis controls the expression of NPH1 (Harmer et al., 2000). It remains open if CRY and NPH1 play a role in the circadian system of *C. reinhardtii*, but both remain prime candidates for an input component.

Over the years, there have been tantalizing suggestions for phytochrome-like transduction in *C. reinhardtii*, but until now neither physiological nor biochemical data provided persuasive support for a red/far-red photopigment like phytochrome (PHY) in *C. reinhardtii*. When the phy A, B, C, D, and E proteins from Arabidopsis were used for the homology search, only very limited similarities could be detected between *C. reinhardtii* proteins with phy A, C, and D, and essentially none to phy B or E. Also, we have used the phytochrome protein sequence from the green alga *Mougeotia scalaris* (NCBI no. P33529) and the PHY C sequence from *Oryza sativa* (NCBI no. Q9ZW19) for the similarity search. But there were no positive hits when the *Mougeotia* and *Oryza* sequences were BLASTed against the translated *C. reinhardtii* nuclear genome sequences. These results suggest that there is indeed no PHY in *C. reinhardtii* or that these genes/proteins have diverged greatly between *C. reinhardtii* and other plant systems. Finally, another phototransduction/photomorphogenesis protein from Arabidopsis is COP1, which shows similarity in its WD40 repeats to C\_1310019.

#### The Melatonin Pathway Seems To Occur in *C. reinhardtii*

Melatonin released by the pineal gland plays an important role within the circadian system regulating

reproduction in vertebrates (Reiter, 1993; Goldman, 2001). However, its presence is not restricted to vertebrates, as it can be also found in nonvertebrates and in plants (Caniato et al., 2003; Hardeland and Poeggeler, 2003; Kolar et al., 2003). In animals, the last two steps in melatonin biosynthesis involve the enzymes *N*-acetyl transferase and hydroxy-indole-*O*-methyltransferase (HIOMT). In *C. reinhardtii*, there are two sequences that show similarity to those animal enzymes (C\_70196 and C\_1870014; Table II). In the case of C\_70196, the area of similarity could be broader than the narrow region reported in Table II because there is a large area of undetermined sequence within scaffold 7 immediately to the right of the C\_70196 position. Thus, the possibility exists that *C. reinhardtii* includes enzymes that could contribute to a melatonin biosynthetic pathway.

#### ONE STEP BEYOND THE GENOME: APPLYING FUNCTIONAL PROTEOMICS TO FIND NOVEL COMPONENTS OF THE CIRCADIAN SYSTEM

*C. reinhardtii* offers excellent characteristics to facilitate the application of functional proteomics. Nuclear, chloroplast, and mitochondrial genome sequences as well as many EST sequences are available (Grossman et al., 2003). Although some proteins can now be identified at the picomole to femtomole range with modern mass spectrometry (MS), many regulatory proteins within a crude extract are not abundant enough to be unambiguously identified by MS. Therefore, enrichment of such proteins is a prerequisite for efficient proteome analysis, and *C. reinhardtii* can be easily and quickly grown in large quantities so as to efficiently implement biochemical purifications. Pioneering proteomic studies have already been carried out in *C. reinhardtii* with regard to the light-harvesting complex, the chloroplast 70S ribosome, and the circadian system (Stauber et al., 2003; Yamaguchi et al., 2003; Wagner et al., 2004).

In a first approach to apply functional proteomics to circadian-expressed proteins from *C. reinhardtii*, basic proteins were enriched by heparin affinity chromatography (Wagner et al., 2004). Cells that were oscillating in constant dim light were harvested throughout the subjective day and night. Comparative analyses were carried out using two-dimensional gel electrophoresis and a normalized spot volume procedure. Two proteins fulfilled a rigorous criterion of significance for a daily rhythm of abundance. These two proteins were digested with trypsin and identified by liquid chromatography-electrospray ionization-MS (Wagner et al., 2004). One of the proteins was a nuclear-encoded protein disulfide isomerase (PDI). Its amount was highest during the middle of the subjective night and lowest at the beginning of the subjective day. PDI is known to be an oxidoreductase that assists in the folding of newly synthesized proteins in the endoplasmic reticulum. It can function as a molecular

chaperone (Freedman et al., 1994). PDI typically catalyzes the formation, reduction, and isomerization of disulfide bonds during protein folding. Interestingly, a chloroplast PDI had already been described in *C. reinhardtii* (named RB60) that is a major component of the *psbA* mRNA-binding protein complex encoding the D1 protein of PSII. Chloroplast PDI is implicated in the redox-responsive regulation of translation in *C. reinhardtii* chloroplasts in a light/dark-dependent manner (Danon and Mayfield, 1994; Kim and Mayfield, 1997; Trebitsh et al., 2001).

The other protein identified in the proteomic search was a TPR (tetratricopeptide repeat) protein (Wagner et al., 2004). Its highest amount also occurs in the middle of the subjective night, but its minimum is in the middle of the subjective day. TPR proteins have been proposed to interact preferably with WD40 domains [tandem repeats of about 40 residues, each containing a central Trp(W)-Asp(D) motif] (van der Voorn and Ploegh, 1992). Notably, the molecular mechanisms of the circadian clocks in *D. melanogaster* and *N. crassa* show a direct involvement of WD40 proteins in the regulation of the circadian oscillator loop (Ko et al., 2002; He et al., 2003). There are also TPR proteins described in *C. reinhardtii* that are suggested to regulate translation/initiation of chloroplast messages and that are part of multiprotein complexes including RNA (Boudreau et al., 2000; Vaistij et al., 2000).

#### CONCLUDING REMARKS

The conjunction of a wide spectrum of molecular, genetic, physiological, and biochemical techniques with the available genome and EST sequences renders *C. reinhardtii* to be an attractive eukaryotic model organism for in-depth studies of the circadian clock. The elucidation of the role of the circadian clock in regulating taxes (phototaxis and chemotaxis) is of particular interest. PP1 and PP2A have been suggested as potential regulatory factors based on the results of the similarity search and their presence within the flagella. Circadian mechanisms that control taxes might also be relevant to humans (e.g. sperm release/movement), especially when one considers that several proteins from the basal apparatus and the flagella of *C. reinhardtii* are well conserved in humans. Defects in the basal apparatus and the flagella of humans cause severe diseases (e.g. changes of left/right symmetry of organs, polycystic kidney disease, Bardet-Biedl syndrome, as well as a syndrome associated with obesity, hypertension, and diabetes [Olbrich et al., 2002; Li et al., 2004; Snell et al., 2004]).

We can make several suggestions based on the comparison of molecular components of the circadian oscillator from model organisms with potential candidates from *C. reinhardtii*. The clock kinases and phosphatases from fungi, plants, flies, and mammals are well conserved in this green alga, and it is possible that they may be also involved in its circadian system. Of

course, this does not rule out the possibility that these kinases and phosphatases may also participate in other cellular processes. The same interpretation holds for the CRY sequences found in *C. reinhardtii*. In this context, it is interesting that there appears to be two CRY proteins, one of which is more closely related to plant CRYs while the other is more similar to animal CRYs. Also, COP1, a negative regulator of photomorphogenesis that interacts with CRY in Arabidopsis and mediates its signaling mechanism (Yang et al., 2001), appears to occur in *C. reinhardtii*. The central clock components that are targets of phosphorylation and are in most cases restricted to a subset of organisms (e.g. KaiC, FRQ, PER, TIM) are not obvious in the *C. reinhardtii* genome. For this reason, this green alga will probably have its "own" central clock components. The evolution of specific phospho-clock proteins that are key components of the central oscillator thus seems to have originated independently in different systems.

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