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Circadian regulation of cell division

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Title:

Light-dependent regulation of cell division in Ostreococcus: evidence for a major transcriptional input

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SUMMARY:

Cell division often occurs at specific times of the day in animal and photosynthetic organisms. Studies in unicellular photosynthetic algae, such as Chlamydomonas or Euglena, have shown that the photoperiodic control of cell division is mediated through the circadian clock. However, the underlying mechanisms remain unknown. We have studied the molecular basis of light-dependent control of cell division in the unicellular green alga Ostreococcus. We found that cell division obeys a circadian oscillator in Ostreococcus. We provide evidences suggesting that the clock may, at least in part, regulate directly cell division independently of the metabolism. Combined microarray and quantitative real time RT-PCR analysis of the main core cell cycle genes expression revealed an extensive transcriptional regulation of cell division by the photoperiod in Ostreococcus. Finally transcription of the main core cell cycle genes, including cyclins and cyclin-dependent kinases, was shown to be under circadian control in Ostreococcus, suggesting that these genes are potential targets of the circadian clock in the control of cell division.
INTRODUCTION:

The molecular basis of cell division has been extensively studied in eukaryotes, including plants (Inze and De Veylder, 2006). Control of cell cycle progression relies mainly on heterodimeric kinases belonging to the cyclin-dependent kinase family (CDK). The activity of CDKs controls the main cell cycle transitions leading to DNA replication, mitosis and cytokinesis. Another challenge, besides unravelling the basic regulation of cell division, is to understand the different levels of integration of cell division both, within multicellular organisms and in response to the environment. Coupling of cell division and development is mediated through the action of cell-cycle regulators at the interface between cell division and differentiation (Gutierrez, 2005). Living organisms face changing daily conditions in their environment, mainly light and temperature, due to the rotation of the earth. As a consequence, many organisms, including plants, have developed an autonomous time-tracker, so-called the circadian clock (Gardner et al., 2006). This clock allows to sense the environment and to respond to the daily changes in light and temperature by anticipating them, in altering the physiological processes and activities.

Day-night rhythms of cell division have been known for long in eukaryotes from unicellular algae, including Euglena, Gonyaulax and Chlamydomonas (Bruce, 1970; Edmunds and Laval-Martin, 1984; Homma and Hastings, 1989) to mammals (Matsuo, 2003; Fu et al., 2005). These rhythms usually persist in constant environmental conditions of light or temperature with a period close to 24 hours. Furthermore they can be entrained by light/dark cycles, so that their period adjusts to the imposed environmental period. Such properties led several authors to the conclusion that cell division is under circadian control in organisms as diverse as dinoflagellates, ciliates, green algae or animals. A recent study in mouse, has shown that in hepatocytes re-entering cell division upon liver ablation, the circadian clock
gates entry into mitosis by regulating the transcription of the master CDK inhibitor Wee1, (Matsuo, 2003).

Plants are photosynthetic organisms, which heavily rely on light as a source of energy. However, little is known about the regulation of the cell division cycle (CDC) by light either directly or indirectly through the entrainment of a circadian oscillator, which in turn would regulate cell division. Much more attention and controversy has been devoted to another organism of the green lineage, the unicellular Chlorophyta Chlamydomonas. Initial studies concluded that cell division is under circadian control in this organism (Bruce, 1970). However these conclusions were challenged in a report by Spudich and Sager (1980), which concluded that, cell division is not under circadian control in Chlamydomonas. Rather, the cells would be forced into a daily periodicity, by the amount of energy available through photosynthesis, independently of the clock. This would also imply that photosynthesis is not under circadian control in Chlamydomonas. Twenty five years after the initial studies by Bruce, it was clearly established that the circadian clock gates cell division in Chlamydomonas (Goto and Johnson, 1995) meeting the main criteria of circadian regulation, including persistence under free running conditions, temperature compensation and entrainment by various photoperiods. The resetting experiments by dark/dim light transition were in good agreement with a stable limit-cycle circadian oscillator. Such an oscillator, formed by at least two state variables (e.g. two genes forming a negative feedback loop) can be perturbed within limits, by light/dark perturbations (resetting) but later it returns by itself to a stable oscillation. Depending on the strength of the perturbation applied (resetting light intensity), the stable variables return to the limit cycle at different time points of the circadian cycle. Furthermore, the light resetting stimulus affects the oscillator in a phase-dependent manner. As a consequence, the same amount of light given at different phases of the circadian time can cause phase shift with different amplitudes and directions. Phase response curves
(PRC) represent such phase shifts as a function of the time when the stimulus is given. Therefore, the shape of the PRC is representative of how the light input influences the sensitivity of the oscillator at different phases. PRCs established using light pulses, usually display delays in the early subjective night, advances at the end of the subjective nights and little phase shift during the day. Regarding circadian regulation of cell division in unicellular algae such as Chlamydomonas, several questions remain: (1) Can intermediate and strong resetting types predicted by Goto and Johnson be observed when varying the intensity of the resetting light? (2) Is the CDC directly controlled by the clock or does the circadian regulation of photosynthesis accounts for the apparent gating of cell division through controlling the amount of energy available along the day? (3) What are the molecular targets of the clock among CDC actors?

Chlamydomonas is a multiple fission cell, that is, cells can divide several times in a raw depending on the growth conditions. This is a limiting factor for studies of cell division. We have chosen a simple system, the green alga Ostreococcus, to investigate the nature of circadian regulation of cell division in the green lineage. This unicellular organism has an extremely simple cellular organisation with only one of each organelle (plastid, mitochondria and Golgi). The small genome of Ostreococcus (12.5 Mb) has been recently sequenced (Derelle et al., 2006). Ostreococcus cells divide by binary fission. The set of the main core cell cycle genes is extremely reduced, most cyclins and CDKs being present as a single copy gene (Robbens et al., 2005). Ostreococcus contains also a canonical CDC25-like not found in higher plants (Khadaroo et al., 2004), as well as a plant specific B-type CDK (Corellou et al., 2005).

In this paper we investigate the nature of the regulation of cell division by light/dark cycles in Ostreococcus. We found that the CDC is under circadian control. Gene expression analysis revealed a major transcriptional regulation of cell division related genes under
entraining conditions as well as under free-running conditions. Resetting experiments using
dark-light transitions, suggest that circadian gating of the CDC are similar in Ostreococcus
and Chlamydomonas. Resetting experiments using various light intensities further support the
predictions of the limit-cycle model proposed by Goto and Johnson (1995). Finally our results
suggest that a direct circadian regulation of cell division, independent of the photosynthetic
capacity, operates in Ostreococcus.
EXPERIMENTAL PROCEDURES

Culture

*Ostreococcus tauri* strain, isolated from the Thau lagoon (Courties et al., 1994), was cultivated in filtered sterile seawater supplemented with Keller enrichment medium (Sigma-Aldrich, Lyon, France) in T-175 tissue culture flasks (Sarstedt, Newton, NC). Cultures were grown under constant gentle agitation at 20°C and subjected to light/dark cycles (35µE.cm-2) or to continuous dim light (15µE.cm-2) with moonlight blue filter (Texen, Toulouse, France).

Flow Cytometry Analysis

One-milliliter cell samples were fixed with 0.25% glutaraldehyde (Sigma-Aldricht, St Louis, MO) for 15 min at room temperature and then stored at 4°C for 1 day. Flow cytometry analysis was performed on a FACScan flow cytometer (FACScalibur; Becton-Dickinson, San Jose, CA). Cells were counted from the appropriate gate FL3-H versus SSC-H. Analysis of the DNA content was performed on fixed 20,000 cells stained with SYBR green I (3,000 x dilution of the commercial solution (Molecular Probes, Eugene, OR). Cell cycle analysis was performed with the Modfit software (Verity Software House, Tophsam, ME).

Preparation of RNA

Cells were harvested by centrifugation in conical bottles (8,000g, 4°C, 8 min), after addition of pluronic acid (0.1%). Pellets were frozen in liquid nitrogen and stored at –80°C until
extraction. Cells were grinded mechanically with 5 mm steal beads using a Tissue Lyser (Retsch, Haan, Germany). RNA extraction was performed with RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. Contaminating DNA was removed using Q1 RNase-free DNase (Promega). Absence of DNA contamination was checked by PCR. Reverse transcription was performed using the PowerScript Reverse Transcriptase synthesis kit (BD Bioscience, Palo Alto, CA).

Quantitative measurements of cell cycle genes expression

Real-time PCR was carried out on a LightCycler 1.5 (Roche Diagnostic, Mannheim, Germany) with LightCycler DNA Master SYBR Green I (Roche Molecular Biochemicals, Mannheim, Germany). Primers were designed with LightCycler Probe Design2 software (Roche Diagnostic, Mannheim, Germany). Results were analyzed using the comparative critical threshold (ΔΔCT) method. The *O. tauri* elongation factor 1 α (EF1α) was used as internal reference. The analyses were performed in duplicate.

Pan-genomic Ostreococcus slides (24K) were manufactured in the Rennes Transcriptome Platform (France). Gene-specific 50-mers oligonucleotides (8096) were designed and synthesized by Eurogentec (Liege Science Park, Belgium). Oligonucleotides were resuspended at a 30 μM concentration in Nexterion™ Spot Solution 2X (Schott Nexterion, Jena, Germany) and spotted in triplicate on Nexterion Slides E (Schott) using a Biorobotics MicroGrid II Spotter (Genomic Solutions Ltd, Cambridgeshire, UK). Positive and negative controls were added and quality of spotting was checked by slide scanning using an Agilent G2565BA Microarray Scanner (Agilent Technologies, Palo Alto, CA, USA). Total RNAs (350ng) were amplified and labelled using a two colour labelling protocol Low Input Linear RNA amplification kit according to the manufacturer recommendations (Agilent,
Palo Alto, CA). Test and reference samples were respectively labelled with Cyanine-5 and Cyanine-3 CTP (10mM, Perkin-Elmer/NEN Life Science, Boston, MA, USA). The reference sample corresponded to a pool of all stages under investigation, so that it represents an average expression of the genome. Cyanine incorporations were monitored using a Nanodrop® ND-1000 Spectrophotometer. Incorporation rates ranged from 1.6 to 2 pmol/µl. Hybridization was performed using an Agilent oligonucleotide microarray in situ Hybridization-Plus Kit. Namely, 1 µg of each test and reference cRNAs were mixed and subjected to fragmentation (30 min at 60°C in the dark). Then, samples were diluted in Nexterion™ Hyb buffer (Schott Nexterion, Jena, Germany) and hybridizations were performed using Gasket Slides in an Agilent Hybridization rotation oven (60°C, 17h at 4 rpm in the dark). Slides were disassembled, washed according to the Schott protocol and dried using a nitrogen-filled air gun. Hybridized slides were scanned with the dynamic autofocus Agilent G2565BA microarray scanner. The Agilent feature extraction software version 9.1 was used to extract data. We used a new web application pPR (PreProcessing on R) developed on the Rennes Transcriptome Platform (http://ouestgenopuces.univ-rennes1.fr/index_en.php) providing academic tools suite for processing Agilent® scanner extracted data. pPR was designed with Perl language (www.cpan.org), R environment (Ihaka and Gentleman, 1996) and Bioconductor packages LIMMA (Smyth, 2005), convert and MArray (Yang et al., 2002). The slide quality was checked (background and signal homogeneity) and for each spot, the background was substracted. Normalization was performed using the print-tip loess method and scaled with the Gquantile method (Yang et al., 2002; Smyth and Speed, 2003). Time courses of gene expression were performed in triplicate, over 27 hours, at 3-hours intervals (9 time points per time-course). Accession numbers of the CDC genes under investigation in this study are: cyclin A (AAV68599); cyclin B (AAV68600); cyclin D (Q5SCB5); cyclin D2 (CAL50505); cyclin H (AAV68602);
CDKA (AAV68595); CDKB (AAV68596); CDKD (AAV68598); CKS (CAL54466); Rb (AAV68604); E2F (AAV68605); DEL (AAV68606); DP (AAV68607); Cdc25 (AAQ16122); Wee1 (AAV68603); Polo Kinase (CAL51478); APC1-Tsg24 (AAV68612); APC2 (AAV68613); APC3-cdc27 (AAV68622); APC4 (AAV68621); APC5 (AAV68614); APC6-cdc16 (AAV68615); APC7 (AAV68618); APC8-cdc23 (AAV68619); APC10 (AAV68616); APC11 (AAV68620); Cdh1 (AAN74839); Cdc20 (AAV68609); MCM2 (CAL55702); MCM3 (CAL52075); MCM4 (CAL52276); MCM5 (CAL51451); MCM6 (CAL50164); MCM7 (CAL52245); RFC1 (CAL52049); RFC3 (CAL53769); RFC4 (CAL55263). The value of each biological time point is an average of the technical triplicates on a slide.
RESULTS

Circadian regulation of cell division in Ostreococcus

To determine whether the CDC is under circadian control in Ostreococcus, cells were entrained under light-dark cycles (LD 12:12) at light intensities of 35 µmole quanta.cm\(^{-2}\). s\(^{-1}\) for five days and then placed under free running conditions of constant light or darkness (supplemental Fig. S1). In LD 12:12, at standard light intensities (35 µmole quanta.cm\(^{-2}\). s\(^{-1}\)), Ostreococcus cells divided once per day, cytokinesis beginning at the time of the light-dark transition (supplemental Fig. S1 and Fig. 1A). When entrained cells were placed in constant darkness, cell division ceased after the first division, since Ostreococcus is an obligate phototroph, which requires light for cell growth (supplemental Fig. S1). When entrained cells were placed under dim light conditions (LL at 15 µmole quanta.cm\(^{-2}\). s\(^{-1}\)), division still occurred rhythmically, at the beginning of the “subjective” night (supplemental Fig. S1 and Figure 1.A). At higher intensities (35 µmole quanta.cm\(^{-2}\). s\(^{-1}\) or 100 µmole quanta.cm\(^{-2}\). s\(^{-1}\)), the rhythms of CDC were lost after 24 hours (supplemental Fig. S1). The red fluorescence of the cells (FL3-H flow cytometer parameter) appeared to be a more precise parameter than direct cell counting to monitor cell division rhythms (Fig. 1A, bottom). As the population progresses through G1 phase during the light phase, cells show an increase in FL3-H corresponding to a synthesis of chlorophyll in the growing chloroplast. At cytokinesis, upon partition of the two chloroplasts, a marked decrease of red fluorescence per cell is observed and is directly correlated to cell division (Fig. 1A). Indeed, when cell division ceases in stationary phase, the decrease in the FL3-H parameter is not detected any longer at the end of the day (data not shown). Entrainment of the clock was tested by varying the photoperiod
during the entrainment (Fig. 1B). Under both long day LD 16:8 and short day LD 8:16, cell division kept occurring periodically 12 hours after light-on, suggesting that the circadian rhythms of cell division are set on mainly by light on. These rhythms of cell division persisted under constant light. The FL3-H parameter was used to determine the period length in constant dim light. Analysis of 5 populations gives a period estimate of 25.97 ± 2.46 h, indicating that these periods are quite variable. For example, in Figure 1 the first period in constant dim light is 27.96±0.65 h while the second period is 22.1±0.98 h. The dim light limiting conditions, which limit cell growth, may explain such variations. The first period in LL is usually longer than the following (eg. Figure 1), probably because cells are not yet adapted to dim light and do not assimilate light efficiently. These data suggest also that the circadian clock restricts cell division to a window of time, rather than to a specific time, the metabolic status being responsible for the period variation within this time window.

**Rhythmicity in the expression of cell division genes**

The main proteins involved in CDC control have been annotated in *Ostreococcus* (Robbens et al., 2005). They include cyclins, CDKs, regulatory kinases such as Wee1 and phosphatases such as CDC25, proteins of the retinoblastoma (Rb) pathway including Rb, E2F and Dp, proteins of the prereplication complex (MCM and RFC) and regulatory proteins of the Anaphase Promoting Complex (APC), which is required for the degradation of cyclins. We have developed a genome-wide microarray which contains 8056 oligonucleotides specific to *Ostreococcus* genes (unpublished results). Three cultures were grown under LD 12:12 at 35 μmole quanta.cm⁻².s⁻¹ (eg Fig. 1) and harvested every 3 hours for 27 hours. The cell cycle stage of the culture was monitored by flow cytometry (Fig. 2, top left). Cells entered in S phase at ZT7 and exited at ZT16. Cells in G2/M were present from ZT10 to ZT19. RNA from
these samples was hybridized to the array (see experimental procedures). Except for cyclin D2, the other 37 CDC genes under investigation displayed a signal/noise ratio which was significantly above the background (2.6 fold) for at least one time point. The expression of most of the cyclins and CDKs under LD 12:12, was confirmed by real time quantitative RT-PCR (QPCR), validating the microarray data (Fig. 3). The expression of cyclin D2 was very low since it was only detected after 32 to 35 cycles of amplification by QPCR. Most genes under investigation (32/37) were rhythmically transcribed, with a more than two fold reproducible induction. Both the mitotic cyclin B and CDKB genes had a peak of transcription at the end of the day with a more than 100 fold induction. The MCM and RFC genes involved in DNA replication were maximally expressed during S-phase. The expression of most genes encoding the Anaphase Promoting Complex subunits (APC) was also transcriptionally regulated. Finally the CDC5/Polo kinase (PLK), the regulatory CDC25 phosphatase and the Weel kinase, which are key regulators of CDKs, had a peak of transcription at the time of mitosis. Among the members of the Rb pathway only E2F did not show a strong diel regulation. Despite overlapping S phase and G2/M phases, hierarchical clustering reveal interesting features of CDC genes transcription (supplemental Fig. S2). Mitotic cyclin B, CDKB and CKS clustered together. Cyclin H and CDKD, which are part of the CDK activating kinase (CAK) are found in the same cluster. Surprisingly cyclin A and cyclin D2 were expressed early during cell cycle, whereas cyclin D was expressed later together with S phase genes. A detailed analysis of these three cyclins revealed that unlike cyclin D, both cyclin A and cyclin D2 both have an Rb-binding motif and, therefore, they may be required for S phase entry whereas cyclin D would be involved later during CDC progression.

The expression of four different cyclin genes (cyclin A, cyclin B, cyclin D and cyclin D2), and three CDKs (CDKA, CDKB and CDKD) was monitored further using QPCR under LD 12:12 (35 µmole quanta.cm⁻².s⁻¹) and dim LL conditions (15 µmole quanta.cm⁻².s⁻¹).
3). In LD 12:12, three patterns of transcription were observed. First, *cyclin A* and *cyclin D2* expression increased from 1 to 4 hours after light-on (Fig. 2 and Fig. 3). Then, from 4 to 7 hours, *CDKA* and *cyclin D* mRNAs were detected. Finally, *cyclin B* and *CDKB* transcripts were observed from 7 hours after light-on.

The culture was then transferred in constant light. Sampling in LL was performed from 24 hours to 72 hours to avoid transient effects often observed the first day in circadian experiments. Because of the diminution of synchrony in cell division of the population grown under low light conditions, oscillations of gene expressions were damped the third day. This loss of synchrony is likely to be due to several reasons. Oscillations in cell division persist only in dim light. However, under these limiting light conditions, only a fraction of the population divides and cell division is delayed, probably due to metabolic limitations (see Figure 1 and supplemental Fig. S1). Furthermore the length of the CDC is short compared to the length of the circadian cycle (2-3 hours versus 26 hours in free running conditions), resulting in a lost of resolution between the different cell cycle stages. Finally, dampening of circadian rhythms is often seen under free running conditions. However, a clear rhythm of transcription was still observed for cyclins and CDKs (Fig. 3B and Fig. 3C).

**Effect of dark-light transition on the circadian regulation of cell division**

To gain further insight into the circadian regulation of cell division the phase dependent response of the circadian clock in response to Dark-Light transitions has been characterised (Fig. 4). If cell division is under circadian control, rather than being directly regulated by light, it is expected that dark-light transitions, using dim light conditions (e.g Fig 1) should have different effects depending on the time when the resetting light is applied. Entrained cells under standard light (35 µmole quanta.cm⁻². s⁻¹) were transferred from
darkness to constant dim light (15 µmole quanta.cm\(^{-2}\). s\(^{-1}\)) at regular time intervals starting from 12 hours after dawn (Fig. 4A). After 72 hours, a persisting rhythm was observed in all conditions but the phase of cell division peak, assayed as the time of maximum red fluorescence of chlorophyll preceding cytokinesis (see Fig. 1), was advanced or delayed compared to the control (standard entraining cycle LD 12:12 rather than constant darkness since cell division rhythms cease in darkness). For example, a phase advance of 5 hours was induced by a light-on signal after 4 hours of darkness. Phase shifts in the circadian rhythm of division are plotted as a function of the circadian time when light is turned on (Fig. 4B). The resulting phase response curve displays a phase-advance at the beginning of the subjective night (positive slope with short nights) and a phase delay at the end of the subjective night and at the beginning of the “subjective” day (negative slope corresponding to decreasing advances and increasing delays with longer nights).

**Transient responses to light of various intensities**

In the next set of experiments, we investigated the transient effect of various intensities of a light-on signal on cell cycle entry (Fig. 5). The purpose of these experiments was to determine, to which extent, the CDC can be uncoupled from the circadian control, when varying the light fluence rates and if the predictions of the limit cycle model can account for the responses observed (Goto and Johnson, 1995). The experimental protocol is the same as described in Figure 4 except that the phase shift was determined the first day after transfer to light of various intensities (dim light at 15 µmol.m\(^{-2}\).s\(^{-1}\), standard light at 35 µmol quanta.m\(^{-2}\).s\(^{-1}\) or bright light at 100 µmol quanta.m\(^{-2}\).s\(^{-1}\)) because the rhythmicity in cell division is lost after one day when cells are exposed to continuous standard to bright light conditions. For standard light or dim light conditions, a phase advance in the timing of cell
division peak, assayed as the time of maximum red fluorescence of chlorophyll, was observed only when light pulses were given at the beginning of the night (ZT12). Transfer to light at the onset of darkness or later during the night had little effect. For bright light conditions, the phase shift was independent of the time of the light pulse, depending only of the time of the dark light transition. The maximum of red fluorescence occurred always 10 hours after light-on. Such fluence rates-dependent responses are in agreement with the fluence rate-dependent responses observed and/or predicted in the limit cycle model of Chlamydomonas (Johnson et al., 1995). Note that, for all light intensities tested, the peak of cell division entry never occurred before 12 hours after light-on, suggesting that the gating of cell division persists the first day, though the circadian regulation of cell division is being lost.

**Time-dependent effect of light exposure on cell division**

Photosynthesis provides energy for plant cell growth. Subsequent cell division occurs when plant cells reach a critical size. The experiment in Figure 6 was designed to evaluate whether the clock controls directly cell cycle progression or operates indirectly, by controlling the amount of energy available through photosynthesis. Cells entrained under LD 12:12 standard conditions were placed into darkness at ZT0. A 6-hour pulse of standard light (35 µmol quanta.m⁻².s⁻¹) was given at different circadian times: the beginning of the subjective day (ZT0-6), the end of the subjective day (ZT6-12), the beginning of the subjective night (ZT12-18) or the end of the subjective night (ZT18-0). Cells entering cell division were scored for each condition. Cell division is represented as the fraction of dividing cells compared to a control population in LD (12:12). A 6-hour light pulse given between ZT0 and ZT6 did not allow cell division. Similarly a light pulse between ZT18 and ZT0 resulted in a low percentage of cell division. However, when light was applied between ZT6 and ZT12 or
between ZT12 and ZT18, a majority of cells were able to divide. These results suggest that the amount of energy available through photosynthesis varies during the day and should be taken into account when studying the regulation of the CDC by the circadian clock in Ostreococcus.
DISCUSSION

Circadian regulation of cell division in Ostreococcus

Our experiments clearly indicate that the CDC obeys a circadian oscillator in Ostreococcus full filling the main criteria of a circadian regulation that is entrainment by different photoperiods and persistence of rhythmicity under free-running conditions (Fig 1). Furthermore, we show that the circadian clock regulates the transcription of the main cell cycle regulators. To better characterise the circadian rhythm of CDC, a phase response curve was established using dark pulses of various durations followed by dim light, similar to the previous experiments in Chlamydomonas (Spudich and Sager, 1980; Goto and Johnson, 1995). Under these conditions, the response is intermediate, with advances when the light is given during the first part of the night. This PRC corresponds to a type 1 phase response curve that displays small phase shifts of less than 6 hours, with continuous transitions between delays and advances. These results are similar to those obtained in Chlamydomonas (Goto and Johnson, 1995). Whether Chlamydomonas cell division is under circadian control or not has been an open question until the work by Goto and Johnson (1995). Upon exposure to intermediate light intensities following dark pulses they observe an intermediate resetting response. This is in apparent contradiction with the previous study, which showed that for bright light, the resetting was so strong in response to light-on, independently of the circadian time, that it was concluded that the CDC is not under circadian control in Chlamydomonas (Spudich and Sager, 1980). The light intensity has clearly an effect on the resetting curve type in Ostreococcus (Fig. 5). For bright light, a complete resetting by the light-on signal is observed, like in Chlamydomonas (Spudich and Sager, 1980). Goto and Johnson (1995) hypothesized that, since the circadian clock is a non-linear limit cycle oscillator, resetting type...
should be highly dependent on the light intensity. Our results in Ostreococcus clearly support this conclusion. Interestingly, the same delay of 12 hours between light-on and cell division entry occurred, independently of the intensity of the resetting light, suggesting that the circadian gating of cell division occurs the first day in LL even for high light intensities. This is in apparent contradiction with the fact that the circadian rhythms of cell division are lost when cells are grown in constant light of moderate to high intensity.

**Metabolic input in light-dependent control of cell division**

A direct regulation of CDC by the circadian clock has been demonstrated in animals (Matsuo, 2003; Fu et al., 2005). In mouse hepatocytes, the central clock gene $Bmal$ controls the transcription of CDC master genes such as the $Wee1$ kinase (Matsuo, 2003). In photosynthetic organisms, light is not only a signal but also a source of energy. A cell will divide only if it has accumulated enough energy to reach a critical size, required for cell division commitment. It is clear from previous studies, that cell division is under circadian control in unicellular algae such as Chlamydomonas or Euglena. The question whether the circadian clock controls directly the CDC or indirectly through the metabolism remains open. Unlike Euglena cells (Hagiwara et al., 2002), Ostreococcus cells commit only in G1 phase, with no cells arrested in S or G2/M phases (Fig. 2; data not shown). From our results in Figure 6, the capacity of G1 cells to commit varies along the day, with a maximum of commitment around subjective dusk. It is clear that the photosynthetic capacity varies along the circadian cycle, being the highest in the afternoon. The circadian regulation of photosynthesis is well documented in higher plants where the transcription of genes like the chlorophyll A binding protein II (CAB2) is under circadian control (Millar et al., 1992). It is therefore possible that the circadian regulation of CDC in plant is an indirect control mediated
by the metabolism. On the other hand, we cannot rule out that metabolism and CDC are controlled independently by the circadian clock. In Ostreococcus, dark-light transitions induce a phase advance between ZT12 and ZT18, while light is well assimilated. The advance observed between ZT12-ZT18 might, therefore, be due to a metabolic process, which will boost cell growth and subsequent division. This would imply, that the circadian regulation of photosynthesis has an amplitude, high-enough to suppress completely cell division in the late night to early day even in the presence of light. On the other hand, while 6 hours of bright light are not sufficient to commit cells between ZT18 and ZT0 (Fig. 6), a phase advance is still observed (Fig. 4), suggesting that, at that time, the CDC is not directly regulated by the amount of energy available. The last and strongest evidence for a direct circadian regulation of the CDC comes from Figure 5. Upon resetting by dark-light transitions, the maximum of entry into cell division occurs at earliest after 10 hours of light independently of the light intensity, suggesting that the circadian clock gates cell division for at least 8-10 hours (Fig. 5). This time-lapse, when cell division never occurs after the light-on signal, would correspond to a closed gate rather than to a metabolic control. In the future, the use of circadian markers independent of the CDC should help to understand the respective involvements of the circadian clock and the metabolism in the regulation of cell division.

Global orchestration of CDC gene transcription in Ostreococcus

Then, the next question is: which core cell cycle genes are the targets of the circadian clock? Microarray and QPCR experiments revealed that a large majority of the genes involved in CDC progression are regulated by the photoperiod in Ostreococcus. Global analysis of gene transcription in Arabidopsis cell suspensions (Menges et al., 2005) and in the unicellular green alga Chlamydomonas subjected to light dark/cycles, suggest that
transcriptional regulations of CDC regulators are important for CDC progression. In agreement with this view, different patterns of expression were observed for Ostreococcus core cell cycle genes. Genes like CDKB or cyclin B, which are involved in the control of mitosis entry (Corellou et al., 2005) are expressed from S phase, several hours after genes likely to be involved in earlier stages of cell cycle progression such as D-type cyclin. Interestingly, the expression of genes like Cyclin D, CKS or Rb (MAT3) does not change significantly under light/dark cycles in Chlamydomonas but it is regulated in Ostreococcus (Bisova et al., 2005). Unlike higher plants and Chlamydomonas, Ostreococcus contains an animal-type CDC25 phosphatase, which has been shown to activate CDKB by dephosphorylation on a conserved tyrosine residue (Corellou et al., 2005). Both CDC25 and Wee1 are regulated at the transcriptional level. Minichromosomes Maintenance genes (MCM2-7) are involved in the formation of prereplication complexes. In Ostreococcus, their transcription levels oscillate over the CDC, increasing during the G1 phase and reaching a maximum at the beginning of the S phase as previously described in budding yeast (Cho et al., 1998). The Anaphase Promoting Complex Cyclosome (APC/C) is required for proteolytic degradation of key CDC regulators such as cyclin B at anaphase. Unlike in budding yeast, where their transcript levels does not oscillate (Cho et al., 1998), we found that in Ostreococcus, all members of the APC (APC 1-8; 10-11) but APC1 are transcriptionally regulated. This is also true for Cdc20 and Cdh1 genes, which encodes activators of the APC and display cell cycle regulated transcription. In summary, almost all CDC genes are regulated by light/dark cycles in Ostreococcus. Among CDC genes, CDKs and cyclins genes under investigation were shown to be regulated not only by the photoperiod but also by the circadian clock, demonstrating that the circadian clock regulates CDC progression at the transcriptional level, even though it cannot be ruled out that postranscriptional regulations operate also in the circadian regulation of CDC progression. In mouse hepatocytes, the
regulation of Weel is mediated at the transcriptional level by Bmal, which regulates the transcription of Weel through binding to a specific E box element in the promoter of Weel. Future studies should focus on regulations of CDC core cell cycle genes by the circadian clock in Ostreococcus to discriminate those under direct circadian control from those, which are regulated when the cells have reached the critical size to divide.

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REFERENCES


**Figure 1**: Circadian regulation of cell division in Ostreococcus cells. A. Cells were entrained for five days in L:D 12:12 (35 µmol.quanta.cm\(^{-2}\).s\(^{-1}\)) and then placed in continuous dim light (15 µmol.quanta.cm\(^{-2}\).s\(^{-1}\)). Cells number (A top) and FL3-H parameter (A bottom) were monitored by flow cytometry. B. Cells entrained for five days under L:D 8:16 (open triangles) or L:D 16:8 (closed rhombus) and then placed in continuous dim light. Cells number (B top) and FL3-H parameter (B bottom) were monitored by flow cytometry. This experiment is representative of 3 independent experiments.

**Figure 2**: Microarray analysis of core cell cycle genes expression. Cells were entrained under L:D 12:12 (35 µmol.quanta.cm\(^{-2}\).s\(^{-1}\)). The percentage of cells in S and G2/M phase were determined by flow cytometry (Top Panel). For each gene, the relative expression (as the log\(_2\) of the ratio of the time point value normalized to the average value of all time points) is plotted as a function of time. An average of biological triplicates is represented. The right scales apply to cyclin B and CDKB, the left scales to other genes.

**Figure 3**: Relative expression levels of the main cyclins and CDKs normalized to EF1-α in synchronized Ostreococcus cells using real time quantitative RT-PCR. A. Sampling strategy. White and black squares represent the day and night periods respectively, dashed squares represent subjective night periods. B. relative mRNA levels of cyclin A (crosses \(\times\)), cyclin B (closed rhombus \(\blacklozenge\)), cyclin D (open squares \([\square]\)), and cyclin D2 (open triangles \(\triangle\)). C. relative mRNA levels of CDKA (open rhombus \(\bigodot\)), CDKB (closed squares \([\square]\)) and CDKD (closed triangles \(\blacktriangle\)). This experiment is representative of two independent experiments.
**Figure 4:** Phase Response Curve (PRC) of the CDC in response to dark-light transitions. Cells entrained in L:D 12:12 (35 µmol.quanta.cm\(^{-2}\).s\(^{-1}\)) were placed into darkness at time 36 h for various durations (0-24 hours) before being transferred to constant dim light (15 µmol.quanta.cm\(^{-2}\).s\(^{-1}\)). Cell division was monitored for 3 days using the FL3-H parameter (red fluorescence). Advances and delays, relative to the control (12 hour dark pulse) were determined the third day. A. A dark pulse of 4 hours induces a phase advance of 5 hours. B. Advances and delays were plotted as a function of the circadian time when the cells were transferred back to light. The example in A is represented by an asterisk.

**Figure 5:** Effect of the intensity of resetting light-on cell division. Cells entrained under L:D 12:12 (35 µmol.quanta.cm\(^{-2}\).s\(^{-1}\)), were transferred to darkness at circadian time ZT12 for various durations from 0 to 12 hrs before being transferred back to light of various intensities for 24 hours: 15 µmol.quanta.cm\(^{-2}\).s\(^{-1}\) (dotted line), 35 µmol.quanta.cm\(^{-2}\).s\(^{-1}\) (continuous line) and 100 µmol.quanta.cm\(^{-2}\).s\(^{-1}\) (broken line). The time of the beginning of cytokinesis, determined as the peak of red fluorescence (FL3-H parameter) after the onset of darkness is plotted as a function of the duration of darkness.

**Figure 6:** Effect of A 6h-long light pulse at various circadian times on cell division. Cells entrained in L:D 12:12 (35 µmol.quanta.cm\(^{-2}\).s\(^{-1}\)) were subjected to a light pulse of 6 hours of the same fluence at different circadian times. Top: The time of cytokinesis is represented by asterisks. Bottom: For each condition, the percentage of dividing cells, relative to the number of dividing cells in L:D 12:12, is represented as the function of the time when the light pulse is given. The number of dividing cells corresponds to the increase in cells number, after 24 hours from the time of transfer to darkness.
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### B

**A**

![Graph A](image1)  
![Graph B](image2)

![Graph C](image3)

### C

**A**

![Graph A](image1)  
![Graph B](image2)

![Graph C](image3)

**Relative mRNA levels vs. time (hrs)**

*Figures A, B, and C depict the relative mRNA levels over time under different conditions.*
A

![Graph showing relative FL3-H fluorescence over time](image)

- 24 hrs
- \(\Delta = +5\) hrs

B

![Graph showing phase shift over time after dawn](image)

- Time after dawn (hrs)
- Phase shift (h)