S-Phase and M-Phase Timing Are under Independent Circadian Control in the Dinoflagellate *Lingulodinium*

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Abstract In many phytoplankton species, cell division (mitosis) usually occurs at defined times of day. This timing is also observed under constant conditions, indicating that it is regulated by a circadian clock rather than by a simple response to the light-dark cycle. For those algae with cell cycles longer than a day, the clock opens a window of opportunity for mitosis at a particular time of day through which cells in an appropriate phase of the cell cycle can pass. Although the timing of mitosis is generally studied due to ease of measurement, for some phytoplankton the timing of S-phase is also circadian. This thus raises the possibility that mitosis is not directly gated by the clock but occurs instead at a defined interval (a constant G2 length) following a circadian controlled S-phase. To determine if the clock exercises independent control over the timing of both S- and M-phase, we measured the timing of both S- and M-phase in cultures of the dinoflagellate *Lingulodinium* grown under a variety of different photoperiods. We interpret the phase angles of both rhythms, in particular those resulting in a change in the length of G2, as an indication that the clock independently regulates the timing of S-phase and mitosis.

Key words cell cycle, circadian rhythm, dinoflagellate, mitosis, S-phase, photoperiod, phytoplankton

The timing of cell division for many phytoplankton species in a light-dark cycle is often fixed at defined times (Chisholm, 1981), presumably to coordinate cellular biochemistry with environmental conditions. While this could be explained in naturally growing populations by a simple response to changes in light intensity, the persistence of cell division timing under constant conditions reveals that there is control exerted by the circadian clock (Chisholm and Brand, 1981). However, for species whose cell cycle is longer than 24 h, clock control is not direct but conditional, and the cell cycle is said to be "gated" by the circadian clock. The gate represents a temporal window through which cells in an appropriate point in the cell cycle can proceed through the cell cycle. Mechanisms that allow the circadian and cell cycle clocks to interact must, therefore, be the logical equivalent of an "and" gate, in which concurrent signals from both are necessary to permit cell cycle progression.

The cell cycle consists of 4 discrete and sequential phases termed G1 (1st gap), S (DNA synthesis), G2 (2nd gap), and M (mitosis). An additional phase, termed G0, is often used to signify quiescent cells that

no longer divide. The decisions to initiate DNA replication or mitosis are both tightly controlled and constitute 2 important regulatory checkpoints where the cell can verify critical elements of the cell state before proceeding (Elledge, 1996). For example, at the G1-/S-phase boundary, the cell confirms that a minimum size has been attained, while at the G2-/M-phase boundary, the cell checks that DNA synthesis has been completed. These 2 important checkpoints share, as a common mechanistic component, enzymes called cyclin-dependent kinases (CDKs; Sanchez and Dynlacht, 2005). This class of kinase requires a cyclin partner for activity, and since levels of cyclin change over the course of the cell cycle, kinase activity can be restricted to the times when cyclin levels are high. Furthermore, in model systems such as budding yeast that have several different cyclins with different expression patterns, the nature of the cyclin partner can influence the substrate specificity of the CDK allowing the same kinase to catalyze entry into different phases of the cell cycle (Loog and Morgan, 2005). For example, S-phase cyclins direct kinase activity toward targets allowing initiation of DNA replication, while mitotic cyclins allow selection of targets whose phosphorylation is required for initiation of mitosis. Most interestingly for the concept of circadian gating, CDKs can also integrate input from other signaling pathways. For example, in addition to the amount of cyclin, CDK activity can be influenced by the presence of CDK inhibitors (CKIs) as well as by phosphorylation of the kinase subunit (Mendenhall and Hodge, 1998). As an example of this latter, passage into M-phase requires removal of an inhibitory phosphate; the equilibrium between phosphorylated and nonphosphorylated forms of the kinase is determined by the balance of Cdc25 phosphatase and Wee1 kinase activites.

As might be expected, therefore, studies on the circadian influence over the cell cycle have revealed direct interactions between circadian clock components and cell cycle regulators. In the regenerating mouse liver model, the clock was shown to gate transcription of the Wee1 kinase, an enzyme normally acting to inhibit the CDK1-cyclin B1 complex required for M-phase entry (Matsuo et al., 2003). Furthermore, the clock regulators RORγ and REV-ERBα have been found to influence expression of the CKI p21[Waf1/CIP1] (Grechez-Cassiau et al., 2008). Last, the checkpoint kinase Chk2 involved in retarding entry into S-phase has been shown to interact with circadian clock components PER and FRQ in mammals and *Neurospora*, respectively (Gery et al., 2006; Pregueiro et al., 2006).

Clearly, precedent exists for coupling the circadian clock and the cell cycle at both S- and M-phases.

A circadian rhythm in cell division in the dinoflagellate *Lingulodinium* was first reported 60 years ago (Sweeney and Hastings, 1958). This is a relatively common observation in phytoplankton (Chisholm and Brand, 1981), with few studies reporting circadian control over S-phase (Homma and Hastings, 1989; Lidie et al., 2005). The observation that S-phase can be under circadian control suggests 2 different mechanisms to account for the circadian rhythm in mitosis: either a circadian gated S-phase entry is followed by a G2-phase of constant length or both S- and M-phases are gated independently by the circadian clock. This question has never been addressed, although constant G2 length when phytoplankton was grown under different nutrient conditions has been reported (Olson and Chisholm, 1986). One method to distinguish between these possibilities is to examine the timing of S- and M-phase in cultures grown under different photoperiods. Light is a major environmental factor synchronizing the circadian behavior of most organisms, and this influence involves changes in the progression of the clock's internal time according to a characteristic phase response curve (PRC). When 2 different rhythms with different circadian phases are compared under different photoperiods, the phase angles of the 2 should show a characteristic pattern related to but not driven directly by the transitions between light and dark (Pittendrigh and Daan, 1976). The change in phase angle as a result of changing photoperiod is a hallmark of circadian regulation (Dunlap et al., 2004) and has been used to demonstrate circadian control over M-phase in *Ceratium furca* (Adams et al., 1984) and *Lingulodinium polyedrum* (formerly called *Gonyaulax polyedra*; Homma and Hastings, 1989). Here, we measure both the phase of S-phase and M-phase concurrently under different photoperiods and find the phase angle of the 2 can vary independently. We conclude from this that the circadian clock is involved in gating passage through both S-phase and M-phase boundaries.

MATERIALS AND METHODS

Cell Culture

Lingulodinium polyedrum cultures (strain CCMP1936; formerly *Gonyaulax polyedra*) were obtained from the Provasoli-Guillard National Center for Culture of

Marine Phytoplankton (Booth Bay Harbor, ME) and grown in f/2 medium (Guillard and Ryther, 1962) at a temperature of 19 ± 1 °C using 6 different light (50 μmol photons/m²/sec cool white fluorescent light)/ dark (LD) regimens (LD 8:16, LD 10:14, LD 12:12, LD 14:10, LD 16:8, and LD 18:6). Cells were grown in each LD cycle for 2 weeks prior to harvesting the cultures. Cultures were diluted 1 in 2 every 3 or 4 days to try and maintain rapid growth, although occasionally the cells may fall back into a lag phase with a temporarily reduced lower doubling time. Although this reduces the magnitude of S-phase it is not thought to affect the timing. For all LD cycles, LD 0 corresponds to the beginning of the subjective day (lights-on).

Flow Cytometry Analysis

Lingulodinium grown under LD 12:12 has S-phase during the dark period (Homma and Hastings, 1989), so for our different photoperiods, samples from the cultures were also taken every hour during the dark phase. For each sample, 150 mL of cell culture was centrifuged at 1000 g for 1 min and resuspended in 15 mL of 70% ethanol. After 15 min, the cells were centrifuged and resuspended in 15 mL of 100% ethanol and then exposed to culture room light for 2 to 5 h with gentle shaking to bleach residual pigments. The samples were stored at 4 °C. Before use, cells were centrifuged and resuspended in 15 mL of 70% ethanol then washed twice with water. The samples in a final volume of 5 mL of water were incubated with 40 mg/mL RNase A for 30 min at room temperature then stained for 30 min with 50 mg/mL propidium iodide (Sigma, St. Louis, MO) in the dark. For each time point 10,000 events were recorded using a FACscan (BD BioSciences, San Jose, CA) equipped with a 488-nm argon laser and a 586-nm filter for the FL2-channel. Data were collected and managed using the CellQuest Pro software supplied by the manufacturer (BD BioSciences). Cellular debris was excluded from the analysis using a gating window in forward against side scatter plots, and cell doublets were excluded using a gating window in FL2-area (FL2-A) versus FL2-width (FL2-W) plots as described (Bertomeu et al., 2007). The percentage of cells in each cellular growth phase (G1, S, and G2) was calculated from histograms of number of cells with defined levels of fluorescence using the ModFit software (BD BioSciences) using the automatic peak finding settings. In essence, the software fits a Gaussian curve under the 2 major fluorescence peaks, which

are generally separated by a factor of ~1.8-fold (representing cells in G0/G1 and those in G2/M, after DNA has been replicated). The number of cells in S-phase represents those cells with intermediate levels of fluorescence that cannot be accounted for in the G1 or G2 peaks. Note that although cells in G0 (quiescent cells) cannot be differentiated from cells in G1, for simplicity we will refer to all these cells as belonging to G1-phase.

RESULTS

Under our culture conditions, *Lingulodinium* cultures have a doubling time of ~5 days, and thus only a small percentage of cells in the population duplicate their DNA in each circadian cycle. It was thus important to establish if flow cytometry could adequately extract cell cycle distributions given the low numbers of cells entering S-phase. The example shown (Fig. 1) demonstrates that despite the low frequency of cell progression through S- and M-phases, the timing of both the beginning and end of S-phase, as well as the time of cell division, can be readily determined. The FACScan records events, only some of which correspond to cells, so it is important to only analyze events that correspond to cells. This restriction is made at 2 steps, first using light scattering characteristics of individual events to include only those of a size and shape consistent with cells (Fig. 1A). Next, the fluorescence characteristics of the individual events are used to include events corresponding to single cells (Fig. 1B). For example, events in which 2 cells pass simultaneously (doublets) can be excluded using FL2-W fluorescence (note that doublets have different fluorescence characteristics from "paired" or dividing cells, as seen using a sample taken at LD 1). The fluorescence characteristics cleanly separate cells in G1 from cells in G2 along the FL2-A axis when DNA is not being replicated (sample at LD 1), or shows cells replicating their DNA (sample at LD 19). For cell cycle analysis, histograms showing the number of cells with discrete fluorescence amplitudes are plotted (Fig. 1C). These histograms show 2 main peaks representing the number of cells in G1 and G2, with cells at intermediate fluorescence levels ascribed to cells in S-phase. The distribution of cells among the different phases of the *Lingulodinium* cell cycle varies over the course of the night and early day of cells grown under a LD 12:12 regimen, with S-phase observed at LD 18 and LD 19 (the number of cells in G2 increase) and M-phase
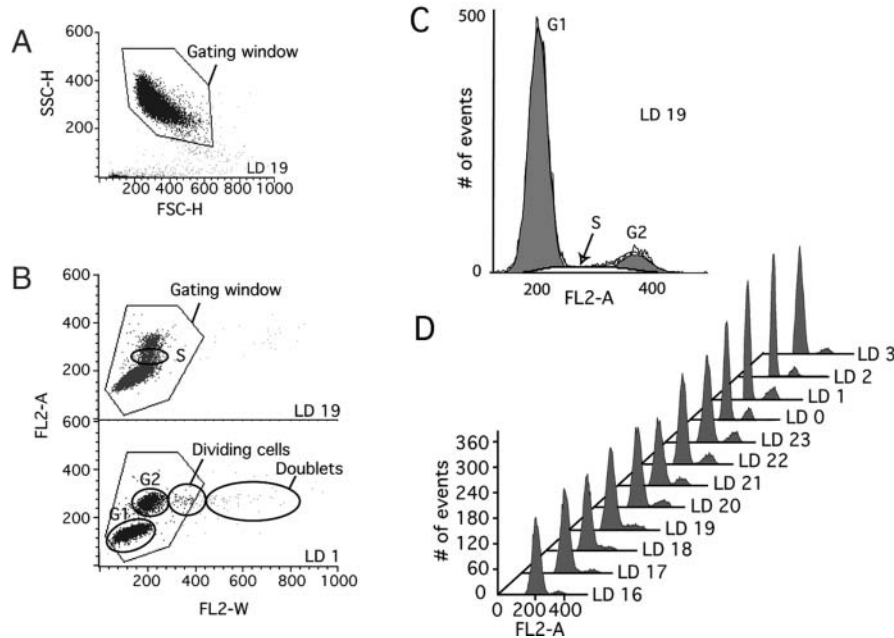
Figure 1. FACScan analysis of cell cycle phases. The FACScan distinguishes cells from other recorded events by first excluding cell debris, as only events within a defined window (polygon) based on side and forward scatter are analyzed further (A), then by excluding doublets (2 cells passing through the detector simultaneously), as only events within a defined window (polygon) in the amplitude and width of the fluorescence peak are analyzed further (B). Note that cells in G1 have lower fluorescence amplitude (FL2-A) than cells in G2, while cells in S-phase have intermediate fluorescence amplitudes (LD 19 sample, upper panel). Note also that dividing cells (often called "pairs") can be distinguished from doublets (LD 1 sample, lower panel). A histogram presentation of the data from LD 19 plots the number of events (cells) in each of 512 fluorescence bins (C). The percentage of cells in the population in G1-, S-, or G2-phases are determined by fitting Gaussian peaks representing G1- and G2-phase cells under the 2 peaks in the histogram, and then assigning the remaining intermediate levels of fluorescence to cells in S-phase. Histograms for cell cultures taken at different times from an LD 12:12 cycle show a systematic variation in the number of cells in G1, S, and G2 over time (D).

between LD 1 and LD 2 where the number of cells in G2 decrease (Fig. 1D).

These cell cycle parameters agree well with previous reports characterizing the cell cycle in LD 12:12 (Bertomeu et al., 2007; Homma and Hastings, 1989). The progression through the cell cycle of cultures grown under a range of different photoperiods was thus also monitored during the night phase and early dawn (Fig. 2). For each of the 6 photoperiods shown, results from at least 3 different experiments were averaged and data presented as the mean ± SD. Generally speaking, the number of cells entering S-phase is typically 10% to 15%, which would correspond to doubling times of 7 and 5 days, respectively. While this value is substantially lower for LD 14:10 and LD 16:8, cell cycle progression can still be determined in these cultures. The low percentage of cells in S-phase in the LD 14:10 cultures is not due to a major change in the timing of cell cycle markers, as additional samples

taken during the day showed no evidence of S-phase (data not shown).

To characterize the progression through the cell cycle as a function of the different photoperiods, the onset and end of S-phase were derived from Figure 2 as the times corresponding to half the maximum percentage of cells in S-phase, while the time of M-phase was estimated as the midpoint of the decrease in the percentage of G2/M cells. While all data sets contain precise measures for the end of S-phase, the data collected for LD 8:16 and LD 10:14 do not allow a precise determination of the onset of S-phase. For both these data sets, S-phase was taken to be a symmetrical peak with the onset thus preceding the 1st time point by half an hour. When both the beginning (filled squares) and the end of S-phase (open squares) are plotted over the range of photoperiods tested (Fig. 3A), these estimated S-phase onsets seem reasonable as they show a generally similar duration of S-phase, as expected from other dinoflagellates (Olson and Chisholm, 1986). Thus, a line drawn through the end of S-phase accounts for all S-phase onsets except for the LD 14:10 sample, in which the fewest cells in S-phase were observed.

The timing of both S- and M-phase is consistent with the action of a circadian clock. In LD cycles containing less than 14 h of light, the timing of S-phase follows the onset of the darkness at a roughly constant interval of ~5 h (Fig. 3A). However, when the LD cycles contain more than 14 h of light, the timing of S-phase appears to follow the onset of the light phase at a roughly constant interval of ~22 h. The timing of M-phase, on the other hand, is slightly different from that seen for S-phase (Fig. 3A). Indeed, a curve representing the timing of S-phase (dotted line) cannot be superimposed on the peaks of M-phase. As a result of
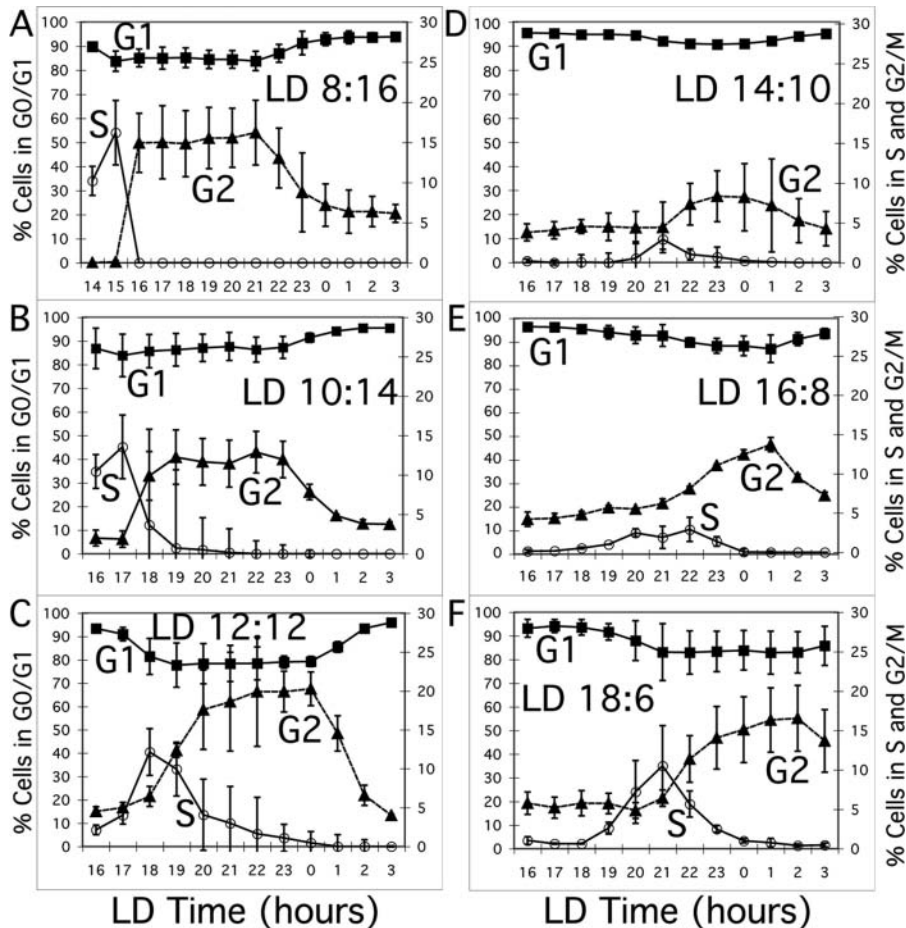
Figure 2.   Cell cycle parameters as a function of photoperiod. The percentage of cells in G1 (filled squares), S-phase (open circles), and G2/M (filled triangles), determined from histograms as indicated in Figure 1C, is shown for each of the 6 photoperiods tested. Each point represents the mean ± SD of 3 experiments, and LD 0 corresponds to lights-on for all photoperiods. Note that scales for the cells in G1 (left) are different from the scales for cells in S and G2/M (right).

we conclude that the circadian clock can independently control both S- and M-phase.

In an alternative graphical method, the same data are replotted with a center around midnight (Fig. 3B). This analysis emphasizes the differences between S- and M-phase timing, as S-phase tracking along dusk and dawn is still clearly visible (here, the lines through the S-phase points are drawn parallel to the slopes of either lights-off or lights-on) while the points indicating the timing of M-phase more closely approximate a vertical line corresponding to LD 1 on a LD 12:12 cycle.

DISCUSSION

We have measured the timing of both S- and M-phase for *Lingulodinium* cultures grown under 6 different photoperiods to test the hypothesis that the circadian clock can gate both S- and M-phase independently. Our data support this hypothesis, with the timing of M-phase varying less in response to the different photoperiods than the timing of S-phase (Fig. 3A, B). In particular, the different length of G2 measured during the different photoperiods (Fig. 3A, inset) clearly rules out a mechanism in which M-phase follows S-phase at a defined interval. It is interesting that circadian control over the cell cycle in this organism is apparently of sufficient importance to warrant regulation at both S-phase and M-phase.

It is important to emphasize that the present experiments were undertaken not to determine if S-phase and M-phase were under circadian control, but to assess if there is independent control over the 2. Both S-phase (Homma and Hastings, 1989) and M-phase (Sweeney and Hastings, 1958) are circadian rhythms, as both continue under constant conditions.
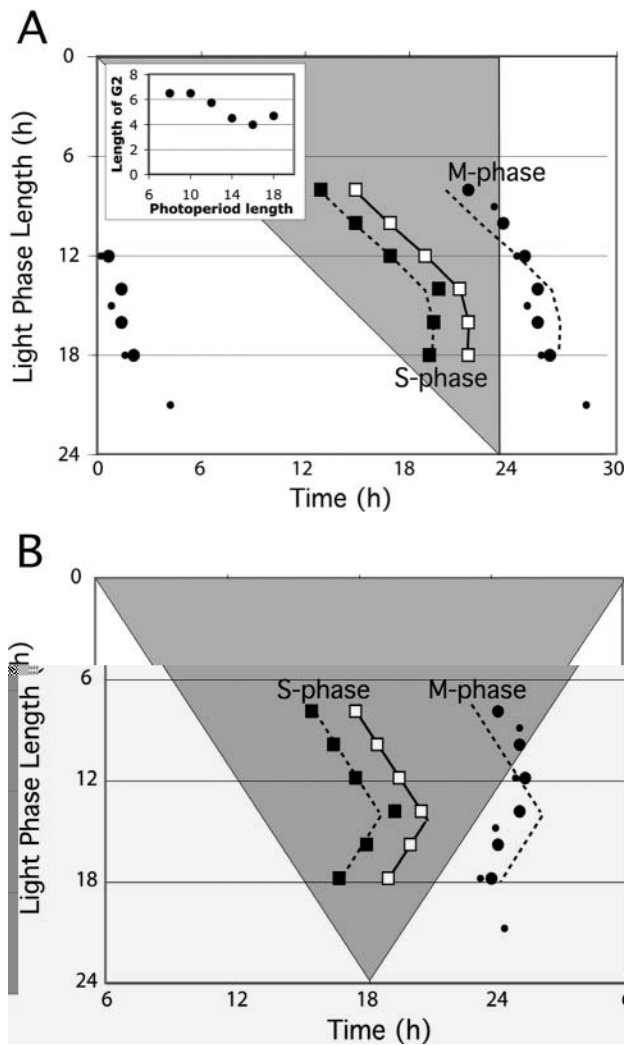
the differential response of S- and M-phase timing to the different photoperiods, the length of G2-phase varies, from a minimum of 4 to a maximum of 6.5 h (Fig. 3A, inset). Clearly, the normal length of G2-phase is secondary to the timing clues provided by the circadian clock. The timing of M-phase observed here agrees well that reported previously (Homma and Hastings, 1989) documenting a complex pattern of phase angle changes in cell division timing under different photoperiods (small circles, Fig. 3A). In this regard, it is reassuring that our data so closely mimic those found previously, as our cultures are grown under a third the light intensity of those reported (Homma and Hastings, 1989) and in consequence grown more slowly (i.e., with a longer generation time). Last, and more importantly, since the timing of M-phase of cells grown under the different photoperiods cannot be explained by a constant length of G2,

Figure 3. **Circadian timing of cell cycle parameters as a function of photoperiod. The cell cycle times for the beginning (closed squares) and end (open squares) of S-phase, as well as for M-phase (filled circles), are shown as a function of the length of the light period (vertical axis). M-phase data from Figure 2 (large circles) and from Homma and Hastings (1989; small circles) are both shown. The 2 graphs present the same data, either with light periods aligned at dawn (A) or with dark periods aligned at midnight (B). The length of G2-phase as a function of the length of the light period is shown as an inset in A. The dotted lines mimic the solid curve, which has been drawn through the points representing the end of S-phase.**

However, independent clock control of both, or clock control over only S-phase (with the timing of M-phase determined by a cell cycle clock), represent 2 alternative hypotheses that could account for the observed circadian behavior of these rhythms. To tease apart control over S- and M-phase, we have monitored the rhythmic properties of both under driving LD cycles

of different photoperiods. Since a difference in the timing of a given rhythm (the phase angle) under different photoperiods is a hallmark of circadian clock control (Johnson et al., 2003), and since the 2 rhythms peak at different times in the circadian cycle and should thus be influenced differentially by the LD cycles, independent control by the circadian clock over both rhythms predicts the phase angle of the 2 would change differently under different photoperiods. Our analysis shows the timing of M-phase does not mirror the timing of S-phase over the different photoperiods (Fig. 3), thus validating this prediction.

To entrain to a 24-h LD cycle, the ~22-h free-running period of the circadian clock under constant light (Nassoury et al., 2001) must achieve a 2-h phase delay each cycle. This recurring phase delay must reflect the ability of the circadian clock to alter its phase or period in response to light (Johnson et al., 2003). While period response curves are not known for *Lingulodinium*, several PRCs are available. The exact shape of the PRC is a matter of debate, as details of the phase responses depend on the quality of light used for both the pulse and the background (Christianson and Sweeney, 1973; Johnson and Hastings, 1989; Johnson, 1990; Roenneberg and Hastings, 1988). For example, blue light produces greater phase advances than delays while red light produces greater phase delays (Roenneberg and Deng, 1997), consistent with the observation that in constant light, increased intensity of red light increases the rhythmic period of bioluminescence while increased intensity of blue light decreases the period (Roenneberg and Hastings, 1988). Overall, it seems likely that maximum delays in the PRC will occur around CT 12, the transition from delays to advances will occur around CT 14, and maximum advances will be found around CT 15. One caveat, however, is that all available PRC curves were obtained using cells maintained on 12:12 LD cycles, and the PRCs under different photoperiods are not known.

To interpret the circadian timing changes of S- and M-phases as a function of the photoperiod, we first considered the finding that the general patterns observed for the 2 rhythms were different. Since the timing of M-phase (circles in Fig. 3A, B) resembles that reported for the fruit fly eclosion rhythm in continuous photoperiods (Pittendrigh and Minis, 1964), it may thus also be explained by nonparametric entrainment (phase shifting by pulses of light at dawn and dusk). In contrast, the S-phase timing appears to track dusk at photoperiods shorter than 14

h and to track dawn at photoperiods longer than 14 h. This rhythm is clearly circadian, given that its phase angle relative to dusk (or dawn) changes with the photoperiod. However, the change from dusk tracking to dawn tracking is difficult to explain by a simple phase shifting model. For example, while accurate tracking of the rhythm with dusk at short photoperiods could be consistent with a clock that arrests during the day and is reset at the light-dark transition, this interpretation does not explain the shift to dawn tracking at longer photoperiods.

An arrest of circadian progression in the light has other difficulties as well, in that it accommodates neither regulation of the timing of daily rhythms such as photosynthesis (Nassoury et al., 2001) nor the circadian timing of cell cycle events in constant light (Homma and Hastings, 1989). An alternative view to the resetting to a defined circadian time is provided by limit-cycle models of the circadian oscillator, in which state variables of the clock in DD compared with LL oscillate in different regions of phase space (Johnson et al., 2003). This modeling allows the clock to complete a circadian cycle during LL, but during LD cycles, also allows the LL cycle to collapse to roughly the same time in the DD cycle. For example, an LL limit cycle centered on the isochron of CT 12 for the DD limit cycle would allow for modest variations ("scalloping") in the circadian timing around the CT 12 isochron of the DD limit cycle. Again, however, this analysis seems incompatible with the observed changes in S-phase timing over the different photoperiods.

An alternative to the simple PRC clock model is that more than 1 oscillator may be involved in regulating passage through the cell cycle. For example, the morning and evening oscillators originally proposed to explain splitting of rhythms in rodents (Pittendrigh and Daan, 1976) have counterparts that control locomotor activity at dawn and dusk in *Drosophila* (Grima et al., 2004). Interestingly, 2 different oscillators have also been found to time bioluminescence and aggregation in *Lingulodinium* (Roenneberg and Morse, 1993) and the 2 have different phase shifting characteristics (Morse et al., 1994). It is possible that a differential impact of the 2 oscillators on the cell cycle under different photoperiods might underlie the change from dusk tracking (a PRC with greater delays than advances) to dawn tracking (greater advances than delays). An involvement of 2 endogenous clocks has been previously proposed to explain the change in timing of mitosis in the dinoflagellate *Ceratium* from

dusk tracking at short photoperiods to dawn tracking at longer photoperiods (Adams et al., 1984), a behavior similar to what we observe here for S-phase timing. Examining the phases of the bioluminescence and aggregation rhythms under different photoperiods could potentially test this hypothesis.

Another alternative to the simple PRC model is that the shape of the PRC itself may differ in different photoperiods. In sparrows, the delay portion of the PRC is much reduced in long photoperiods compared with short photoperiods (Binkley and Mosher, 1986). Similarly, long photoperiods reduce the amplitude of phase responses in hamsters when compared with short photoperiods (Pittendrigh et al. 1984). Since a PRC in which delays are greater than advances is capable of tracking dusk, while a PRC in which advances are greater than delays is capable of tracking dawn, a change from a predominantly delay form PRC at short photoperiods to a predominantly advance form PRC at long photoperiods may allow for the shift from dusk tracking to dawn tracking observed for the timing of S-phase. A test of this hypothesis for the cell division rhythm would be labor intensive, although it may be feasible using automated bioluminescence rhythm measurements.

Independent of the mechanism used to regulate the timing of cell cycle progression in *Lingulodinium*, it is interesting that S-phase and M-phase are regulated independently. This allows the length of G2 to vary under different photoperiods, and is thus quite different from what was seen when growth of the dinoflagellate *Amphidinium* was limited by light or nutrients, as in this case only the length of G1 was found to change (Olson and Chisholm, 1986). It is also noteworthy that the *Lingulodinium* clock ensures S-phase is restricted to the dark period under all photoperiods tested. While this superficially seems to have evolutionary significance, by placing DNA replication at a time when light is unlikely to cause mutations, S-phase has been observed during the day in the dinoflagellates *Amphidinium operculatum* (Leighfield and Van Dolah, 2001; Van Dolah and Leighfield, 1999) and *Karenia brevis* (Van Dolah et al., 2007). It will be interesting to see if S-phase is similarly clock regulated in these other dinoflagellates.

The independent clock regulation of entry into both S-phase and M-phase indicates that the biochemical components of the circadian and cell cycles clocks must be able to interact twice. Based on the circadian controls elaborated in studies of mammals and fungi, we propose that for both regulatory events the cell

cycle clock provides input in terms of cyclin levels (either S-phase or mitotic cyclin). The circadian clock component influencing entry into S-phase could then involve inactivation of a CKI (Grechez-Cassiau et al., 2008; Pregueiro et al., 2006), while regulation of the Wee1 kinase (Matsuo et al., 2003) could represent the circadian clock influence over entry into M-phase. As a consequence, the molecular signals regulating the onset of S-phase and mitosis remain distinct for both cell cycle and circadian inputs, thus reducing the possibility of confusion between the 2 regulatory events.

ACKNOWLEDGMENTS

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