

# Is the Cell Division Cycle Gated by a Circadian Clock? The Case of *Chlamydomonas reinhardtii*

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**Abstract.** Circadian oscillators are known to regulate the timing of cell division in many organisms. In the case of *Chlamydomonas reinhardtii*, however, this conclusion has been challenged by several investigators. We have reexamined this issue and find that the division behavior of *Chlamydomonas* meets all the criteria for circadian rhythmicity: persistence of a cell division rhythm (a) with a period of  $\sim 24$  h under free-running

conditions, (b) that is temperature compensated, and (c) which can entrain to light/dark signals. In addition, a mutation that lengthens the circadian period of the phototactic rhythm similarly affects the cell division rhythm. We conclude that a circadian mechanism determines the timing of cell division in *Chlamydomonas reinhardtii*.

CYCLIC phenomena play crucial biological roles. Two such oscillations are the cell division cycle (CDC) and circadian rhythms. The CDC is responsible for cellular cycles of asexual reproduction. Circadian rhythms are an adaptation of organisms to daily environmental cycles, and they exhibit  $\sim 24$ -h periods in continuous conditions (Pittendrigh, 1960). Because many cells also divide on a 24-h cycle, it is understandable that these two rhythms might sometimes be confused in cases in which cells divide in 24-h multiples. In such cases, an important question arises: is cellular division timed by a circadian oscillator or by a cell division oscillator that is completely independent of a circadian clock or, is the timing the result of interaction between two oscillating mechanisms, one circadian and the other which directly controls replication, mitosis, and cytokinesis? This paper addresses a specific case in which the distinction between the CDC and the circadian clock requires clarification, namely in the alga *Chlamydomonas*.

Recent studies of the CDC have focused on its molecular mechanism and identified many key components including cyclins and kinases (Nurse, 1991; Murray and Hunt, 1993; Pines, 1994). Our current understanding of circadian pace-making mechanisms is much less concrete. It may be that the molecular mechanism of circadian clocks has been evolutionarily conserved. On the other hand, we may eventually discover that natural selection has devised many different

mechanisms to accomplish circadian timing in different organisms. Either way, circadian rhythms are defined by three major phenomenological criteria which are well established (Pittendrigh, 1960), and not by their molecular mechanism. The first criterion is that circadian rhythms persist in constant conditions (i.e. constant temperature and either constant light or constant darkness) with a period of  $\sim 24$  h. To demonstrate persistence, it is necessary to assay the rhythm for at least several consecutive cycles. The second criterion is that these endogenous rhythms of  $\sim 24$  h can be entrained to exactly 24 h by daily cues in the environment, such as light/dark cycles, temperature cycles, and so on. This characteristic allows a circadian clock to establish a specific phase relationship to the entraining day/night cycle. Finally, the third criterion is that these rhythms are temperature compensated, so that they proceed at almost the same rate (= same period) no matter what the ambient temperature may be ( $Q_{10}$  values of circadian rhythms range from 0.8 to 1.4; see Sweeney and Hastings, 1960). Changes of the ambient temperature usually entail changes of growth rate, so that the period of a circadian rhythm of cell division should be relatively unchanged even when the growth rate of a culture is altered by different temperatures. In addition, circadian rhythms often exhibit transients, after-effects, and a dependency of the period on the intensity of light in continuous illumination (LL) (Pittendrigh, 1960).

A circadian rhythm of cell division was first reported in the dinoflagellate alga *Gonyaulax* (Sweeney and Hastings, 1958), and the phenomenon has since been most extensively studied in the algal flagellate *Euglena* by Edmunds and his colleagues (Edmunds, 1988; Carré and Edmunds, 1993). In these and other organisms, the timing of cell division exhibits the major characteristics of circadian rhythms spec-

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1. **Abbreviations used in this paper:** 137c, wild-type strain *Chlamydomonas*; CDC, cell division cycle; LD, light/dark cycle; LL, continuous illumination; WW, white LL; YY, yellow LL.

ified above. In the cases of *Euglena* and dinoflagellate algae, there is no disagreement that circadian oscillators are somehow involved in the timing of cell division (Sweeney, 1982; Edmunds, 1988). In the case of *Chlamydomonas*, however, the issue of circadian control of cell division has become controversial. From his study of photoautotrophic cultures under batch and continuous conditions (see Materials and Methods for descriptions of these culturing conditions), Bruce concluded that a circadian oscillator "gated" division in *Chlamydomonas* (Bruce, 1970). He reported a persisting 24-h rhythm of daughter cell liberation in wild-type cells in light/dark (LD) cycles and in continuous white light (white LL of  $\sim 40 \mu\text{mol m}^{-2}\text{s}^{-1}$ ) at 23°C. The cell division rhythm appeared to synchronize to the LD cycle, but entrainment was not rigorously tested. Bruce did not report an assessment of temperature compensation of the cell division rhythm. Straley and Bruce (1979) later reported that batch cultures of wild type and a long-period mutant, *per-4*, display persistent daily rhythms of cell division and hatching with the same periods as those for the well-established circadian rhythm of phototaxis.

Later studies challenged Bruce's conclusion that a circadian mechanism is involved in the timing of the division and daughter cell liberation in photoautotrophic cultures of *Chlamydomonas*. Spudich and Sager (1980) explained the light-dark synchronization of division by suggesting that CDC progression was forced into a daily periodicity by the diurnal availability of energy via photosynthesis. More recently, John and coworkers modelled the decision-making process in *Chlamydomonas* as a composite of "sizer" and "timer" mechanisms (Donnan and John, 1983; Donnan et al., 1985; McAteer et al., 1985; John, 1987). The sizer forbids cells that are too small from dividing. The timer allows division to occur at a specific phase relative to synchronizing LD cycles. John and his coworkers further explicitly stated that the timer is not an endogenous circadian timer but postulated that it is composed of two hourglass-type timers: a commitment timer and a postcommitment timer (Donnan and John, 1983; Donnan et al., 1985; McAteer et al., 1985; John, 1987). John and coworkers presented data which they believed directly argued against circadian clock control over cell division (summarized in the Discussion).

We have resurrected the question of whether a circadian oscillator is involved in timing cell division in *Chlamydomonas*. Does an authentic circadian oscillator time cell division in *Chlamydomonas*, as suggested, but not rigorously proven, by Bruce (1970)? Or, do the experimental results of Spudich and Sager (1980) or of John and coworkers prove that a circadian pacemaker is not involved in timing cell division in this alga? Our results fully support the initial conclusions of Bruce. Under the conditions we used, the timing of cell division in *Chlamydomonas* behaved in a manner which is consistent with the model of circadian gating of cell division and liberation. This circadian mechanism is distinct from the CDC mechanism, yet it is responsible for the timing of cell division by virtue of its phasing of the cell division cycle. Apparently, the conclusions of Spudich and Sager (1980) and John and coworkers are either based on experiments performed under conditions in which the circadian control is not expressed (perhaps high light intensity and/or very rapid growth), or upon invalid assumptions about how circadian oscillators respond to various environmental conditions.

## Materials and Methods

### Strains and Medium

We used two different strains of *Chlamydomonas reinhardtii*: wild-type (137c, mating type +, stock no. CC-125 of the *Chlamydomonas* Genetics Center) and Bruce's period mutant *per-1* (mating type +, stock no. CC-1117 of the *Chlamydomonas* Genetics Center). The *per-1* mutant is due to a single-gene mutation of 137c so that it expresses a significantly longer free-running period of phototaxis than does wild type (Bruce, 1972, 1974). The cells of both strains were cultured photoautotrophically in high salt medium (HS medium, see Harris, 1989).

### Phototaxis Rhythms

Before assay of the circadian phototaxis rhythm, cells were grown in continuous white light (1,000 lux,  $14 \mu\text{mol m}^{-2}\text{s}^{-1}$ ) until the cultures reached a density of  $\sim 1 \times 10^6$  cells/ml. Under these conditions, this cell density corresponds to a growth stage of late log to early stationary stage. At that density, the cells were given a 12-h dark pulse to synchronize the clocks in individual cells, and then released into free-running conditions of continuous white or yellow LL ( $8.5 \mu\text{mol m}^{-2}\text{s}^{-1}$ ). The phototaxis behavior was monitored by a computerized system which has been described previously (Kondo et al., 1991; Johnson et al., 1991).

### Cell Division Rhythms

Cells were grown in two liter bottles containing HSM, bubbled with air at a flow rate of  $\sim 500$  ml/min, and stirred. Cultures were illuminated either from the top or from the side using an array of cool-white fluorescent bulbs. The highest light intensity was 6,000 lux ( $90 \mu\text{mol m}^{-2}\text{s}^{-1}$ ). To present yellow light to the cultures, the culture bottles were wrapped in a blue cut-off filter (Rosco SUPERGEL no. 10, 50% transmission at 480 nm); this filter reduced the light intensity to 80% of that measured from unwrapped white fluorescent light bulbs. In most experiments, cultures were synchronized with 24-h light-dark cycles of LD 12:12 (12 h white light followed by 12 h darkness) before release into continuous white (white LL) or yellow light (yellow LL). In some experiments, however, the synchronization protocol was modified (e.g., see Fig. 4). Cells were collected with an automatic sampling device:  $\sim 7$  ml of the culture were collected every 2 h into a test tube containing 0.5 ml of 20% neutralized formalin plus 5% KCl. The cell number was then measured with an electronic particle counter (Coulter Corp., Hialeah, FL).

Two different kinds of culturing methods were employed: batch and continuous cultures. For batch cultures, the bottles containing medium were inoculated with cells at the beginning of the experiments and the cell density increased monotonically thereafter. For continuous cultures, however, the cell suspension was exchanged for fresh medium at a rate such that the culture volume was maintained at a constant volume of one liter and the cell density was maintained at an almost constant concentration. The rate of exchange was adjusted to compensate for any changes in the average growth rate. The division rate is calculated from the rate of dilution.

We found that both wild-type and *per-1* strains display daily rhythms of daughter cell liberation. *Chlamydomonas* cells can undergo multiple fissions within one cell division cycle, so that a single mother cell can divide into 2, 4, or 8 (or sometimes even 16) daughter cells, depending upon the size of the mother cell (Donnan and John, 1983; John, 1984). This multiple-fission characteristic of *Chlamydomonas* provides a mode of growth that permits rapid proliferation of cell numbers while retaining a cycle that is attuned to the daily environmental cycle. All mitotic events occur within a few hours, and liberation of daughter cells from the mother cell occurs 2–3 h after the completion of all daughter cell cleavages in this multiple-fission alga (Howell, 1974; Harris, 1989 [p. 115]). Because daughter cell liberation occurs soon after the final daughter cell division in *Chlamydomonas reinhardtii* in a strict temporal sequence, we will hereafter use the term "daughter cell liberation" to be equivalent to the final stage of cell division.

## Results

### Phototaxis Rhythm

The phototaxis rhythm of *Chlamydomonas* satisfies the key criteria of circadian rhythms (Bruce, 1970; Johnson et al., 1992). Fig. 1 depicts a phototaxis experiment performed in

constant light (in this particular experiment yellow LL, YY) at an intensity of  $8.5 \mu\text{mol m}^{-2}\text{s}^{-1}$  ( $21^\circ\text{C}$ ). Under these conditions, the free-running phototaxis rhythm of wild type has a period of 23.4 h, while the single-gene mutation *per-1* lengthens the period to 28.2 h (Bruce, 1972, 1974; Johnson et al., 1992). The periods of these free-running circadian rhythms were not affected by the presence of 0.4% ethanol (Fig. 1), as had been assumed by McAteer et al. (1985). These phototaxis rhythms entrain to light/dark cycles (Bruce, 1970), and are also phase-shifted by single pulses of light (Kondo et al., 1991; Johnson et al., 1991, 1992) or darkness (see Fig. 4). Finally, the phototactic rhythm is temperature compensated; for cells in white LL (WW), the  $Q_{10}$  for the period is 0.88 (as calculated from the data in Table I). The fact that the  $Q_{10}$  is less than 1 means that the oscillator actually runs faster at colder temperatures. This over-compensation of the free-running circadian rhythm has been discovered in several organisms (Sweeney and Hastings, 1960; Berger et al., 1992).

### Persistence of Cell Division Rhythm

*Chlamydomonas* is an excellent organism to use for examining the interaction between circadian and cell division cycles because the relationship between the timing of daughter cell liberation and the G<sub>1</sub>, S, nuclear division, and cytokinesis stages of the CDC have been mapped under both LD and LL conditions (Chiang and Sueoka, 1967; Howell, 1972, 1974; Rollins et al., 1983; Harper and John, 1986; Harris, 1989). Therefore, the progression of the CDC can be determined from observations of the timing of daughter cell liberation.

Our data show that both wild-type and *per-1* strains display daily rhythms of cell division that are equivalent in all important ways to the corresponding phototaxis rhythms, which are well documented to be bona fide circadian rhythms. The circadian rhythm of daughter cell liberation in *Chlamydomonas* persisted in photoautotrophic culture conditions un-

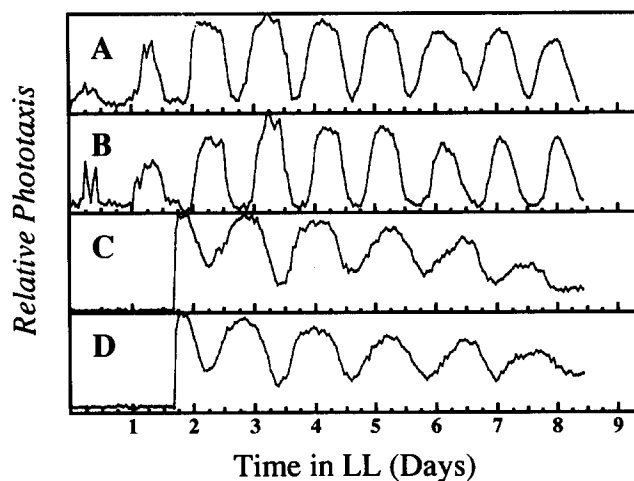


Figure 1. The circadian rhythms of phototaxis expressed by 137c and *per-1* in yellow LL ( $8.5 \mu\text{mol m}^{-2}\text{s}^{-1}$ ) at  $21^\circ\text{C}$ . (A) wild-type (137c); (B) wild-type (137c) plus 0.4% ethanol (87 mM); (C) *per-1*; (D) *per-1* + 0.4% ethanol (87 mM). Note that the wild-type cells were placed in the measuring apparatus at the beginning of data collection, while measurements of the *per-1* cells were started 1.6 d later.

der a variety of light, temperature, and culturing conditions. For example, the rhythm persisted at light intensities from  $50\text{--}90 \mu\text{mol m}^{-2}\text{s}^{-1}$  in white or yellow LL (WW or YY, respectively). These persisting rhythms of daughter cell liberation were expressed whether the culturing conditions were batch or continuous cultures (Fig. 2). The *per-1* mutant displayed circadian daughter cell liberation in batch cultures with a period of 26.9 h at  $18^\circ\text{C}$  in YY, and in continuous cultures with a period of 24.0 h at  $16^\circ\text{C}$  in WW (Figs. 2, A and B and 3 A, Table I). Figs. 2 C and 3 B show the results from *per-1* in batch cultures at 20, 22, and  $25^\circ\text{C}$ . Wild-type cells (137c) expressed a 22.6 h period at  $18^\circ\text{C}$  in YY (Table I).

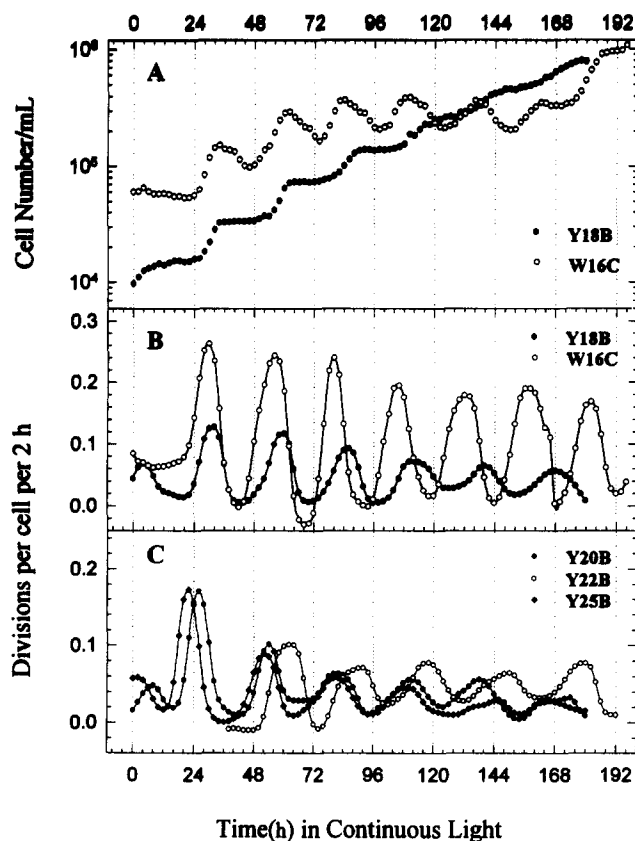
Table I. Comparison of Periods for Different Strains and Rhythms

	Period $\pm$ SEM		
	Phototaxis Rhythm*	C.D. Rhythm "Batch"†	C.D. Rhythm "Continuous"‡
Wild type (137c <sup>+</sup> )			
Yellow LL			
18°C	-----	22.6 $\pm$ 0.5 n = 2 (10)	-----
21°C	23.40 $\pm$ 0.080 n = 17	-----	-----
25°C	-----	24.8 $\pm$ 0.4 n = 3 (20)	-----
White LL			
18°C	23.55 $\pm$ 0.015 n = 53	-----	-----
21°C	23.70 $\pm$ 0.005 n = 69	-----	-----
25°C	25.67 $\pm$ 0.011 n = 53	24.9 $\pm$ 0.9 n = 1 (7)	-----
Long period ( <i>per-1</i> )			
Yellow LL			
16°C	-----	24.3 $\pm$ 0.6 n = 2 (8)	24.8 $\pm$ 0.5 n = 1 (5)
18°C	-----	26.9 $\pm$ 0.3 n = 9 (44)	30.0 $\pm$ 0.8 n = 4 (4)
20°C	-----	27.6 $\pm$ 0.5 n = 4 (16)	29.2 $\pm$ 1.0 n = 1 (5)
22°C	28.2 $\pm$ 0.24 n = 11	27.1 $\pm$ 0.8 n = 4 (18)	28.7 $\pm$ 1.8 n = 1 (3)
25°C	-----	29.0 $\pm$ 0.5 n = 6 (22)	-----
White LL			
16°C	-----	27.0 $\pm$ 1.0 n = 1 (4)	24.0 $\pm$ 0.5 n = 2 (10)
18°C	-----	-----	24.4 $\pm$ 0.7 n = 1 (5)
20°C	-----	27.4 $\pm$ 0.7 n = 1 (7)	28.3 $\pm$ 1.0 n = 1 (6)
22°C	-----	-----	24.0 $\pm$ 0.8 n = 1 (4)
25°C	-----	30.7 $\pm$ 0.5 n = 2 (14)	26.7 $\pm$ 1.8 n = 1 (3)

C.D. rhythm, rhythm of cell division (daughter cell liberation).

\* In phototaxis rhythms, *n* means the number of independent cultures whose period was measured. Each sample comprises 5–10 circadian cycles.

† C.D. rhythm, rhythm of cell division (daughter cell liberation). In cell division rhythms, *n* means the number of independent cultures whose period was measured, and the total number of cycles used for the period estimate is shown within the parentheses.

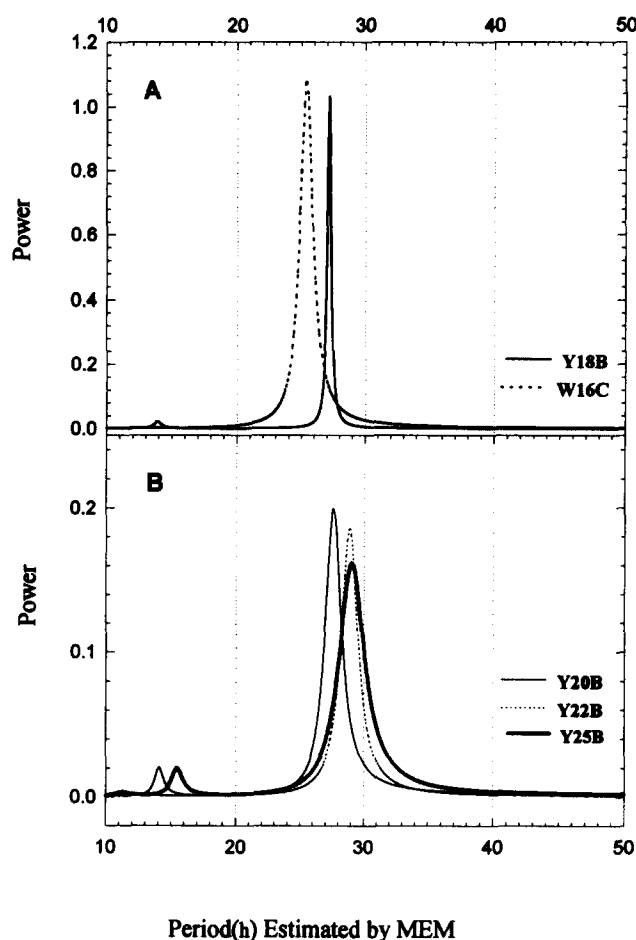


**Figure 2.** Daughter cell liberation rhythms of the period mutant *per-1* of the multiple fission algae, *Chlamydomonas reinhardtii*. (A and B) Circadian rhythm of cell division either in batch culture under yellow LL ( $50 \mu\text{mol m}^{-2}\text{s}^{-1}$ ) at a constant temperature of  $18^\circ\text{C}$  (closed circles, Y18B) or in continuous culture under white LL at a constant temperature of  $16^\circ\text{C}$  (open circles, W16C). In continuous culture, the volume was maintained at 1 l and at a narrow range of cell titer by continuous dilution. The data are plotted in two ways. The first, shown in A, is an ordinary growth curve and the second, shown in B, is the rate of cell division (divisions per cell per 2 h, which was calculated from the difference of the logarithm of the cell numbers of two successive batch samples and, in continuous cultures, from the dilution rate). (C) Comparison of the circadian cell division rhythm in batch cultures under yellow LL at 20, 22, and  $25^\circ\text{C}$  (plotted as in B). Before release into LL and assay, all the cultures were entrained to LD 12:12 and preadapted to the various temperatures for several cycles.

In general, the cell division rhythm persisted with substantial peak-to-trough amplitude for more cycles in *per-1* than in the wild-type 137c. This improved persistence allowed us to make more precise measurements of period and phase in *per-1* than in 137c. Nevertheless, 137c shared all of the circadian characteristics exhibited by *per-1* that are described in this paper.

#### Temperature Compensation

The cell division rhythm persisted in batch and continuous cultures over the range of  $16$  to  $25^\circ\text{C}$ , and clearly shows temperature compensation of period (Table I). Temperature compensation was observed for the batch cultures and for the continuous cultures in white or yellow LL (Figs. 2 and 3, Ta-

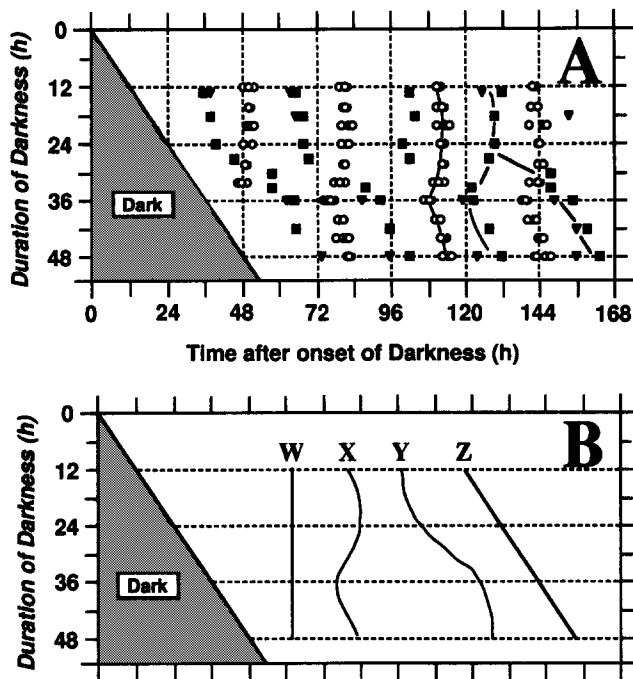


**Figure 3.** Analysis of cell division data of Fig. 2 using the maximum entropy method (= MEM; Akaike, 1969a,b). (A) is the analysis of the period of cell division of the raw data in Fig. 2 A and B, while B is the analysis of the period of cell division of the raw data in Fig. 2 C. Abscissa, period estimated by MEM; ordinate, power (significance) of period estimates.

ble I). Based on the data listed in Table I, the  $Q_{10}$  of the cell division rhythm for wild-type cells in batch culture (YY) was 0.88, and of the *per-1* mutant, the  $Q_{10}$  was 0.87 (batch cultures in WW), 0.89 (continuous cultures in WW), 0.82 (batch cultures in YY), and 0.78 (continuous cultures in YY, the last two  $Q_{10}$  values were smaller because of the abrupt shortening of the period at  $16^\circ\text{C}$ ). Therefore, like the circadian phototaxis rhythm (Table I), the cell division rhythm is over-compensated for temperature, i.e., it runs faster at colder temperatures.

#### Entrainment

To demonstrate the entrainment properties of the circadian rhythms of cell division and phototaxis, we performed a series of experiments that were essentially a repeat of Spudich and Sager's (1980) and McAteer et al.'s (1985) dark-pulse duration experiment, except that we measured the cell division rhythm for many subsequent cycles and compared its response with that of the phototaxis rhythm under the same protocol. Our data are shown in Fig. 4 A. The dark pulses clearly elicited rhythmic behavior from previously arrhythmic



**Figure 4.** Synchronization of circadian rhythms of daughter cell liberation and phototaxis to dark pulses of various durations. (A) Raw data. *Per-1* cells were grown in LL, then placed into darkness at time 0 for various lengths of time from 12 to 48 h (see ordinate). After the dark pulse was over, the cells were returned to LL at the times indicated by the diagonal line and the rhythms were subsequently assayed for up to a week. The rhythms of cell division and phototaxis were not measured from the same cultures, because the optimal illumination conditions are different between these two rhythms and because the apparatuses for measurement are mutually exclusive. In the case of the phototaxis rhythm, LL was white fluorescence light at an intensity of  $8.5 \mu\text{mol m}^{-2}\text{s}^{-1}$  (400 lux) at  $22^\circ\text{C}$ . The times of the peaks of the phototaxis rhythm are plotted in the figure as open circles. Each cluster of phototaxis peaks includes at least three, and sometimes four points (phototaxis experiment, PT159). In the case of the cell division rhythm, LL was white (inverted solid triangles) or yellow (solid squares) fluorescence light at an intensity of  $90 \mu\text{mol m}^{-2}\text{s}^{-1}$  (6,000 lux) at  $25^\circ\text{C}$ . The symbols mark the times of maximum cell division. In some cases, the triangles are obscured by nearby squares, but at some point in the free-running rhythm the phases of the triangles and squares are separated sufficiently to be distinguished. (B) Predictions of the responses of rhythms to the dark pulse protocol based on different models. Each of the curves is a prediction of the phases of the rhythm for one cycle only, in the real data, these predicted patterns should recur periodically. Curve W assumes that lights-off resets the rhythm completely, and that lights-on has no effect. Curve Z makes the opposite assumption, namely that the phase of the subsequent rhythm is set completely by lights-on. Curves X and Y are based on limit cycle modelling (Peterson, 1980) of circadian oscillators: curve X assumes a weak resetting response, whereas curve Y assumes a strong response. All predictions are based on a period of  $\sim 30$  hours, which is similar to that of *per-1* at  $25^\circ\text{C}$  (Table I).

mic populations of cells for the timing of both division and phototaxis. The phases of the rhythms show consistent relationships to the phase of the dark pulses. In other words, the dark pulses have acted as an entraining stimulus. In the case of the phototaxis rhythm, the timing of the light-to-dark transition appears to be a more important determinant of subsequent phase, since the peaks of phototaxis line-up nearly

**Table II. Multiple Regression Analysis of the Period of the Daughter Cell Liberation Rhythm Against Five Independent Variables**

Variables	Partial		
	Beta	Correlations	p-level
Batch vs. continuous cultures	-0.063	-0.063	0.480
White vs. yellow LL	0.085	0.078	0.380
Temperature	0.524	0.495	<0.001
Average cell titer	0.039	0.033	0.711
Rate of daughter cell liberation	-0.068	-0.057	0.523

The higher the absolute value of Beta or partial correlations for an independent variable, the more it contributes to determining the dependent variable, circadian period. The data in this table are derived from free-running rhythms of 30 independent cultures of *per-1*, which together include 132 cycles.

vertically, i.e., parallel to the light-to-dark transition (see the data between 108 and 120 h after onset of darkness). The data are not perfectly vertical, however, and appear to vary consistently around the vertical.

The behavior of the cell division rhythm is somewhat different. In this case, the phases of cell division peaks can be interpreted in either of two ways, as indicated by the interpretive lines on data points between hours 120 and 168; the data are intermediate between rhythmic fluctuations around the light-to-dark transition (vertical) or around the dark-to-light transition (diagonal). The interpretation of these results will be addressed in the Discussion.

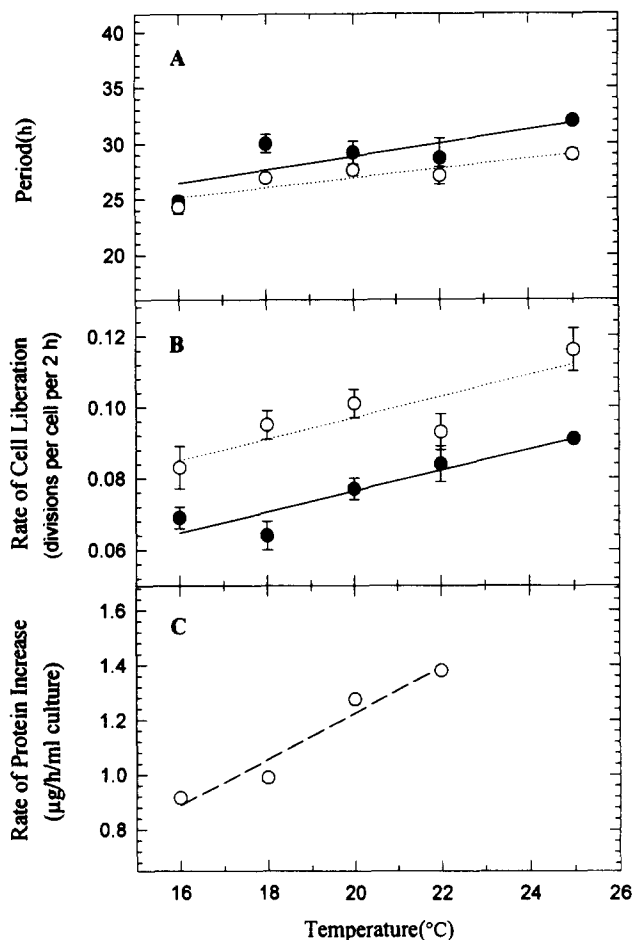
### Factors which Influence the Period of the Cell Division Rhythm

We have modulated the temperature and light intensity over a wide range in our experiments; in so doing, we have affected the growth rate. Table II shows a multiple regression analysis of period of the cell division rhythm versus five independent variables: temperature, color of the constant illumination, average cell titer, batch versus continuous culturing, and the rate of daughter cell liberation for 132 cycles of *per-1* data. Most of the independent variables analyzed and summarized in Table II (batch versus continuous culturing, color of LL, and average cell concentration) had no consistently significant impact upon the period of the cell division rhythm. Temperature does have a significant effect on period, but in the opposite direction from its effect upon growth rate. For example, Fig. 5 shows data for batch and continuous cultures of *per-1* in YY; raising the temperature significantly increases the growth rate (Fig. 5, B and C), but the period of the cell division rhythm is actually slowed (i.e., the period increases) by the temperature increase (Fig. 5 A), consistent with a  $Q_{10}$  less than 1 (Table I). Therefore, as growth rate increases, the number of daughter cells divided from each mother cell increases in this multiple-fission alga (Donnan and John, 1983; John, 1984), but the timing of that division is invariant. Thus, the period of the cell division rhythm is conserved even while generation time/growth rate varies.

### Discussion

#### Circadian Control of the Cell Division Cycle

It is clear that cell division of *Chlamydomonas* cells is con-



**Figure 5.** Growth rate and circadian period of *per-1* cells in constant yellow light (YY) at different temperatures. Closed symbols are for data obtained from continuous cultures, while open symbols are for data obtained from batch cultures. The lines are linear regressions of the data points, and the bars are SEM. (A) Circadian period versus temperature (same data as in Table I). (B) Average growth rate versus temperature. In this panel, average growth rate is expressed as the rate of cell liberation (rate of increase in cell number per every 2 h). For each datum, the increase of cell number was averaged over several days. (C) Average growth rate versus temperature. In this panel, average growth rate is expressed as the rate of increase of protein per ml of culture ( $\mu\text{g}$  protein/h/ml of culture; data from batch cultures only). In C, points are the averages of duplicate measurements. Because the cell density of batch cultures increases more rapidly at higher temperatures and because cultures at high density grow more slowly due to depletion of medium and due to decreased effective light intensity by mutual shading of cells, the data from batch cultures are calculated from those days in the middle of the time-course in which growth rate is rapid (before the growth rate slows due to high cell density).

trolled by a circadian oscillator, at least under the conditions employed in this study. The data described herein conclusively show: (a) self-sustained daily oscillations of daughter cell liberation in batch and continuous cultures (Figs. 2 and 3); (b) independence of the period length from the growth rate (Table II, Fig. 5); (c) entrainability of these rhythms by dark pulses of different durations with phase relationships predictable by a circadian limit cycle model (Fig. 4); (d) temperature compensation of the period, in fact, overcompensation ( $Q_{10}$  is between 0.8 and 0.9; Table I); and (e)

single-gene mutations that have a similar impact on the cell division rhythm as on the well-characterized circadian rhythm of phototaxis (Figs. 1 and 2; Table I). The evidence is overwhelming.

However, previous researchers presented data which they believed directly argued against circadian clock control over cell division (Spudich and Sager, 1980; Donnan and John, 1983; Donnan et al., 1985; McAteer et al., 1985; John, 1987). One piece of anti-circadian evidence was the observation that pulses of darkness delay the timing of the first division after the pulse by a fixed amount, and that the timing of cell division could be forced to follow light/dark cycles of noncircadian duration (Spudich and Sager, 1980; McAteer et al., 1985). Second, they assumed that ethanol should affect circadian oscillators, and then showed that ethanol has no effect on the timing of cell division (McAteer et al., 1985). Third, they found that temperature steps from 30 to 20°C can delay the first cell division after the transition without affecting the timing of later divisions (McAteer et al., 1985; John, 1987). Fourth, cells whose growth was slowed by deprivation of  $\text{CO}_2$  had cycles longer than 24 h, for example, cells bubbled with 0.02%  $\text{CO}_2$  divided about every 72 h (John, 1987) (note that 72 is a multiple of 24). Fifth, cell division could be synchronized by pulses of  $\text{CO}_2$  deprivation in the light without the light-dark transition presumed to be required to entrain a circadian oscillator (McAteer et al., 1985; John, 1987).

It is possible that the exclusion of a circadian oscillator from the mechanism timing cell division in *Chlamydomonas* by these other investigators is attributable to different conditions in their experiments as compared with ours, e.g., a higher light intensity which may have uncoupled the CDC from its usual circadian control. On the other hand, the discrepancy between our conclusions and those of the other workers may be due to invalid assumptions by them about how circadian oscillators behave. One example is Spudich and Sager's criticism that the timing of cell division could be forced to follow light/dark cycles of non-circadian duration. However, it is well known that circadian oscillators can entrain to a wide range of light/dark cycles, including those outside of the circadian range (Pittendrigh, 1960; Hastings, 1964; Bruce and Bruce, 1981). In the experiments of neither Spudich and Sager (1980) nor John and coworkers (Donnan and John, 1983; Donnan et al., 1985; McAteer et al., 1985; John, 1987) was an established circadian behavior (e.g., phototaxis) of *Chlamydomonas* measured to determine its response to the conditions they used to exclude a circadian contribution to the timing of cell division. Our data in Fig. 4 show that both the phototaxis and cell division rhythms entrain to dark pulses of varying durations (also see below). Another example is their assumption that ethanol will perturb circadian oscillators, but we clearly show in Fig. 1 that ethanol does not affect the circadian phototaxis rhythm of *Chlamydomonas*. Yet another example is their assumption that  $\text{CO}_2$  deprivation will not affect circadian pacemakers; there is no reason to believe a priori that  $\text{CO}_2$  deprivation will not affect the circadian clock of a photosynthetic organism whose metabolism is dependent upon  $\text{CO}_2$ .

The differing conclusions between ourselves and the other researchers could also be due to the fact that circadian rhythms must be monitored for many cycles after any perturbation. It is well known that circadian rhythms often show transient behavior in the first few cycles after light/dark or

temperature transitions (Pittendrigh et al., 1958; Pittendrigh, 1960). In fact, the significance of transients was initially misinterpreted by Kalmus (1935) in the case of *Drosophila* eclosion rhythms (Pittendrigh et al., 1958; Pittendrigh, 1960). Transient behavior could reconcile the observations of growth-dependent timing and of temperature-shift induction of delayed division by John and coworkers (McAteer et al., 1985; John, 1987) with our conclusion that a circadian oscillator is the overriding controller of cell division. In Fig. 4 A, note that in the first cycle after the lights-on transition, the phases of the cell division rhythm appear to line up diagonally (as did the data of Spudich and Sager [1980] and John and coworkers [Donnan and John, 1983; McAteer et al., 1985]), suggesting strong resetting. After a few cycles of free-run, however an intermediate response is observed, as described above. This change is typical of transients in the circadian literature. In fact, a recent report on the circadian rhythm of cell division in *Euglena* describes such transients after perturbations (Carré and Edmunds, 1993). Therefore, because Spudich and Sager (1980) and John and coworkers almost always monitored cell division for only one, or at most two, cycles after perturbations, their conclusions could be based on transient phenomena.

#### *Period of Cell Division Rhythm Is Independent of Growth Rate*

A piece of evidence against the circadian gating model in *Chlamydomonas* has been the observation that the timing of cell division is dependent upon the growth rate (= doubling time) and that it can assume periods which are quite different from circadian periods (Donnan and John, 1983). Although not a specific critique of the *Chlamydomonas* situation, Vaulot and Chisholm (1987) warned that apparent circadian division timing can be simply due to generation times that are close to circadian periods without a circadian pacemaker being directly involved in the timing of cell division. We think their explanation is the most rigorous modelling of the idea that many cell cycle workers have in mind when they have criticized the interpretation that the circadian clock gates cell division. On the basis of their modelling, Vaulot and Chisholm (1987) state that "the best criterion for distinguishing between the clock-controlled and transition point-controlled models of the cell cycle is the free-running behavior. In cells which are not clock controlled, the rhythm will not persist indefinitely, and the period length will be equal to the mean generation time of the population." Therefore, their model can be tested by the very simple experiment of assaying self-sustained cell division cycles for many cycles within a range of conditions over which the generation time varies; if the period of cell division is always directly correlated with generation time, then a non-circadian timing mechanism is probable.

It is precisely these criteria in which circadian clock control of division is so obvious in *Chlamydomonas*. The free-running rhythm does persist indefinitely (given that the rhythm will slowly damp in a population of cells with slightly different periods). Most importantly, the period is restricted within a circadian range which is independent of the growth rate/generation time (Table II, Fig. 5). We have modulated the temperature and light intensity in our experiments so that we have affected the growth rate. As growth rate changes, the

number of daughter cells divided from each mother cell changes in this multiple-fission alga (Donnan and John, 1983; John, 1984), but the timing of that division is invariant. This independence not only excludes the idea that the circadian cell division rhythm is a manifestation of the population average of generation times close to 24 h, it also reveals that the circadian clock is a mechanism separate from and controlling the CDC timer. Although there have been previous reports of no apparent correlation between average generation time and circadian period (Bruce, 1970; Anderson et al., 1985; Edmunds, 1988), none of those studies shows the statistical evaluation of the data that we have included in Table II.

#### *Response to Dark Pulses*

One of the criticisms of the circadian gating model has been based on the response of cell division to dark pulses of various durations. Spudich and Sager (1980) and McAteer et al. (1985) reported that the first cell division burst after dark pulses of various durations occurs at a fixed interval after lights-on (equivalent to curve Z in Fig. 4 B). Both groups use this result as a key support for their noncircadian models for CDC timing in *Chlamydomonas*. They apparently assume that the phase of circadian clocks will be set by the lights-off transition independently of the duration of the dark pulse (curve W in Figure 4 B).

Because circadian clocks are nonlinear oscillators, however, the means by which their phase is determined is considerably more complex. The responses of circadian oscillators to the dark pulse experiment can be predicted based on limit cycle models which were originally designed to explain circadian behavior of insects (Winfree, 1970; Pavlidis, 1973; Peterson, 1980). Extensive evidence suggests that circadian oscillators are limit-cycle oscillators (Winfree, 1970; Pavlidis, 1973; Peterson, 1980), including data obtained from *Chlamydomonas* (Johnson and Kondo, 1992).

Specifically, we would predict that a circadian oscillator will respond to the dark pulse protocol in the following fashion: for relatively dim LL (weak stimuli), the phase of the clock will be only slightly shifted by lights-on (curve X in Fig. 4 B; see Fig. 4, a-d in Peterson, 1980), whereas for bright LL (strong stimuli), the phase of the clock will be dramatically shifted by lights-on, so that the timing of subsequent events could appear to be a fixed interval from the lights-on transition (curve Z in fig. 4 B), but there should still be an oscillation around this fixed interval which may or may not be experimentally detectable (curve Y in Fig. 4 B; see Figs. 3 and 4 of Peterson, 1980). For LL of intermediate intensity an intermediate response is expected: subsequent events do not occur at a simple fixed interval, but their phasing is significantly reset in a phase-dependent manner by lights-on (somewhere on a continuum between curves X and Y in Fig. 4 B).

Do the rhythms of phototaxis and cell division in *Chlamydomonas* conform to these predictions for circadian limit-cycle oscillators? In the case of the phototaxis rhythm, the intensity of LL was relatively weak ( $8.5 \mu\text{mol m}^{-2}\text{s}^{-1}$ , 400 lux). Therefore, we would expect this rhythm to respond to the dark pulse protocol as in curve X of Fig. 4 B: as shown in Fig. 4 A, this prediction is upheld by the data (this pattern indicates that lights-off has synchronized the oscillators to a



certain phase, and that lights-on later provides a weak additional phase shifting effect).

In the case of cell division, both our data and the data of Spudich and Sager (1980) and John and coworkers (Donnan and John, 1983; McAteer et al., 1985) are entirely consistent with a limit-cycle model. They used very bright light ( $200\text{--}240\ \mu\text{mol m}^{-2}\text{s}^{-1}$ ), and their data conforms to the limit-cycle prediction of strong resetting (curve Z). In our studies of cell division, the intensity of LL was much higher ( $90\ \mu\text{mol m}^{-2}\text{s}^{-1}$ , 6,000 lux) than that used in the phototaxis measurements ( $8.5\ \mu\text{mol m}^{-2}\text{s}^{-1}$ , 400 lux), but still dimmer than that used by Spudich and Sager (16,000 lux,  $240\ \mu\text{mol m}^{-2}\text{s}^{-1}$ ) and John and coworkers ( $200\ \mu\text{mol m}^{-2}\text{s}^{-1}$ ). As predicted by the limit-cycle model, the dark pulses in our study provoke an intermediate response (see the interpretive lines on data points between hours 120 and 168 in Fig. 4 A) which wavers between the predictions of curves X and Y in Fig. 4 B (see Figs. 3 and 4, e and f, in Peterson, 1980). Therefore, all the available data support the hypothesis that cell division in *Chlamydomonas* is gated by a circadian limit-cycle oscillator.

### Possible Mechanisms of Circadian Control

The timing of cell division in *Chlamydomonas* is clearly under circadian control. Does this exclude a contribution of transition points (Spudich and Sager, 1980) or commitment timers (Donnan and John, 1983)? Not at all. The biochemical mechanism of circadian gating is totally unknown and it is quite possible that the gate itself may be a kind of transition point, or that the circadian clock may trigger a commitment timer. Therefore, a sizer and timer model (Donnan and John, 1983) for controlling the timing of cell division in *Chlamydomonas* is possible, but the over-arching command timer that determines the period must be a circadian one, at least, under the conditions of our study.

A sizer can interact with a circadian timer of cell division in the same way as proposed to explain the circadian timing of eclosion of *Drosophila* pupae (Pittendrigh, 1960): the gating hypothesis. This model is that a circadian pacemaker opens a gate every cycle which allows all cells over a certain size to commit to division and proceed with replication and subsequent events of division (Sweeney and Hastings, 1958). In a multiple-fission cell like *Chlamydomonas*, the size-sensing mechanism determines not only whether a cell is big enough to proceed with replication at the clock-specified gate, but it also determines how many rounds of replication and cytokinesis should occur in each cycle to conserve a particular nucleus/cytoplasm ratio.

Models of circadian gating of the CDC are usually conceived as systems composed of a self-sustained circadian oscillator that regulates the CDC process, which in turn can be either an independent oscillator or an hourglass timer. Yet another possibility is that the oscillating mechanism of the CDC and the circadian clock are intrinsically intermeshed (Edmunds, 1988; Honma and Hastings, 1989). The data of Table II and Fig. 5, which show the independence of period versus liberation rate, indicate that the latter model (i.e., intertwined oscillators) is less likely to be true for *Chlamydomonas* than the model in which a circadian oscillator gates the CDC processes. The central point is that the cell division behavior reported herein conforms to every criterion of circadian rhythmicity, and therefore, by definition, a circadian

oscillator is the overriding controller of cell division in *Chlamydomonas reinhardtii* as it is in many other organisms.

Despite the recent explosion in our knowledge about the biochemical mechanism of cell division (Dirick and Nasmyth, 1991; Nurse, 1991; Tyson, 1991; Murray and Hunt, 1993; Pines, 1994), we understand CDC control best in yeast and in rapidly dividing embryos. In other cells, the number of components (especially cyclins and CDKs) and their interactions has proliferated so that the control pathways are labyrinthine. Much remains unknown about the control of cell division in somatic cells of multicellular organisms, or even in unicellular organisms like *Chlamydomonas* (Murray and Hunt, 1993; Pines, 1994). Models have been proposed to explain the biochemical basis of circadian gating of cell division in *Euglena* (Carré and Edmunds, 1993), but we still do not know (even phenomenologically) the nature of the gating event. For example, does the circadian clock permit (or forbid) a commitment timer to proceed? How many checkpoints are controlled by the circadian oscillator? At what circadian phase does the event occur? An adequate understanding of cell division must inevitably include an understanding of the key role played by circadian pacemakers. In the temporal dimension we have much to learn.

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