Circadian gating of cell division in cyanobacteria growing with average doubling times of less than 24 hours

(biological clock/Synechococcus/flow cytometry)

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ABSTRACT To ascertain whether the circadian oscillator in the prokaryotic cyanobacterium Synechococcus PCC 7942 regulates the timing of cell division in rapidly growing cultures, we measured the rate of cell division, DNA content, cell size, and gene expression (monitored by luminescence of the PpsbAI::luxAB reporter) in cultures that were continuously diluted to maintain an approximately equal cell density. We found that populations dividing at rates as rapid as once per 10 h manifest circadian gating of cell division, since phases in which cell division slows or stops recur with a circadian periodicity. The data clearly show that Synechococcus cells growing with doubling times that are considerably faster than once per 24 h nonetheless express robust circadian rhythms of cell division and gene expression. Apparently Synechococcus cells are able to simultaneously sustain two timing circuits that express significantly different periods.

Circadian rhythms and cell division cycles (CDCs) constitute two important cyclic biological systems. These rhythmic phenomena are usually not independent in unicellular eukaryotic organisms, where the circadian system often controls the timing of the cell division cycle (1–3). The nature of this control appears to be via “gating” of cell division such that the circadian oscillator specifies certain phases in which cell division is “allowed” to occur and other phases in which it is “forbidden,” even if the cells have attained sufficient size (1). Thus, the circadian oscillator acts in addition to other “checkpoints” in determining when cells divide.

Recently, it has been discovered that prokaryotic cyanobacteria express authentic circadian rhythms of gene expression (4–10). We wondered whether these prokaryotes would exhibit circadian control over the timing of cell division, especially in cases where the doubling times are much shorter than 24 h. In unicellular eukaryotic organisms, the relationship between cell division and circadian expression has come to be encapsulated in the so-called “circadian-infradian rule,” which states that circadian rhythms are expressed only in cells that are dividing once per day or more slowly, i.e., in the “infradian” mode; in cells dividing more rapidly than once per day, cellular processes are thought to become uncoupled from circadian oscillator control (1, 11). This rule implies that there is an interdependency between these two timing circuits such that when cell division is more rapid than once per day, the cells are unable to maintain an independent circadian oscillation. It has been suggested therefore that a circadian clock that provides temporal programming is only adaptive to organisms whose generation time is as long or longer than a day (12).

There have been a few investigations with Synechococcus species of cyanobacteria indicating circadian rhythms of cell division (4, 13), but these have been limited to strains and/or growth conditions that allowed only relatively slow growth (doubling times slower than once per 24 h). We decided to reexamine these issues using a strain (Synechococcus sp. strain PCC 7942) that exhibits bona fide circadian rhythms (6) and can grow rapidly. Our goal was to test whether cyanobacteria that are dividing more rapidly than once per 24 h nevertheless exhibit circadian regulation over cell division. We achieved this objective using Synechococcus cultures that were continuously diluted with fresh medium to attain cultures that were maintained at an approximately equal cell density. The results we report herein indicate that the circadian clock controls the timing of cell division, cell size, and the average cellular DNA content, even in cyanobacterial cells that are growing with doubling times that are much faster than once per 24 h.

MATERIALS AND METHODS

Synechococcus sp. PCC 7942 Strains. The strains used in this study were wild-type (PCC 7942), AMC149, SP22, and LP27. The strain AMC149 is a bacterial luciferase reporter strain that contains a PpsbAI::luxAB translational fusion integrated into a neutral site of chromosome of the wild-type strain (6). The ppsbAI gene encodes the D1 protein of photosystem II. Strains LP27 and SP22 are circadian period mutants isolated after chemical mutagenesis of AMC149 (14).

Culture Conditions. Cells were cultured phototrophically at 30 ± 0.5°C in BG-11 medium in 1-liter bottles as described (6). For growth of strains AMC149, SP22, and LP27, the culture medium was supplemented with spectinomycin (40 μg/ml). For growth of wild-type Synechococcus cells, BG-11 medium was used without spectinomycin. Cell suspensions were bubbled with air and stirred. Illumination was provided from the side of the culture bottle by a cool-white fluorescent light bulb standing parallel to the culture bottle. The light intensity in all experiments reported herein was 125 μE/perm² per s. Cultures were synchronized with light-dark cycles of 12 h of light followed by 12 h of darkness (LD 12:12) and then released into free-running conditions of continuous light (LL).

For continuously diluted cultures, cells were initially grown in LD 12:12 in a batch culture. Cultures were then released into LL and continuous dilution of culture was started. Cell cultures were maintained at an approximately constant cell density and volume by replacing cell suspension at a constant rate with fresh medium. The fresh medium was continuously supplied to 500 ml of cell suspension in a culture bottle using a peristaltic pump at a flow rate of about 30 ml/h. Therefore, approximately 30 ml of cell suspension was withdrawn from the culture each hour and replaced with 30 ml of fresh medium, resulting in a rate of dilution that was almost constant at about 30/500 per h (0.06 h⁻¹). A portion of the cell suspension that was removed from the culture was automatically saved into one of

Abbreviations: CDC, cell division cycle; LD, light-dark cycle; LL, continuous light; FALS, forward-angle light scatter; DT, doubling time.

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a series of test tubes in a fraction collector for measurements of cell number and luminescence and for flow cytometric analyses (see below).

**Monitoring Cell Division.** The portion of the cells for determination of cell number was automatically collected and diluted 1:1 with iodine solution (iodine at 2 mg/ml and potassium iodine at 1 mg/ml) so that cells were fixed immediately. The samples were kept at room temperature or 4°C. The cell numbers in the suspensions were measured with an electronic particle counter (model ZF, Coulter) with a 30-μm diameter aperture tube. Periods of cell division rhythms were calculated by the maximum entropy method (15, 16). Microscopical examinations of cells from rapidly dividing cultures indicated that each mother cell divided into only two daughter cells (i.e., clusters of two, but not of three or more, daughter cells were observed from dividing cultures).

**Flow Cytometry.** For flow cytometry, a portion of the culture (3 ml) was saved every 3 h and centrifuged at 20,000 × g (Sorvall SS-34, 12,700 rpm) for 10 min at 4°C. The pellet was resuspended in 5 ml of 70% ethanol and then centrifuged again as above. The resulting pellet was resuspended in 3 ml of absolute ethanol and stored at −15°C. Aliquots of the ethanol-fixed samples were resuspended in phosphate-buffered saline (pH 7.5) and stained with the DNA-specific fluorochrome Hoechst 33342 (final concentration = 0.5 μg/ml). Stained samples were analyzed on a Coulter EPICS-753 flow cytometer using 150- to 300-mW UV excitation and a “biosense” flow cell. Hoechst fluorescence, which is proportional to DNA content, was measured between 408 and 470 nm; phycocyanin fluorescence, used to unambiguously identify cyanobacterial cells, was measured through a 680-nm bandpass filter (40-nm band width); and forward-angle light scatter (FALS) was measured with a custom-installed photomultiplier tube through a 320-nm bandpass filter. All parameters were collected as linear pulse-integrated values. A control sample, derived from a stationary-phase *Synechococcus* PCC 6301 culture, was repeatedly analyzed along with each batch of experimental samples and served as a staining standard for those samples. Approximately 30,000 cells were measured for each determination of DNA content and FALS. Genome numbers were assigned to peaks in the DNA frequency distributions as described (17). FALS data were normalized to the control sample and expressed as relative values.

The relationship between FALS and cell size is complex and depends on the refractive index of the cells and the optical configuration of the instrument (18). For a given cell type analyzed on a given instrument, however, it is reasonable to assume that changes in FALS reflect changes in cell size, at least qualitatively (19), as we confirmed microscopically.

**Measurement of Luminescence.** Every 3 h, 1 ml of culture was transferred into a 20-ml glass scintillation vial, and then a capless 1.5-ml microcentrifuge tube containing 300 μl of 10% n-decanal in soybean oil was placed in the vial. The vial was sealed to allow the n-decanal vapor to equilibrate and then immediately placed into a luminometer apparatus and incubated at room temperature (21 ± 1.5°C) in darkness. After a 30-min incubation, light emission from the culture was measured with the photomultiplier tube (model 931B, Hamamatsu Photonics, Hamamatsu City, Japan) of the luminometer. Light emission from samples was calibrated to a standard curve determined with C-14 standards (20).

**RESULTS**

**Circadian Rhythms of Cell Division and Luminescence in PCC 7942 and AMC149.** As shown in Figs. 1 and 2, *Synechococcus* cells growing with doubling times less than 24 h displayed circadian rhythms of cell division in continuous-dilution cultures. Fig. 1 shows three cultures diluted continuously with fresh medium to maintain the culture in exponential growth. The doubling times for these cultures, as calculated from the cell numbers and the dilution rate are 10.3 h for the PCC 7942 culture, 11.8 h for the upper AMC149 culture, and 10.9 h for the lower AMC149 culture. Under these conditions, cell division occurred in the subjective day and late subjective night (increasing or constant cell numbers in the face of dilution), while the cells slowed or stopped dividing early in the subjective night (decreasing cell numbers). The periods of the cell division rhythms in these cultures were 24.0 h (upper trace), 25.2 h (middle trace), and 24.2 h (lower trace). The two
entained by the prior LD cycles, a diagnostic characteristic of circadian rhythms. The upper AMC149 trace has also been plotted as a logistic growth curve (Fig. 1B) and as an instantaneous rate of increase in cell number as compensated for the rate of medium dilution (Fig. 1C).

Because AMC149 is a transformed strain that carries a foreign gene set in its chromosome, it is conceivable that its growth characteristics could be altered. A previous study found no evidence for such an effect (9), and the clear circadian rhythm expressed in the wild-type strain Synechococcus sp. PCC 7942 in this experiment likewise indicates that the AMC149 rhythms reported in this paper are not artifacts of the genetic alteration in AMC149.

Fig. 2 illustrates another continuous-dilution experiment in which cell number, luminescence, cell size (as FALS) and DNA content per AMC149 cell were monitored for several days. After growth in LD 12:12 (the last 1.5 days are shown in Fig. 2), the AMC149 culture was placed in LL and continuously diluted to maintain cell concentration between 2.6 and 4.5 x 10^7 cells per ml for more than 96 h. As in the experiments shown in Fig. 1, this continuous-dilution culture maintains a circadian rhythm of the timing of cell division in LL (period = 23.3 h), with maximal division rates in the subjective day/late subjective night and slow or arrested division early in the subjective night (Fig. 2B). The raw data in Fig. 2B have been recalculated in Fig. 2C to include the rate of dilution so as to present an instantaneous rate of increase in cell number.

Luminescence and DNA content per cell in the continuous-dilution culture of Fig. 2 also exhibited circadian rhythmicity. Luminescence cycled with peaks in the early subjective day and troughs in the early subjective night (Fig. 2A), indicating a rhythm of psbAl gene expression as has been observed (9). Average DNA content per cell also showed a distinct circadian rhythm (Fig. 2E). Synechococcus cells are known to contain multiple copies of the chromosome (17). These changes in mean cellular DNA content reflect changes in the relative proportion of cells in the population containing 2, 3, 4, 5, etc. genome equivalents (Fig. 3A). In this experiment, the average number of chromosomes per cell oscillated between 3.9 and 5.4 genomes per cell, with peaks in the early subjective night phase and troughs in the late subjective day phase (Fig. 2E).

Average cell size as reflected by average FALS showed an oscillation that paralleled that of cellular DNA content (Fig. 2D). We obtained similar results by microscopically measuring cell size directly (data not shown). In addition, there was a significant positive correlation between mean FALS and DNA content in this and other experiments (Fig. 3B), indicating that larger cells do indeed have higher DNA content.

To determine whether the rate of new DNA synthesis also exhibited a circadian rhythm, the DNA content per cell and the rate of medium dilution were used to calculate the instantaneous specific rate of increase of DNA and plotted in Fig. 2F. The data have an average slope of zero, suggesting that DNA synthesis initiates and proceeds at a constant rate, even though the cells are dividing rhythmically. Such uncoupling of DNA synthesis and cell division has been hypothesized to occur in other species of Synechococcus as well (17, 21). One interpretation of these data is that the cells are attempting to maintain a constant cellular chromosome content under conditions in which the rate of cytokinesis is slightly faster than the rate of DNA replication. In such a case, there must be an interval in which cytokinesis is slowed to allow the DNA content per cell to catch up. Apparently the circadian clock controls the timing of this slowdown interval. This same logic may apply to cell growth as well: if new biomass is produced at a constant rate, we would expect cell size to oscillate such that the cells will be largest at the end of the slowdown interval, just before cytokinesis begins again. This prediction is upheld by the FALS data in Figs. 2 and 4. The strong correlation between
**DISCUSSION**

In rapidly growing batch cultures, circadian rhythms of gene expression (luminescence and mRNA abundance) have been observed in the AMC149 experiment. Line shows the least-squares regression taking DNA and FALS as the dependent and independent variables, respectively ($r = 0.89; P < 0.01$).
observed in cultures of the AMC149 strain of *Synechococcus* that correspond with the expectation of a circadian rhythm of luminescence in each cell and an exponential increase in the number of cells in the culture (T. Kondo, T.M., N.V. Lebedeva, S. Aoki, M. Ishiura & S.S. Golden, unpublished data). We wanted to determine whether that result could be obtained with continuous cultures in which the cell concentrations were precisely measured to discern circadian gating. In this study, we used continuously diluted cultures to maintain a constant growth environment. In batch cultures, nutrient levels are constantly depleted over the course of the experiment. More importantly, the growth of batch cultures entails a continual decrease in the effective light intensity as the heavily pigmented cells "self-shade" each other. This self-shading can be very significant in *Synechococcus* cultures at cell densities greater than an OD\textsubscript{750} of 0.1 (23), which corresponds to a cell concentration of about $3 \times 10^7$ cells per ml. Consequently, the effective light intensity decreases progressively in batch cultures at densities above $3 \times 10^7$ cells per ml, which in turn progressively restricts the only source of energy available to these cells.

Our data clearly show that *Synechococcus* PCC 7942 cells growing with doubling times that are considerably faster than once per 24 h nonetheless express robust circadian rhythms of cell division (exemplified by "slowdowns" of division rate), DNA content, and *psbA1* gene expression (monitored by luminescence of the *PsbA1:luxAB* reporter). A previous study (10) reported that the circadian clock globally regulates promoter activity in *Synechococcus* PCC 7942. Possibly some of the promoters that we have observed to express circadian rhythms of gene expression are most directly related to cell division; in other words, that gene expression which might be most directly involved in cytokinesis exhibits circadian properties because the timing of cell division is dictated by the circadian oscillator.

Apparently *Synechococcus* cells are able to keep track of two timing circuits that are partially independent of each other. More specifically, the circadian clock—as gauged by the timing of cell division and luminescence—seems completely independent of the cell division cycle, since its period is essentially the same in cultures with quite different doubling times (T.M., unpublished observations). On the other hand, the cell division cycle does not seem to be completely independent of the circadian clock, since there are phases of the circadian cycle in which cell division slows or stops, implying some inhibitory gating control of the circadian clock over the CDC timer.

These observations contradict the usual formulation of the circadian-infradian rule. What does this mean? It might be argued that the relationship between the circadian clock and the CDC timer is fundamentally different in eukaryotic unicells versus cyanobacteria. On the other hand, the propositions of the circadian-infradian rule have only been adequately tested in a few eukaryotic organisms (11). It has therefore been instructive to test the implications of the rule in a different type of organism—in this case, a prokaryote—to show that some organisms are able to maintain circadian timing in the face of rapid cell division cycling.

Fig. 5 illustrates a perspective on cell division timing that suggests that the regulation in *Synechococcus* might not be fundamentally different from that in eukaryotic unicells. The figure illustrates that each of three organisms have phases during their circadian cycle in which division is "allowed" or "forbidden." The allowed phases are those during which a "gate is open," or in other nomenclature, during which the circadian checkpoint is satisfied. The forbidden phases are those in which the "gate is closed," or in which the circadian checkpoint is not satisfied. In the case of *Euglena*, there is only a brief allowed "gate," and during this time, a single mother cell may divide into only two daughter cells in each cycle. The gating in *Synechococcus* is not so very different conceptually except that it is the forbidden zone (the phase of division "slowdowns") that is brief. In *Synechococcus* PCC 7942, division of the mother cell may occur up to four times in a daily cycle (for a doubling time of 6 h). The eukaryotic multiple-fission alga *Chlamydomonas* is an intermediate case; the allowed zone is brief, but several cycles of nuclear replication and cytokinesis can occur during this brief interval. During that allowed zone, the period of the nuclear replication cycle may be as rapid as 0.5-1 h. A single mother cell can divide into as many as 32 daughter cells per cycle. Consequently, even though the cell division cycle is gated by a circadian clock in these cells (3), the data from *Chlamydomonas* show that the period of the circadian cycle is not required to mesh with the period of a single round of nuclear replication. Thus, while there may be environmental factors that act to limit cell division to only certain phases of the daily cycle (12), there may not be mechanistic constraints that prevent cells from keeping track of two timing circuits with different periods.

These considerations suggest that perhaps the circadian-infradian rule is not as accurate a descriptor of the relationship between the circadian oscillator and the cell division cycle as we once thought, even in eukaryotic unicells. In addition to the case of *Chlamydomonas*, which was discussed in the preceding paragraph, there has been other evidence that the circadian-infradian rule might not always apply to eukaryotic unicells.
some marine diatoms have been observed to exhibit daily rhythms when dividing more rapidly than once a day (24) and also to entrain to LD cycles with variable phase relationships that are difficult to reconcile with the simplest version of the circadian-infradian rule (25). Whether or not we will ultimately determine that eukaryotic cells are also able to maintain circadian expression and CDC gating in cells growing with a doubling time of less than 24 h, our data from *Synechococcus* clearly indicate that these cyanobacteria are able to maintain circadian expression in rapidly dividing cells and further that this daily clockwork exerts some gating control over the timing of division by interposing brief slowdown zones that recur with a circadian periodicity.

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