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chapter 16

Circadian control of cell division in unicellular organisms

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Cell division cycles and circadian rhythms are major periodic phenomena in organisms. Circadian oscillators control biochemical, physiological, and behavioral events in a wide range of living systems including almost all eukaryotes that have been tested and some prokaryotes–in particular, the cyanobacteria. Gating of cell division is one of the key processes that has been reported to be regulated by circadian clocks in many organisms. We survey studies of the circadian control of cell division in eukaryotic microorganisms and introduce recent progress on understanding the interaction between circadian rhythms and cell division cycles in cyanobacteria.

Recent developments in non-equilibrium physics and chemistry show that the "arrow" of time is a source of order (1). Life is only possible in a universe that evolves spontaneously towards increasing complexity; self-reproduction, which is one of fundamental characteristics of non-equilibrium living systems (2), is inevitably rhythmic. Cell division is one such rhythmic program for self-reproduction whose period can depend upon many parameters, including developmental and environmental factors. Some of these environmental factors are a result of astronomical movements of the planet Earth and its satellite the Moon that cause daily, tidal and seasonal changes on the Earth. It is reasonable that natural selection allowed the evolution of an internal rhythm to co-ordinate metabolic networks and/or hierarchical structures in living systems so that they are optimally adapted to the rhythmic changes of environment.

Daily rhythms in biochemical, physiological and behavioural phenomena have been reported in many organisms from algae to molds to plants to animals, including humans (3-5). These rhythms continue to oscillate in constant environmental conditions devoid of time cues with a period that is close to–but not exactly–24 hours. In addition, the period of these rhythms is not significantly altered when tested at different ambient temperatures within the physiological range. Finally, when the organisms are subjected to the environmental cycles of light/dark and temperature, they entrain to the environmental cycle so that their period becomes equal to the period of the environmental cycle. These three characteristics–persistence in constant conditions, temperature compensation, and entrainment are diagnostic for the endogenous biochemical oscillators that have come to be called "circadian clocks". These clocks appear to be limit cycle oscillators. Scientists believe that these clocks rhythmically control many cellular events so as to adapt the organism to daily environmental changes. In this article, we review the evidence that two major biological rhythms, namely the cell division cycle (CDC) and circadian rhythms, interact within living organisms.

CIRCADIAN RHYTHMS OF CELL DIVISION IN EUKARYOTIC MICROORGANISMS

In many unicellular organisms, circadian rhythms of the timing of cell division have been reported. The marine dinoflagellate *Gonyaulax polyedra* was the first organism for which persuasive evidence of circadian cell division was demonstrated (6, 7). As shown in Figure 1A (right curve), *Gonyaulax* divides at the end of darkness when the cells are in a 12-h light and 12-h dark cycle (LD 12:12). When cells grown in LD 12:12 are transferred to continuous light conditions (LL), the periodicity of cell division continues. In continuous bright light, the rhythmicity of cell division persists for at least 6 days, whereas in continuous dim light it continues for at least 14 days (Figure 1B). The rhythms have almost the same period (approximately, but not exactly, 24 h) at different temperatures in populations of cells dividing with different generation times. Those observations clearly indicate that the rhythm is under the control of a circadian clock in *Gonyaulax*. Another famous example is that of *Euglena* (4, 8, 9). As shown in Figure 1C (right curve), *Euglena* cells dividing in constant light display obvious "gating" of cell division by the circadian clock: cells are allowed to divide in the night (gate is open), but not in the day (gate is closed). Other eukaryotic unicellular organisms for which circadian gating of cell division has been demonstrated include *Paramecium* (10, 11), *Tetrahymena* (12), and *Chlamydomonas* (13, 14).

However, when populations of eukaryotic cells are grown in conditions that allow them to divide with a doubling time of less than 24 hours, the daily periodicity of division disappears (Figure 1A, left curve for *Gonyaulax*, Figure 1C, left curve for *Euglena*). From observations in eukaryotic microorganisms (especially *Gonyaulax*, *Euglena* and *Tetrahymena*), an empirical rule called the "circadian-infradian rule" was introduced (4, 15-17). The circadian-infradian rule stated that circadian

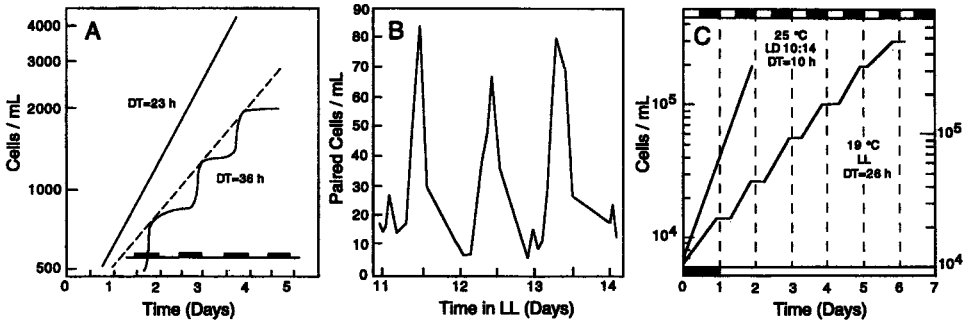

Figure 1. Entrainment of the circadian rhythm of cell division and its persistence under constant conditions in the eukaryotic unicells *Gonyaulax* and *Euglena*. (A) Increase of cell density in a population of *Gonyaulax polyedra* in continuous light (DT = 23 h curve) and in 24-hour light-dark cycles (DT = 36 h curve). (B) Circadian rhythm of cell division in *Gonyaulax* persists for at least for 2 weeks under continuous dim light. Cells were grown in LD 12:12 and then transferred to continuous dim light. The numbers of paired cells (i.e., cells that are in the last stages of cytokinesis) in the population on 12th, 13th and 14th day in continuous dim light are traced. (C) Population growth of a photosynthetic mutant of *Euglena gracilis* grown in LD 10:14 at 25 °C (upper abscissa shows light treatment) and in continuous LL at 19 °C (lower abscissa shows light treatment; these cells had been in constant darkness prior to the transfer to LL). For all panels: DT = doubling time; LL = constant illumination; black bars on abscissae indicate intervals of darkness. (Panels A and B were modified from ref. 6, and panel C was modified from ref. 9).

rhythms are expressed only in cells that are dividing once per day ("circadian mode") or more slowly ("infradian mode"). In the "ultradian growth mode" (i.e., cells dividing more rapidly than once per day), the circadian oscillator was postulated to be either (i) speeding along with the same period as the CDC oscillator, (ii) running normally with an approximately 24 h period but no longer controlling the cell division cycle and other cellular processes, or (iii) stopped. The rule implied that there is an interdependency between the CDC and circadian timing circuits such that when cell division is more rapid than once per day, the cells are unable to maintain an independent circadian oscillation.

While there have been some results with eukaryotic unicells that did not match the predictions of the circadian-infradian rule (18-21), the rule became generally accepted. More recently, the rule was thought to be consonant with molecular models of circadian clocks, which propose that negative feedback regulation of clock gene expression is a crucial cog in the circadian clockwork. In those models, temporal control over intercompartmental transport of the products of clock genes is essential, and it would therefore be expected that the processes of cell division would affect circadian time-keeping. Therefore, the tenets of the circadian-infradian rule seemed to fit with models of the circadian clock mechanism in eukaryotic organisms.

CIRCADIAN RYTHMS IN CYANOBACTERIA

While the circadian-infradian rule may still hold for eukaryotic organisms, recent experiments with prokaryotic cyanobacteria have proven that circadian clocks *can* be expressed in cells dividing in an ultradian mode. Previously called blue-green algae or cyanophyta, cyanobacteria are the largest group of oxygenic photosynthetic bacteria and physiologically they resemble eukaryotic green algae and higher plants. Fossil records indicate that cyanobacteria appeared on Earth over 2 billion years ago, and it is believed that cyanobacteria were the largest contributor to the transformation of Earth's environment, namely the change in atmospheric oxygen levels from 0.1% to 20%. The predominance of oxygen in the atmosphere enabled (i) the formation of an ozone layer in the stratosphere that helped to protect living organisms from ultraviolet light, and (ii) the evolution of oxygen as a terminal acceptor of energy metabolism (22). Cyanobacteria are gram-negative bacteria that have nucleoids, or non-membrane-bounded fibrils of DNA, but no nuclear envelope. The ancestors of cyanobacteria have been suggested to be the origin of the plastids that became the chloroplasts of plant cells as permanent symbionts (23).

Until about ten years ago, it was a dogma in the circadian rhythm field that prokaryotes did not have circadian oscillators (24). Reports suggesting the existence of circadian rhythms in cyanobacteria began to surface in the mid-1980s. In the cyanobacteria *Oscillatoria* and *Synechococcus* spp., activity of the primary enzyme responsible for nitrogen fixation (nitrogenase) was observed to cycle in continuous illumination without any time cues (25, 26), although the authors of those reports did not interpret their data in terms of circadian control. Because cyanobacteria are prokaryotes, it was dogmatically assumed that they grow in direct response to environmental conditions (light, temperature, nutrients etc) without an endogenous timekeeper of about 24 h, so it is not surprising that these daily rhythms were not immediately thought to be circadian.

Huang and his colleagues were apparently the firstto recognise that the rhythms displayed by cyanobacteria were controlled by a circadian clock (27). Their pioneering work with the cyanobacterium *Synechococcus* RF-1 clearly demonstrated circadian rhythms of nitrogen fixation, photosynthetic activity, amino acid uptake, and general gene expression that are entrainable by both light-dark and temperature cycles and is also temperature-compensated (27-33). The phase relationship between the rhythms of nitrogen fixation (peaking in the night) and photosynthesis (peaking in the day) in cyanobacteria has been interpreted to be a circadian temporal program that has possible adaptive significance. In particular, nitrogen fixation is catalysed by nitrogenase and nitrogenase is irreversibly inactivated by oxygen, but photosynthesis produces oxygen. So, how can a "simple" unicellular, photosynthetic organism accomplish nitrogen fixation? The current explanation is that these two incompatible processes are separated in time; photosynthesis in the day, nitrogen fixation at night (24, 26).

In a variety of cyanobacterial species, circadian rhythms in nitrogen fixation, photosynthesis activity/capacity and the expression patterns of many genes have also been found (34-43). Recently, genes related to circadian time-keeping were cloned from *Synechococcus* sp. strain PCC7942 (44), and their putative homologs have been found in other cyanobacterial species (45) and archaea (46-49). For further information, recent progress on the study of cyanobacterial circadian rhythms is reviewed in several articles (24, 50, 51).

CELL DIVISION RHYTHMS IN CYANOBACTERIA

Circadian rhythms in the timing of cell division have been observed in slowly growing cyanobacteria (52). What about in rapidly growing cyanobacteria, such as the species we study, *Synechococcus* sp. strain PCC7942? Figure 2 represents a typical growth curve of a culture of this single fission unicellular cyanobacterium in batch cultures. Under light-dark cycles, this photosynthetic bacterium divides in the light phase and stops dividing in darkness. After being grown in 24-h light-dark cycles, the cultures were placed in constant light (LL). In LL, the wild-type cells continue to divide rhythmically (Figure 2). The rate of division is high in the "subjective" day and slows in the "subjective" night. The period of this division rhythm is approximately 24 hours–i.e., it is *circa*-24 h. In contrast, division of the clock-null strain in which the circadian clock gene *kaiC* (44) is genetically deleted (T. Mori, unpublished), does not continue to cycle after being placed in continuous light conditions (Figure 2). These data show that *Synechococcus* cells divide rhythmically in constant light, and that this rhythm is abolished by eliminating the circadian clock system that is controlled by *kai* gene products.

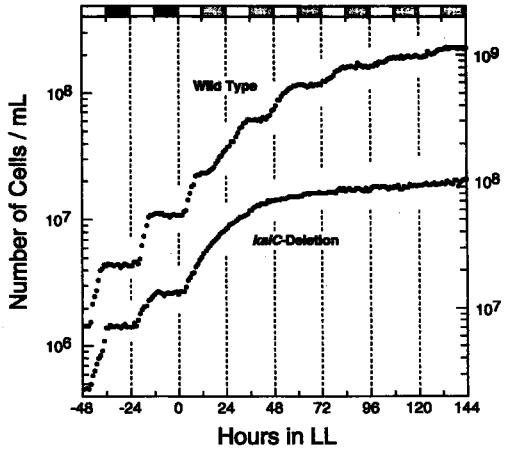

Figure 2. Circadian rhythm of cell division in batch cultures of *Synechococcus* sp. strain PCC7942. Cell number data for the wild-type strain (•) and a clock-null strain *kaiC*-deletion (o). To create the clock-null strain the circadian clock gene *kaiC* (44) in the chromosome of the P*psbAI*::*luxAB* reporter strain AMC149 (34) was replaced by a chloramphenicol resistance gene (T. Mori, unpublished). This deletion strain exhibits no circadian rhythm of bioluminescence. The wild-type and *kaiC*-deletion strain were grown in LD 12:12 and transferred into LL (45 $\mu E/m^2/s$) at time 0. The last two LD cycles preceding LL are illustrated by the bars on the upper abscissa (white = light, black = dark, grey = subjective night phases of LL). The left ordinate is for the wild-type strain, and the right ordinate is for the *kaiC*-deletion strain.

In cultures that were continuously diluted with fresh medium to maintain a constant average cell concentration in a population that was doubling more rapidly than once every 24 hours, the cyanobacteria also exhibited rhythms of cell division (53). The cells slowed or stopped dividing in the early subjective night (Figures 3 and 4). Since the average doubling time was less than 12 hours in the continuously diluted cultures (at some phases, the instantaneous rate was as high as once every 6 hours) and this cyanobacterium divides in single fission mode, the cells divided at least twice on average every circadian period (about 24 hours). We have observed that the period of the cell division rhythm is essentially the same in both batch and continuous cultures that are growing with different doubling times ranging from 9 to 90 hours. Therefore, circadian timekeeping in cyanobacteria is obviously independent from the cell division cycle. These data indicate that the circadian timekeeping system is neither based on the cell division cycle itself nor is generated in relationship to the period of the cell division cycle. On the other hand, cell division in this cyanobacterium is regulated, or "gated," by a circadian timekeeper that is not influenced by the rate of cell division. Therefore, in the hierarchy of these rhythms, the circadian clock gates the CDC, but the CDC does not modulate the circadian oscillator.

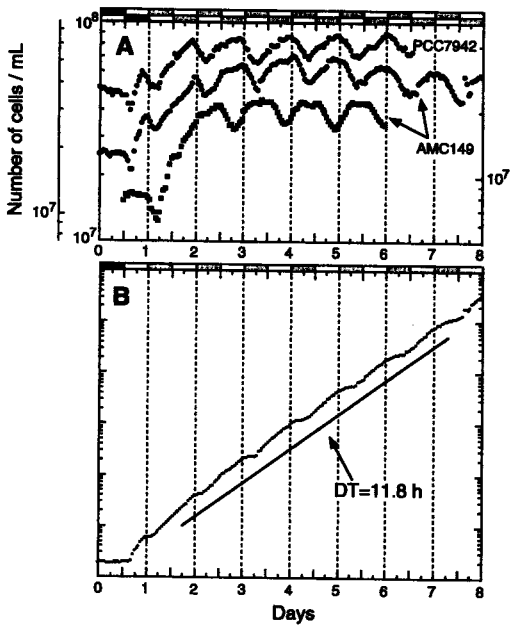

Figure 3. Cell division rhythms in continuously-diluted cultures of *Synechococcus* sp. strain PCC7942. In this figure, data from the wild-type strain is labelled "PCC7942," and data from the wild-type strain transformed with a luciferase reporter construct (34) is labelled "AMC149." **(A)** Cell number data for PCC 7942 and AMC149 cultures. The uppermost trace is for wild-type *Synechococcus* PCC 7942; the two AMC149 traces are from cultures that were previously entrained to LD cycles (125 $\mu E/m^2/s$ in light) which were 12 hours out of phase with each other compared to local clock time. Abscissae: the last LD cycle preceding LL is illustrated by the bars on the upper abscissa (upper bar for top and middle traces, lower bar for lowest trace; white = light, black = dark, grey = subjective night phases of LL). Ordinates: the leftmost ordinate is for the middle trace, the ordinate on the left side of the frame is for the lowest trace, and the right ordinate is for the top trace. After the entrainment, the cultures were released into LL (125 $\mu E/m^2/s$) and continuously diluted as described. The dilution rates of the cultures were 0.0658 hr^{-1} (upper trace), 0.057 hr^{-1} (middle trace), and 0.0618 hr^{-1} (lower trace). The periods of the cultures estimated were 24.0 hours (upper trace), 25.2 hours (middle trace), and 24.2 hours (lower trace). **(B)** The data in panel A for the middle trace are replotted as a cumulative increase in cell number (i.e., a logistic growth curve, calculated from the rate of dilution and the cell number data of panel A). The diagonal line indicates a doubling time (DT) of 11.8 hours. (Adapted from ref. 53).

We also demonstrated that not only the cell division cycle but also the promoter activity of the photosynthetic gene, *psbAI* (monitored by a luciferase reporter as in ref. 34), exhibited rhythms in rapidly dividing cyanobacteria in continuous cultures (Figure 4C). That observation has been confirmed in batch cultures in which the generation time was as short as about 6 hours. In batch cultures at low cell density, the rhythm of *psbAI* promoter activity grows exponentially in a pattern that can be modelled accurately by assuming (i) a circadian rhythm of *psbAI* promoter activity in every cell, and (ii) an exponential increase in the number of cells (54). Our observations clearly demonstrate that circadian rhythms (at least, of cell division and expression of the *psbAI* gene) are expressed on time in cyanobacteria regardless of the phase of the growth cycle (exponential or stationary). These data are completely at odds with the circadian-infradian rule that was developed to describe the CDC/circadian interface in eukaryotic micro-organisms.

THE MECHANISM OF CIRCADIAN CONTROL OF CYANOBACTERIAL CELL DIVISION

There have been many studies on the cell division cycle in bacteria, especially in *Escherichia coli* (55, 56) that might be applicable to the cell division cycle in cyanobacteria and its circadian control. In continuous cultures of the cyanobacterium *Synechococcus* sp. strain PCC7942, we found a circadian rhythm of cell division (cytokinesis) (Figures 2-4 and ref. 53). In this species, each cell contains multiple copies of the chromosome, and we found that under continuous culture conditions, the DNA content of the cells oscillated between an average of 4 and an average of 5.5 chromosomes per cell (Figure 4D). The average DNA content of the cells was strongly correlated to the average cell volume. From the data on DNA content and cell volume (Figures 4D and 4E, respectively), we calculated that the rate of DNA synthesis appears to be constant and it is the periodic cell division that leads to a rhythm of DNA content per cell (53). In other words, in constant exponential growth the cyanobacterial cell mass (represented by cell volume) grows continuously as in other bacteria and the DNA synthetic rate is constant, but the timing of cell division is rhythmic.

In a closely related cyanobacterial species, *Synechococcus* sp. strain PCC 6301, genome replication times have been reported to vary between 1 hour and 12 hours under different conditions (43, 57, 58). Variance in genome replication times is probably not the limiting factor responsible for the rhythm of DNA content per cell in our species because the DNA synthetic rate is constant and the DNA content/cell is strongly correlated with cell size in LL (53). We found that the initiation of DNA replication progresses at a constant rate in LL and probably depends upon the overall rate of cell growth (i.e., cell mass), but is independent of the timing of cell division, which is controlled by the circadian system. This rhythm of division causes the average cell size and DNA content per cell to be rhythmic.

Therefore, it is the timing of cell division alone that appears to be controlled by the circadian clock. How does the internal circadian oscillator control division? Can we infer anything about how this might happen mechanistically by analogy with what is known about cell division in other bacteria? Fluctuations in the

expression of some genes related to cell division have been studied in *E. coli* (59). In particular, the abundance of the early division factor, FtsZ (60), oscillates over the cell division cycle by about 50%, and it has been demonstrated that the expression of the *ftsZ* gene also fluctuates through cell division cycle in *E. coli*.

Could the circadian clock of cyanobacteria control cell division by regulating the expression of cell division proteins? We have found that the cyanobacterial *ftsZ* gene is expressed rhythmically. A luminescence reporter construct reveals that the activity of the promoter driving cyanobacterial *ftsZ* expression peaks in the early night and is minimal in the early day (unpublished). Based on the functions of FtsZ and the expression patterns of *ftsZ* in other bacteria, the circadian expression pattern of the *ftsZ* gene in cyanobacteria does not suggest a direct correlation between *ftsZ* expression and the circadian control of cell division. Other genes that are related to cell division in other bacteria have not yet been analysed in cyanobacteria. For example, a promising class of candidate molecules responsible for the circadian control would be division inhibitor proteins. In *E. coli*, some proteins known to inhibit division have been identified. For example, the SulA protein is produced as a part of the SOS response and reversibly inhibits cell division by interacting with the FtsZ protein (61-63). A homolog of the *sulA* gene has been found in the cyanobacterial species for which the complete genomic sequence is available, *Synechocystis* sp. strain PCC 6803 (45). Therefore, it might be worthwhile to determine if the *sulA* homolog is rhythmically expressed in cyanobacteria and whether the phase of that expression is consistent with a role in controlling the circadian rhythm of cell division.

WHY HAVE CIRCADIAN GATING OF CELL DIVISION?

In eukaryotic unicellular organisms, Pittendrigh suggested that a selective pressure that aided the evolution of circadian organisation may have been the deleterious effects of solar irradiation (5, 64). Sunlight contains many different wavelengths of light and even relatively innocuous visible light can be absorbed by pigmented molecules (e.g., cytochromes) and thereby affect cellular metabolism. An even more potent component of sunlight is ultraviolet light that may mutate DNA, and some eukaryotic cells are known to be sensitive to UV irradiation during early S phase and/or M phase of the CDC (65). Therefore, it is reasonable to postulate that an adaptive strategy to evade the harmful effects of light would be to use a rhythmic gating system to forbid light-sensitive phases of the CDC from occurring during the day and to allow them at night, as may be true for the eukaryotic unicells *Euglena* and *Chlamydomonas* (Figure 5).

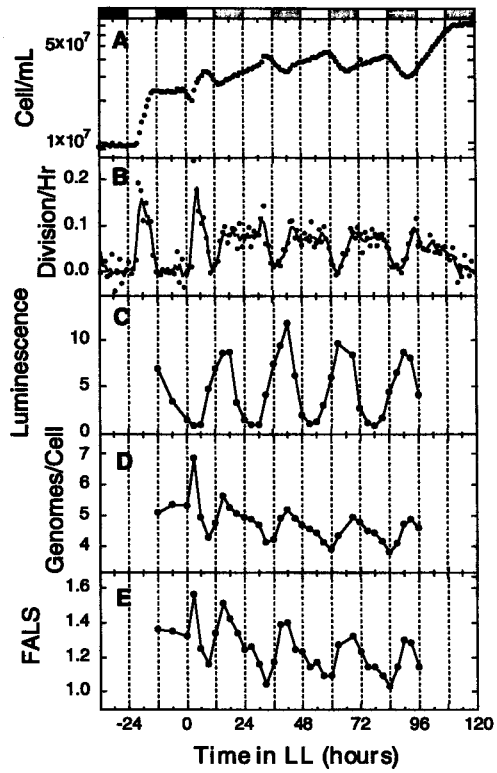

Figure 4. Cell number, luminescence, DNA content and cell size in continuously-diluted cultures of the AMC149 strain of *Synechococcus* sp. strain PCC7942. Cells were entrained to LD 12:12 in batch cultures and then released into LL at time 0, at which time continuous dilution began and was maintained until hour 96. The dilution rate was 0.061 hr^{-1} (DT = 10.5 hours). **(A)** Number of cells per ml of culture (raw data). Estimation of period in LL was 23.3 hours. **(B)** Instantaneous rate of increase in cell number (in divisions per hour) corrected for the rate of medium dilution. The points in panel B are the rate of change between each successive pair of points in the data of panel A, and the line is a three-point moving average. On this graph, a value of "0" means that the cell number did not increase at that time. **(C)** Luminescence expressed by the luciferase reporter construct (a P_{psbAI}::*luxAB* transcriptional fusion). *In vitro* luminescence is represented in units of 10^7 quanta/sec/ml of culture. **(D)** Average number of genomes per cell (DNA content/cell) as estimated by flow cytometry. **(E)** Average cell size as measured by forward-angle light scatter (FALS) per cell as estimated by flow cytometry (larger value = larger cell size). (Adapted from ref. 53).

But what about the cyanobacterium *Synechococcus*? It appears to violate the "evasion of light" hypothesis since the cells divide in the daytime. Although we do not know the molecular mechanisms of circadian control of its cell division, it may be possible to infer why the cell division cycle is controlled by a circadian clock in *Synechococcus* to occur in the day. Under continuous illumination, these cells stop or slow their division rate in the early "subjective" night and they divide in the subjective day and late subjective night phases (Figure 5). And in constant light, the chromosomal DNA is replicated

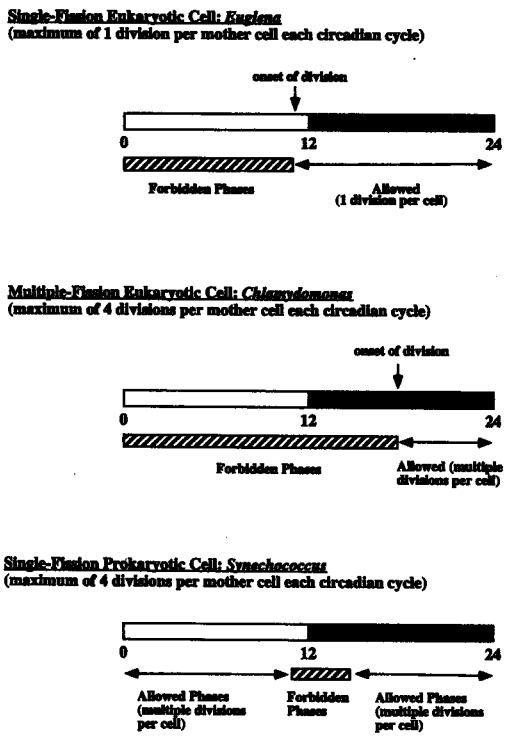

Figure 5. Model for circadian gating of cell division in the photoautotrophic unicellular organisms *Euglena*, *Chlamydomonas*, and *Synechococcus*. *Euglena* and *Chlamydomonas* are eukaryotes and *Synechococcus* is a prokaryotic cyanobacterium. The cell division timing of all three organisms is characterised by a "forbidden phase" in which cell division is not allowed to proceed, and an "allowed phase," in which cells of sufficient size are able to divide. The circadian oscillator gates these allowed and forbidden phases. In each panel, the white/grey bar indicates the circadian time course: white for subjective day, grey for subjective night. Numbers under the bar (0, 12, 24) indicate circadian time (0 & 24 = dawn; 12 = dusk). (Adapted from ref. 53).

continuously, during both subjective day and night. When the cells are in natural sunlight/darkness cycle, are the dividing cells not harmed by UV-induced mutations in the day? It may be that energy considerations are the most important in this obligate photoautotroph and that the cells circumvent the UV damage by other mechanisms.

For example, the size of the genome is small in this cyanobacterium and each cell has multiple copies of the chromosome (2-6 copies), so the rate of mutation per genome per generation is probably not high. Even after a mutation occurs in a gene on one of the several copies of the chromosome, it could be complemented by the wild-type genes on other chromosomes in the same cell and it might not immediately impede the efficiency of cell reproduction. After several generations, the daughter cells to which the mutated DNA segregates may die, but most of the daughter cells remain "wild-type" and grow normally. Alternatively, the presence of multiple wild-type copies of a mutated gene may allow a template for accurate repair.

Why do cells stop dividing in the night of a light/dark cycle? Because the cyanobacteria are obligate photoautotrophs, the simplest explanation is that they grow and divide when energy (light) is abundant and they do not have an energy storage system that allows them to continue DNA replication and cell division in darkness. In LL, the circadian system still "expects" the night to come and in anticipation of that event, the clock shuts down cell division. But under the unnatural conditions of LL, energy continues to flow into the cells in the subjective night, and so growth continues. Apparently, initiation of DNA replication is linked to cell growth and so it also continues in LL. Therefore, Pittendrigh's "evasion of light" hypothesis may not apply to *Synechococcus* sp. strain PCC7942, where the need to efficiently utilise the rhythmic energy source (sunlight) is the top priority. Nevertheless, that hypothesis could be true for other organisms that can store energy for the night and never have more than two copies of the genome per cell.

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