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Independence of Circadian Timing from Cell Division in Cyanobacteria

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In the cyanobacterium *Synechococcus elongatus*, cell division is regulated by a circadian clock. Deletion of the circadian clock gene, *kai*C, abolishes rhythms of gene expression and cell division timing. Overexpression of the *ftsZ* gene halted cell division but not growth, causing cells to grow as filaments without dividing. The nondividing filamentous cells still exhibited robust circadian rhythms of gene expression. This result indicates that the circadian timing system is independent of rhythmic cell division and, together with other results, suggests that the cyanobacterial circadian system is stable and well sustained under a wide range of intracellular conditions.

Two particularly important periodic biological events are circadian rhythms and the cell division cycle (CDC). The CDC operates in all growing organisms, and circadian rhythms are widely found in organisms from prokaryotic cyanobacteria to essentially all eukaryotes, including protista, fungi, plants, and animals up to human beings (7, 13, 20, 38, 41). Fundamental characteristics of circadian rhythms which define and distinguish them from other periodic phenomena in living organisms are (i) they are endogenous and genetically determined, (ii) the rhythms continue with a ~24-h period under constant conditions that is entrainable by environmental cycles, and (iii) the period length is compensated for changes in ambient temperature over a wide range of physiologically relevant temperatures (3, 12, 37, 42). Despite the fact that circadian rhythms display features that are not shared by the CDC oscillator (such as temperature compensation), some researchers have suggested that there might be a bidirectional interdependent linkage between these two oscillating systems (13, 14, 23). In the case of other circadian rhythms, there is evidence of such bidirectional linkage in that some outputs can feed back onto the central oscillator (36). For circadian rhythm-CDC coupling, other researchers have favored an alternative hypothesis that the circadian clock mechanism oscillates independently of the status of the CDC, but the CDC is dependent on the phase of the circadian clock such that cell division is gated to occur only in specific circadian phases (13, 14, 16, 35).

Cyanobacteria are the simplest organisms in which circadian rhythms have been clearly documented (20). Over the past 15 years, many circadian rhythms including rhythms of photosynthetic activity, nitrogen fixation, global gene expression, and—most relevant for this study—cell division have been found in several cyanobacterial species. In fact, among photosynthetic organisms, our knowledge of clock components and interactions is most highly advanced in the unicellular cyanobacterium *Synechococcus elongatus*. The *kai* genes that are intimately involved in circadian timekeeping in *Synechococcus* have been cloned (18), and their homologs have been found in other cyanobacterial species as well as in archaea (6, 20, 21, 22, 29, 33). Deletion of any one of the *kai* genes does not affect viability (in single-strain cultures) but causes arhythmicity. As had been suggested for other model organisms such as mice, flies, and fungi (17, 28, 30, 43), Ishiura *et al.* (18) proposed for cyanobacteria that transcriptional and/or translational control of circadian clock genes by their own products (negative feedback regulation) is essential for circadian timekeeping. Biochemical and biophysical analyses of the processes by which the *kai* genes and their products are involved in circadian timekeeping are under way (19, 20).

We previously reported that cell division in *S. elongatus* is gated by a circadian oscillator (35). In light-dark (LD) cycles, division occurs only in the light phase. In constant light (LL), where growth is apparently continuous, the cells divide in the subjective day and late subjective night but are kept from dividing in the early subjective night by the circadian oscillator. In this study, we demonstrate that the same *kai*-dependent clock that regulates gene expression also controls cell division. The bacterial cell division gene *ftsZ* is expressed with a circadian pattern in *Synechococcus*. Most importantly, overexpression of FtsZ protein stops the cells from dividing while they continue to grow (resulting in filamentous cells) but does not affect circadian rhythms of gene expression. These results indicate that the circadian pacemaker that gates cell division and gene expression in *Synechococcus* oscillates stably and independently of feedback from the CDC.

MATERIALS AND METHODS

Strains and culture conditions. *S. elongatus* PCC 7942 (wild type; also known as *Anacystis nidulans* R2 or *Synechococcus* sp. strain PCC 7942) and derivative strains were grown in modified BG-11 medium (15) at 30°C. Depending on the antibiotic resistance of each strain, spectinomycin (20 μg/ml), kanamycin (12.5 μg/ml), and/or chloramphenicol (10 μg/ml) was added to the medium. Continuous culturing and measurement of cell density of cultures were performed as previously described (35, 46).

Cloning and sequencing of *ftsZ*. Plasmid isolation, restriction digestion, ligation, transformation, and Southern blotting were performed essentially as described by Sambrook et al. (40). To make the *ftsZ* gene probe for screening genomic libraries, genomic PCR was performed with the primers FZTM1 (5′-

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CCT GAA TTC AAY ACN GAY GNC ARG C-3′) and FZTM2 (5′-CCT GAA TTC GTN CCN GTN CCN CCN CCC CAT-3′). These primers were the same as those used by Zhang and coworkers for cloning *ftsZ* from *Anabaena* (11, 47). PCR mixtures of 100 μl contained 1 μmol of Tris-HCl (pH 9), 5 μmol of KCl, 150 nmol of $MgCl_2$, 1 μg of Triton X-100, 20 nmol of each of the deoxynucleoside triphosphates, 50 pmol of each primer, 75 ng of genomic DNA, and 2.5 U of *Taq* DNA polymerase (Promega, Madison, Wis.). PCR was performed on a Perkin-Elmer DNA thermal cycler (Applied Biosystems, Foster City, Calif.) with an initial hot start at 95°C followed by 30 cycles at 95°C (1 min), 55°C (1 min), and 74°C (2 min) and a final polymerization step of 74°C for 7 min. One major 220-bp DNA fragment was amplified by PCR. This PCR product was cleaved with *Eco*RI, cloned into the *Eco*RI site of pBluescript II vector (Stratagene, La Jolla, Calif.), and sequenced. The resulting sequence showed strong similarity to known *ftsZ* sequences, and the PCR product was used as a probe for genomic Southern analyses and for screening a cosmid library. To screen the cosmid library (provided by Susan Golden), hybridization was performed to a digoxigenin-labeled PCR probe (~20 ng/ml) in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% *N*-lauroylsarcosine–0.2% sodium dodecyl sulfate–1% blocking reagent (Roche, Indianapolis, Ind.) at 68°C for 16 h, and hybridization of the probe on the filters was detected by the immuno-chemiluminescence method as recommended by the manufacturer (Roche). DNA fragments containing the *ftsZ* gene were isolated from identified cosmid clones, subcloned into a plasmid vector (pMT111 or pMT115), and sequenced. Plasmid templates for DNA sequencing were sequenced from the insert ends with T3, T7, M13 primers or with custom oligonucleotide primers. All double-stranded DNA templates were prepared with a Wizard Plasmid Miniprep kit (Promega) or by PCR and sequenced on an ABI377 DNA sequencer (Applied Biosystems). Database searches for similarity with other proteins were performed with BLASTP and TBLASTN (1) at the National Center for Biotechnology Information, National Institutes of Health, via the Internet.

RNA analyses. Cells were grown in 24-hour LD cycles (LD 12:12) and harvested. The culture was mixed with crushed ice to immediately chill the cells, and then the cell suspension was centrifuged at 15,000 × *g* at 4°C for 5 min. The cell pellets were frozen in liquid nitrogen and stored at −80°C. Total RNA was isolated by a modification of the method of Chomczynski and Sacchi (9). Five milliliters of TRI-Reagent (Molecular Research Center, Cincinnati, Ohis) was added to the frozen cell pellet in 13-ml centrifuge tubes, and the samples were vortexed with 2 g of glass beads for 5 min at room temperature. The homogenates were centrifuged at 10,000 × *g* at 4°C for 10 min; then the supernatants (~3.6 ml of each) were transferred into new tubes to which 360 μl of 1-bromo-3-chloropropane was added and vortexed vigorously for 15 s. The mixture was incubated at room temperature for 10 min and then spun at 10,000 × *g* for 15 min (4°C). About 2.2 ml of the aqueous phase was transferred to a new tube, and 2.2 ml of isopropyl alcohol was added and kept on ice for 10 min. Total RNA was collected by centrifugation at 12,000 × *g* at 4°C for 8 min, washed with 75% ethanol, dried, dissolved in water, and stored at −80°C.

For Northern analyses, RNA was separated by electrophoresis in 1.0% agarose gels under denaturing conditions. After blotting onto a nylon membrane, an *ftsZ*-specific RNA probe was used to hybridize to *ftsZ* mRNA on the membrane.

For primer extension, the 5′ ends of primers FZPE1 (5′-ATC GAA CCC CGA CAG AGA GCC GTC AC-3′) and FZPE2 (5′-ATC GGC ATA GGG TCG GTC AT-3′) were labeled with [γ-^{32}P]ATP (<5,000 Ci/mmol; NEN Life Science Products, Boston, Mass.) using T4 polynucleotide kinase (New England Biolabs, Beverly, Mass.). Ninety-eight micrograms of total RNA and 2.4 pmol of ^{32}P-labeled primer (~10^6 cpm) in 30 μl of hybridization buffer (40 mM piperazine-*N,N*′-bis[2-ethanesulfonic acid] [PIPES], 1 mM EDTA, 0.4 M NaCl, 80% formamide [pH 6.4]) were denatured by heating at 85°C for 10 min and then incubated at 32°C overnight. RNA-primer hybrids were recovered by ethanol precipitation, air dried, and dissolved in 20 μl of reverse transcriptase reaction mixture containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 20 U of RNase inhibitor (Ambion, Austin, Tex.), and 100 U of SuperScript II RNase H$^-$ reverse transcriptase (Life Technologies, Gaithersburg, Md.). The reaction was performed at 42°C for 1 h and terminated by heating at 70°C for 10 min. RNA in the mixture was removed with RNase A. Ten micrograms of salmon sperm DNA was added as a carrier, and cDNAs were extracted with phenol-chloroform-isoamyl alcohol (25:24:1), precipitated with ethanol, and then analyzed by electrophoresis through 6% polyacrylamide–7 M urea gels. The sequencing ladders to determine the endpoints of primer extensions were prepared with the same ^{32}P-labeled primer using pMT115 as the template by cycle sequencing (Seq Therm EXCELL II kit; Epicentre Technologies, Madison, Wis.). The gels were dried and then exposed to X-ray film (X-Omat AR; Eastman Kodak, Rochester, N.Y.) without intensifying screens at −80°C for 1 to 3 days.

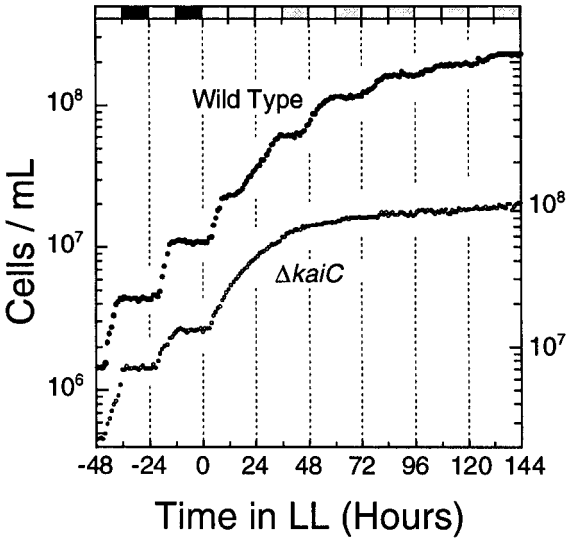

FIG. 1. Circadian rhythm of cell division in batch cultures of *S. elongatus*. Cell number data for the wild-type strain (●) and a clock-null Δ*kaiC* strain (○). The wild-type and Δ*kaiC* (46) strains were grown in LD 12:12 and transferred into LL (45 μE/m^2/s) at time zero. The last two LD cycles preceding LL are illustrated by the bars on the upper abscissa (white, light; black, darkness; gray, subjective night phases of LL). The left ordinate is for the wild-type strain, and the right ordinate is for the Δ*kaiC* strain.

Construction of the luciferase reporter strains and *in vivo* luminescence measurement. To construct *ftsZ* promoter (*ftsZ*p)-bacterial luciferase transcriptional fusions, the vectors pAM1580 (2) and pAM1583I (2) (modified by M. Izumo [unpublished data]) were used for construction of the *luxAB* reporter strain. These vectors carry a promoterless *luxAB* gene set and transfer inserts to the neutral sites of the *Synechococcus* chromosome by homologous recombination. The upstream region of the *ftsZ* gene was isolated after restriction digestion of the *ftsZ* clone or amplified by PCR using *Pfu* DNA polymerase (Stratagene) and cloned into the unique *Stu*I site upstream of the *luxAB* genes in the vectors. The constructs were linearized with *Nde*I to avoid homologous single recombination and then introduced into neutral site I (8) or neutral site II (2) of the wild-type *Synechococcus* chromosome by homologous double recombination (2, 15). Antibiotic-resistant colonies were selected, and correct transformations were confirmed by genomic PCR or Southern analysis. In vivo luminescence was measured as previously described by Kondo et al. (24, 26) and Mori et al. (35).

Overexpression of *ftsZ* in *E. coli* and cyanobacteria. Using primers TRCFZ1 (5′-TCG AGC TCA AGG AGG AAT AAC ATA TGA CCG ACC CTA TGC CGA TC-3′) and TRCFZ2 (5′-TGG GAT CCC ATA TGC TAG GGT CGG TTT TGA ATT TTC CG-3′) and the cosmid 8B5 as a template, we amplified a 1,215-bp *Sac*I/*Bam*HI fragment which contains the *ftsZ* gene (underlines denote created *Sac*I and *Bam*HI sites). An alternative ribosome binding site was introduced just upstream of the *ftsZ* open reading frame (ORF). This 1,215-bp *Sac*I/*Bam*HI fragment was inserted into the *Sac*I-*Bam*HI site downstream of the *trc* promoter of p322-Ptrc-ΔNdeI, a derivative of p322-Ptrc (27), yielding plasmid pMT411. A 3.4-kb *Bgl*II segment of pMT411 that contains *lacI*^{q}, *trc*p::*ftsZ*, and an *rrnB* operon region (as a transcription terminator) was isolated and inserted into the *Bam*HI site of either pAM1313 for targeting to neutral site I (2, 24) or pTS2K-ΔNdeI, a derivative of pTS2K (27), for targeting to neutral site II. The constructs were introduced into either neutral site I or II on the chromosome of cyanobacterial strains by double crossover. The FtsZ protein was overexpressed in cyanobacterial strains in liquid or on solid (1.5% agar) BG-11 medium containing 0.5 or 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The cells were observed under a light microscope, and the microscopic images (bright field with 20× or 40× objectives) were captured by a charge-coupled device camera.

Nucleotide sequence accession number. The nucleotide sequence of the *S. elongatus ftsZ* gene and flanking regions has been deposited in the DDBJ/EMBL/GenBank databases under accession number AF076530.

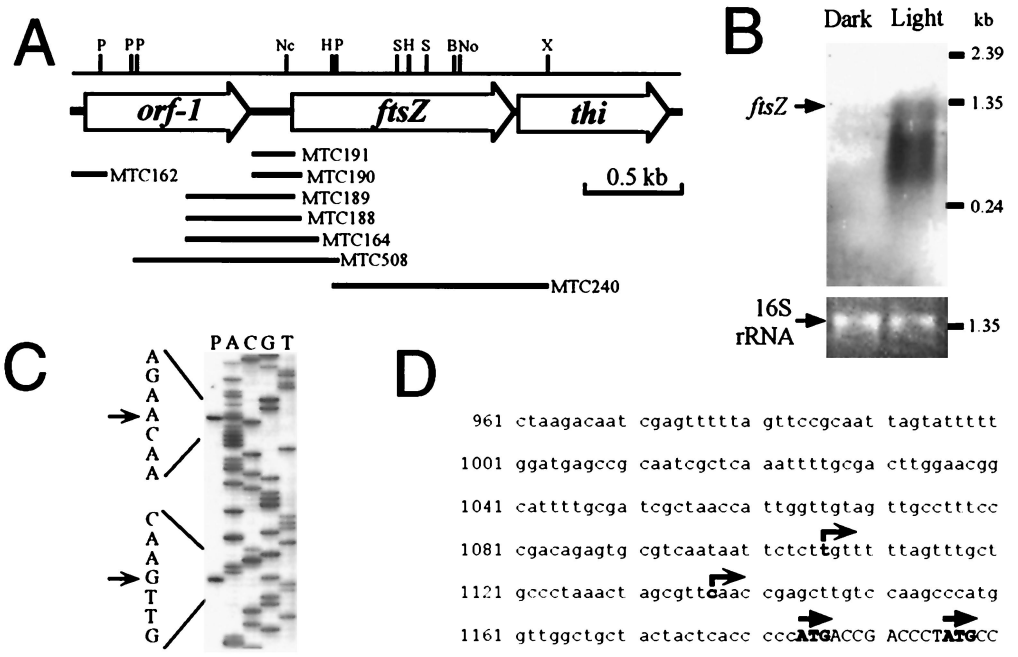

FIG. 2. Primary structure and expression of the *S. elongatus ftsZ* gene. (A) Physical map of the *ftsZ* gene and its flanking regions. Segments used for constructing *luxAB* reporter strains are indicated by horizontal bars with the names of the transformed strains. P, *Pst*I; Nc, *Nco*I; H, *Hin*dIII; S, *Sal*I; B, *Bss*HI; No, *Not*I; X, *Xho*I. (B) Northern blot analysis of the *ftsZ* gene. To show equivalent loading of the two lanes in the gel, ethidium bromide staining of the 16S rRNA band is shown. Cells were grown in LD 12:12 cycles and harvested in the day and night. In LD cycles, cyanobacteria divide only in the day (Fig. 1). (C) Determination of putative transcriptional start sites of the *ftsZ* gene by primer extension. The transcription start site was confirmed by S1 mapping (not shown). (D) Sequence of the promoter region of *ftsZ*. Putative transcriptional (right-angle arrows) and translational (straight arrows) start sites are indicated.

RESULTS AND DISCUSSION

Circadian rhythm of cell division regulated by *kaiC*-dependent clock. In a previous study (35), we showed that *S. elongatus* cells exhibit a rhythm of cell division cycling in continuous cultures of rapidly growing cells. The period length of the cell division rhythm is altered in the same manner by mutations (25) that change the period length of the promoter activity rhythm of the *psbAI* gene (24, 35). The mutations in strains C22a and C27a (previously named SP22 and LP27, respectively), which altered period lengths of circadian rhythms of both cell division (35) and *psbAI* promoter activity (25), have been mapped to the circadian clock gene *kaiC* in the cluster of *kai* genes (18). We therefore examined the cell division phenotype of a *kaiC*-deficient strain that is arhythmic for the *psbAI* promoter rhythm. The wild-type (AMC149) and *kaiC* deletion (Δ*kaiC* [46]) strains were grown in batch cultures. In both the wild-type and Δ*kaiC* strains, cell division occurred in the day phases of LD 12:12 cycles (Fig. 1). After transfer to LL, the wild-type strain exhibited a stepwise growth curve; the cells divided in the subjective day and stopped dividing in the early subjective night (Fig. 1), as we reported previously (35). In contrast, in the Δ*kaiC* strain, cell density increased without apparent rhythmicity until the culture reached stationary phase (Fig. 1). Lack of a cell division rhythm in the Δ*kaiC* strain indicates that the same *kaiC*-dependent clock that regulates global gene expression regulates cell division and supports our prior finding using point mutations of the *kaiC* gene (35).

Cloning the *ftsZ* gene from *Synechococcus*. Although the circadian gating of cell division in cyanobacteria has been clearly demonstrated (35) (Fig. 1), the molecular bases for gating the CDC are still largely unknown. The protein FtsZ is ubiquitous in bacteria and is also found in chloroplasts. In bacteria, a cytoskeletal element called the Z ring is formed in the middle of the cell (5, 39), and septation occurs through the action of the Z ring. The FtsZ proteins assemble into the Z ring and are known to be essential for cell division (4, 10). Our previous study indicated that DNA replication occurs continuously and randomly throughout the circadian cycle within a population of rapidly growing cells but that cytokinesis is gated by the circadian clock such that division is forbidden in the early subjective night (35). Consequently, we hypothesized that genes related to cytokinesis (or septation) might play a role in the circadian gating of cell division; therefore, we cloned the *ftsZ* gene from *Synechococcus* and investigated its temporal expression patterns.

To make the *ftsZ* gene probe for screening genomic libraries, PCR was performed with the primer set that was used for cloning *ftsZ* from *Anabaena* (11, 47). One major 220-bp DNA fragment was amplified by genomic PCR. This PCR product was sequenced and found to be very similar to a region of known *ftsZ* genes. Therefore, the PCR product was used as a probe for genomic Southern analysis and for screening a cosmid library. Genomic Southern analyses confirmed that the *ftsZ* gene was a single-copy gene (data not shown). Thirteen positive clones were obtained from the 650 clones of the cosmid library, and cosmid 8B5 was used for further characterization. The *ftsZ* ORF was localized within cosmid 8B5 and sequenced. The predicted amino acid sequence of *S. elongatus*

FtsZ shows strong similarity to FtsZ proteins of other bacteria (74% identity to *Anabaena* FtsZ, 72% identity to *Synechocystis* sp. strain PCC 6803 FtsZ, and 48% identity to *E. coli* FtsZ) and chloroplasts (61% identity to *Arabidopsis* plastid FtsZ). DNA sequence analysis upstream and downstream of *Synechococcus ftsZ* demonstrated that the *ftsZ* gene is flanked by an unknown gene (homologous to ORF sll1632 located just upstream of *ftsZ* in *Synechocystis* sp. strain PCC 6803 [21]) and a homolog of the *thiD* gene (37% identity to the putative amino acid sequence of the *thiD* gene from *Salmonella enterica* serovar *Typhimurium* [45] encoding phosphomethylpyrimidine kinase) (Fig. 2A). Unlike in *E. coli* (39), the *ftsZ* genes of *S. elongatus* and *Synechocystis* sp. strain PCC 6803 are not organized in a cluster with other cell division genes (e.g., *ddlb-ftsQ-ftsA-ftsZ-envA*). Northern blotting analysis shows the maximum size of detectable *ftsZ* transcript is 1.3 kb and the *ftsZ* gene is expressed more strongly in dividing cells during the day (Fig. 2B). Primer extension indicated at least two putative transcriptional start sites (Fig. 2C and D), and those sites were confirmed by S1 analysis (data not shown). Reporter analysis (see below) indicated that essential promoter elements are located within the 167-bp 5′ region (strain MTC191). These findings indicate the lack of conservation between cyanobacteria and other bacteria in the flanking regions of the *ftsZ* gene.

Circadian rhythms of *ftsZ*p activity. To monitor the expression patterns of *ftsZ*, we constructed transcriptional fusion strains with the *ftsZ* promoter linked to a bacterial luciferase gene set. The upstream regions of the *ftsZ* gene were isolated and transcriptionally fused to the *luxAB* gene set (the DNA segments fused to *luxAB* are indicated in Fig. 2A), and the reporter constructs were introduced into neutral sites of the wild-type *Synechococcus* chromosome. Rhythmic fluctuations of *in vivo* luminescence from the *luxAB* reporter strain MTC508 are shown in Fig. 3B and D. The rhythms of luminescence peaked at the end of the subjective day (or the beginning of subjective night), with troughs near the subjective dawn both in slowly growing batch cultures (doubling time [DT] > 24 h [Fig. 3B]) and in continuously diluted cultures of rapidly dividing cells (DT ~ 12 h [Fig. 3C and D]). The phasing of this promoter rhythm was similar to that expressed by the *kaiBC* promoter, a class I gene (18, 31, 32). In batch cultures of the other reporter strains (MTC164, MTC188, MTC189, MTC190, and MTC191) in which shorter pieces of the 5′ region of *ftsZ* were fused to *luxAB* (Fig. 2A), luminescence rhythms were as observed from strain MTC508 (data not shown). Therefore, the activity of the *ftsZ* promoter appears to be under the control of the circadian clock in *Synechococcus*.

We observed that *ftsZ*p activity was highest when cells in the population were not dividing, which is somewhat different from the case for *E. coli* (5, 34) and other bacteria (39). This result suggests that expression of *ftsZ* might not be a rate-limiting step of the cell division cycle in *Synechococcus*. Alternatively, assuming that the turnover of the FtsZ protein is rapid enough that its abundance patterns are similar to that of *ftsZ*p activity, FtsZ may negatively regulate cell division in cyanobacteria just as overexpression of FtsZ inhibits cell division in *E. coli* (44).

Overexpression of FtsZ in cyanobacteria halts division. The FtsZ protein was overexpressed in *trc*p::*ftsZ* transformants in liquid BG-11 medium containing 0.5 mM IPTG. As was also found for *E. coli* (44), overexpression of FtsZ causes the cyanobacterial cells to become filamentous (Fig. 4A). When the FtsZ protein was overexpressed in *trc*p::*ftsZ* cells growing on solid (1.5% agar) BG-11 medium containing 1 mM IPTG, single cells formed a long filament (Fig. 4C), whereas colonies formed from single cells of the *trc*p::null strain on the same medium (Fig. 4B). Microscopic examination confirmed that the filamentous cells did not form septa. Figures 4A and C indicate that cell division, but not cell growth, was stopped when FtsZ was overexpressed from the *trc* promoter.

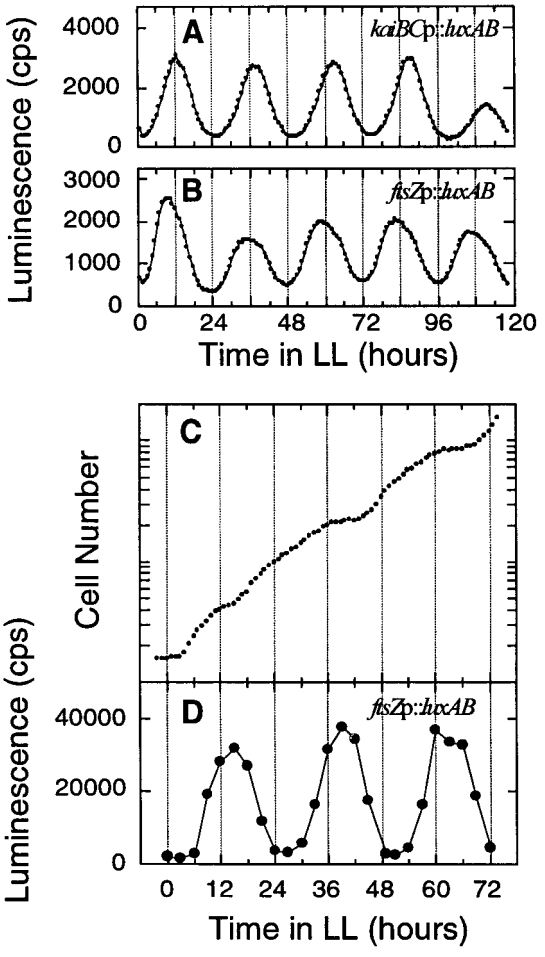

FIG. 3. Rhythms of luminescence in *ftsZ*p::*luxAB* reporter strain MTC508. (A and B) In vivo luminescence from 3-ml batch liquid cultures of *kaiBC*p::*luxAB* (A) and *ftsZ*p::*luxAB* (B) strains was measured as described by Kondo et al. (24, 26). (C and D). The *ftsZ*p::*luxAB* cells were grown in LD 12:12 (125 μE/m^2/s) and then released into LL, and continuous dilution of the culture was started. Cell density of the culture was monitored every hour, and a growth curve calculated from the actual cell density and the dilution rate (dilution rate = 43.3 ml of medium exchanged every hour in a total volume of 780 ml) is plotted in panel C. The average doubling time of this culture was 12.1 h. From the culture in panel C, 1 ml of cell suspension was withdrawn every 3 h and for measurement of luminescence (D) as described by Mori et al. (35).

Circadian rhythms of gene expression in nondividing cyanobacteria. To determine whether circadian rhythms of gene expression persist in nondividing cells, we transformed three different *luxAB* reporter strains with the *trc*p::*ftsZ* construct. Figure 5 shows luminescence patterns that report rhythms of

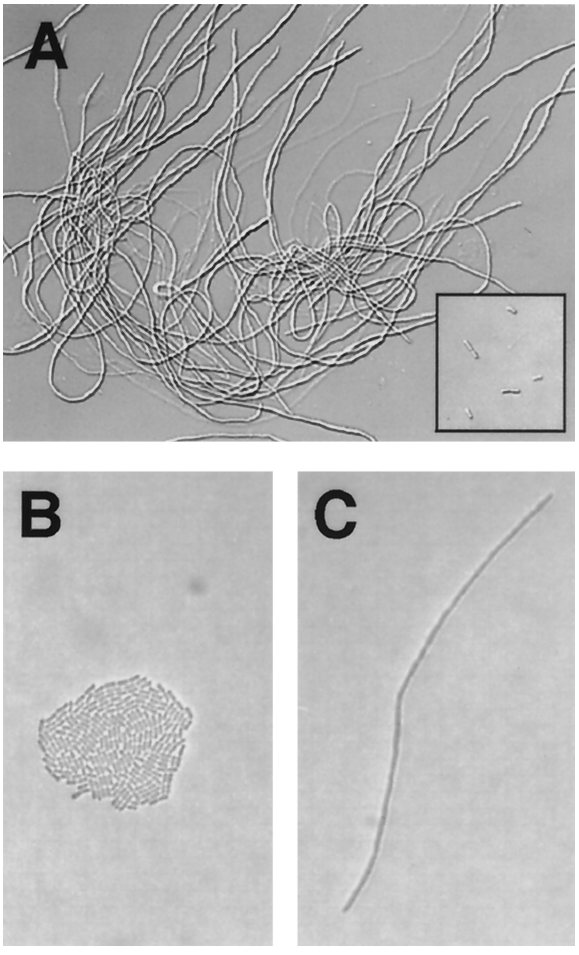

FIG. 4. Cell division of growing cyanobacteria is stopped by overexpression of FtsZ, resulting in filamentous cells. (A) The *trc*p::*ftsZ* strain was grown for 101 h in liquid BG-11 medium supplemented with 0.5 mM IPTG. The insert in the bottom right corner of panel A shows *trc*p::null cells as a control under the same conditions. (B and C) The *trc*p::*ftsZ* and *trc*p::null cells were also grown on solid (1.5% agar) BG-11 medium supplemented with 1 mM IPTG for 48 h. A colony of *trc*p::null cells (B) and a filamentous *trc*p::*ftsZ* cell (C) are shown. Presumably both the colony in (panel B) and the filament in (panel C) were derived from a single initial cell.

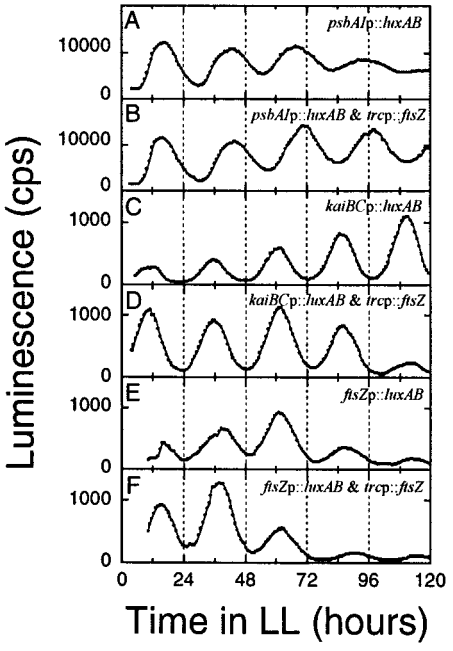

FIG. 5. Luminescence rhythms in dividing and nondividing cyanobacteria in liquid cultures. In vivo luminescence was monitored as described by Kondo et al. (24) in the following reporter strains: (A) *psbAI*p::*luxAB*; (B) FtsZ overexpression in the *psbAI*p::*luxAB* strain; (C) *kaiBC*p::*luxAB*; (D) FtsZ overexpression in the *kaiBC*p::*luxAB* strain; (E) *ftsZ*p::*luxAB*; (B) FtsZ overexpression in the *ftsZ*p::*luxAB* strain. FtsZ protein was overexpressed continuously with 0.5 mM IPTG in panels B, D, and F, and filamentous morphology in those cultures was confirmed microscopically.

promoter activities of the *psbAI, kaiBC*, and *ftsZ* genes in *Synechococcus*. Whether or not cell division was stopped by overexpression of FtsZ, the expression patterns of all three genes maintained robust circadian fluctuations for at least 4 to 5 days. Rather surprisingly, overexpression of FtsZ protein did not appear to affect the level of *ftsZ*p activity, implying that there is little or no feedback of FtsZ abundance on the *ftsZ* promoter.

It might be hypothesized that there is a different clockwork that regulates the expression of these three promoters from the clockwork that gates cell division, and therefore the overexpression of FtsZ would not be expected to impinge on the gene expression patterns. However, the data in Fig. 1 show that cell division is regulated by the same *kaiC*-dependent clock that controls the gene expression patterns (18). Therefore, the data in Fig. 4 and 5 demonstrate that the circadian clock that regulates gene expression and gates cell division is not affected by halting cell division.

The results described herein have several important ramifications. First, there does not appear to be feedback from the cell division output rhythm back upon the central oscillator in *Synechococcus*. This is different than the case with some circadian rhythms (e.g., locomotor activity) in higher organisms, where induced activity of an output can change the phase or period of the central oscillator (36), Second, the periodicity of the circadian system is precise and stable whether the cells are dividing rapidly (DT = 12 h [see also references 26 and 35]), dividing slowly, or not dividing at all. These different division states must have an important impact on the intracellular milieu, but the circadian mechanism appears to be unaltered. This result is consistent with our previous observation that the circadian timing mechanism of *S. elongatus* is impervious to conditions of metabolic repression, either by extended darkness or by inhibition of protein synthesis (46). Apparently, the circadian clockwork is well buffered and stable against significant changes of the intracellular milieu. Finally, the persistence of the gene expression rhythms when cell division is stopped clearly indicates that the circadian clockwork gates cell division, but its timing circuit is not dependent on the CDC in cyanobacteria.

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coccus genomic DNA and the *luxAB* vectors, J. Lutkenhaus for providing *E. coli* anti-FtsZ antibodies, and S. Kutsuna, M. Ishiura, and T. Kondo for providing p322-Ptrc and pTS2K vectors. We are grateful for advice from T. Kondo and M. Ishiura in early stages of this project.

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