Circadian Expression of the *dnaK* Gene in the Cyanobacterium *Synechocystis* sp. Strain PCC 6803

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The expression of the *dnaK* gene in the cyanobacterium *Synechocystis* sp. strain PCC 6803 was continuously monitored as bioluminescence by an automated monitoring system, using the bacterial luciferase genes (*luxAB*) of *Vibrio harveyi* as a reporter of promoter activity. A *dnaK*-reporting bioluminescent *Synechocystis* strain was constructed by fusing a promoterless segment of the *luxAB* gene set downstream of the promoter region of the *Synechocystis dnaK* gene and introduction of this gene fusion into a *Bgl*II site downstream of the *ndhB* gene in the *Synechocystis* chromosome. Bioluminescence from this strain was continuously monitored and oscillated with a period of about 22 h for at least 5 days in continuous light. The phase of the rhythm was reset by the timing of the 12-h dark period administered prior to the continuous light. The period of the rhythm was temperature compensated between 25 and 35°C. Thus, the bioluminescence rhythm satisfied the three criteria of circadian rhythms. Furthermore, the abundance of *dnaK* mRNA also oscillated with a period of about 1 day for at least 2 days in continuous light conditions, indicating circadian control of *dnaK* gene expression in *Synechocystis* sp. strain PCC 6803.

Circadian rhythms are found ubiquitously in organisms from cyanobacteria to humans and are thought to be an adaptation to daily changes in environmental conditions on the earth, such as light and temperature (4), although the molecular mechanism that generates the rhythms (circadian clock) remains to be solved. Temperature is lowest just before dawn and highest about noon. Circadian rhythms of heat resistance have been observed in many plants (12, 18, 26, 32). Consistent with temperature change, heat resistance is maximal about noon and minimal during the night (12, 18). Heat stress transiently increases the rate of synthesis of heat shock proteins in various organisms from *Escherichia coli* to humans (8), and the proteins may be directly involved in acquisition of heat resistance (11, 17, 30, 39). The heat inducibility of heat shock protein synthesis is controlled by a circadian clock in *Neurospora crassa* (7), pea (*Pisum sativum* L. [24]), and cotton (*Gossypium hirsutum* L. [26]). Accumulation of mRNAs for several heat shock proteins in *P. sativum* L. is also under the control of the clock (24).

DnaK is one member of a well-conserved group of heat shock proteins and plays a protective role in supporting growth at high temperatures beyond the normal physiological range of *E. coli* (16). DnaK, DnaJ, and GrpE are known to be key proteins involved in regulation of heat shock gene expression in *E. coli* (8, 9, 34, 36). Thus, whether the expression of the *dnaK* gene is under the control of a circadian clock is an interesting question.

In previous studies, we monitored the expression of the *psbAI* gene, which encodes D1, a major protein component of the photosystem II reaction center, in the unicellular cyanobacterium *Synechococcus* sp. strain PCC 7942 by using the bacterial luciferase *luxAB* gene set from *Vibrio harveyi* as a reporter. We found that the expression of the *psbAI* gene, monitored as bioluminescence, is controlled by a circadian clock in this *Synechococcus* strain (13–15).

We took advantage of the fact that the *dnaK* gene has been isolated from the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803 and characterized (6) to monitor the expression of the *dnaK* gene in this study. Another advantage of using this *Synechocystis* strain for circadian studies is that there is a glucose-tolerant derivative of the *Synechocystis* strain which is able to carry out light-activated heterotrophic growth in glucose-supplemented medium in near darkness (1). As in the case of the *Synechococcus* strain used previously (14), the *Synechocystis* strain also has a naturally occurring gene transfer system and is amenable to molecular analyses (38). We fused a promoterless segment of the *luxAB* gene set downstream of the promoter segment of the *Synechocystis dnaK* gene and introduced the gene fusion into a specific site of the *Synechocystis* chromosome. We show here that bioluminescence of the *dnaK*-reporting *Synechocystis* transformant exhibited a daily oscillation which satisfied the criteria of circadian rhythms: persistence in constant conditions, phase resetting by light-dark signals, and temperature compensation of the period. The abundance of *dnaK* mRNA also exhibited a daily oscillation in constant conditions.

MATERIALS AND METHODS

Bacterial strains, media, cultures, enzymes, and manipulation of DNA. Wild-type cells of *Synechocystis* sp. strain PCC 6803 (gift of T. Ogawa) were maintained in BG-11 liquid medium (27) or on BG-11 agar that contained BG-11, 1 mM sodium thiosulfate, 10 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES, pH 8.2), and 1.5% Bacto-Agar (Difco Laboratories, Detroit, Mich.) at 30°C under continuous illumination (LL; intensity was about 35 μmol m⁻² s⁻¹ from white fluorescent lamps). Bioluminescent reporter strains of the *Synechocystis* sp. constructed in this study were maintained in BG-11 medium supplemented with 40 μg of spectinomycin sulfate per ml. Enzymes were purchased from Takara Shuzo Co. (Kyoto, Japan), New England Biolabs, Inc. (Beverly, Mass.), Promega Co. (Madison, Wis.), and Gibco BRL (Gaithersburg, Md.). *Escherichia coli* cells were maintained in Luria-Bertani broth (LB) or on LB agar that contained 1.2% agar in LB (Shouei Kanten Co., Tokyo, Japan). Plasmids were propagated in *E. coli* HB101 or DH5α. Handling of *E. coli* and manipulation of DNA for molecular cloning were carried out as described by Sambrook et al. (29).

Isolation of a genomic clone of the *dnaK* gene. We constructed a library of *Synechocystis* genomic DNA with the λ DASH vector (Stratagene Cloning Systems, La Jolla, Calif.). A 2.1-kb DNA segment containing the coding region of the *dnaK* gene (6) was amplified by PCR with genomic DNA as a template and

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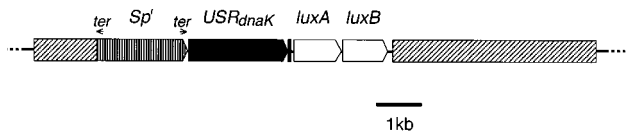
FIG. 1. Schematic representation of the *dnaK* reporter plasmid pCF5. The open rectangles with arrowheads represent a promoterless segment of the luciferase *luxAB* genes from *V. harveyi* and the Ω fragment carrying the spectinomycin/streptomycin resistance gene (Sp'). An upstream region segment of the *dnaK* gene (USR_{dnaK}) is followed by an amino-terminal coding region of the *dnaK* gene (solid rectangle with arrowhead), which is fused out of frame with the *luxA* gene. The arrows indicate the direction of transcription. The direction of the terminators (*ter*) of the Ω fragment is also shown by the arrows. The hatched boxes surrounding the USR_{dnaK}::*luxAB* gene fusion and the Ω fragment represent *Synechocystis* DNA sequences flanking the integration site in the chromosome. The pUC18 portions of the plasmid were not integrated into the chromosome and are not shown.

used as a hybridization probe. The λ clone λDNAK2, which carries the *dnaK* gene, was isolated by plaque hybridization. A 7.6-kb *Bam*HI fragment of this λ clone was subcloned into the *Bam*HI site of pUC118 (37) to produce pDNAK. The *Xcm*I site of pDNAK was converted to a *Bam*HI site by removing the protruding 3′ nucleotide of the *Xcm*I terminus with T4 DNA polymerase (DNA blunting kit; Takara Shuzo), followed by the addition of a *Bam*HI linker (d[pCG GATCCG]; Takara Shuzo) to produce pDNAKb.

Construction of reporter plasmids and *Synechocystis* reporter strains. pAM977 (gift of S. Golden) carries a P_{psbAI}::*luxAB* gene fusion, namely, a promoter region of the *Synechococcus psbAI* gene (P_{psbAI}) connected to the coding region of the *V. harveyi* luciferase genes (*luxAB*) (14), and the Ω fragment, which contains the spectinomycin/streptomycin resistance gene (25). A 3.4-kb *Hin*dIII-*Eco*RI fragment of pAM977 which carried the Ω fragment and the P_{psbAI}::*luxAB* gene portions of the P_{psbAI}::*luxAB* gene fusion was ligated with a 3.9-kb *Hin*dIII-*Eco*RI fragment of pLAV1 (gift of T. Baldwin) (2, 14) which carried the *luxB* gene portions of the *luxAB* genes. A 4.6-kb *Hin*dIII-*Sma*I fragment of the resulting plasmid, pCF1, which carried the Ω fragment and the P_{psbAI}::*luxAB* gene fusion was removed to *Bgl*II fragment by the filling-in reaction with Klenow enzyme, followed by the addition of a *Bgl*II linker (d[pC AGATCTG]) and digestion with *Bgl*II. The fragment was ligated into the unique *Bgl*II site of plasmid A71 (gift of T. Ogawa) (23); this site is located downstream of the *Synechocystis ndhB* gene. The unique *Spl*I site of the resulting plasmid (pCF2), located 54 bp upstream of the translation start site of the *luxA* gene, was filled in with Klenow enzyme and converted to a *Bam*HI site by the addition of a *Bam*HI linker (d[pCGGATCCG]). A 230-bp *Bam*HI fragment of this plasmid (pCF3) which contained the *psbAI* promoter segment was removed to produce a promoterless reporter plasmid (pCF4). Then, a 2.2-kb *Bam*HI fragment of pDNAKb which carried a 2.16-kb upstream region of the *dnaK* gene (USR_{dnaK}) followed by a segment of the *dnaK* amino-terminal coding region (13 codons plus 2 bp) was inserted into the unique *Bam*HI site of pCF4. From this insertion, a plasmid which had the *dnaK* upstream segment in the same direction as the *luxAB* genes was selected as a *dnaK* reporter plasmid (pCF5). Another *dnaK* reporter plasmid which carried an 846-bp *Bst*EII-*Xcm*I segment of pDNAK was also constructed (pCF6). In this case, the *Bst*EII-*Xcm*I fragment of pDNAK was converted with T4 DNA polymerase to a blunt-ended fragment which carried a shorter 805-bp upstream region of the *dnaK* gene followed by a segment of the *dnaK* amino-terminal coding region (13 codons plus 2 bp) and inserted into the unique *Bam*HI site of pCF4 which had been converted to a blunt-ended site with Klenow enzyme.

Wild-type *Synechocystis* cells were transformed with plasmids pCF4, pCF5, and pCF6 by the procedure described by Williams (38), and spectinomycin-resistant transformant colonies were selected. Transformants resulting from homologous recombination between the reporter plasmid and the host chromosome were selected by Southern blotting analysis. All transformants tested showed that the predicted recombination events had occurred. The CFC2 and CFC3 strains obtained by transformation with pCF5 and pCF6, respectively, carried the USR_{dnaK}::*luxAB* (longer and shorter USR_{dnaK}, respectively) and the Ω fragment at the *Bgl*II site of the chromosome and lacked vector sequences (Fig. 1). The CFC1 strain obtained by transformation with pCF4 carried the Ω fragment and the coding region of the *luxAB* gene but did not include the *dnaK* promoter segment. Three kinds of transformants showed normal morphology and growth rate. Thus, the insertion of the reporter constructs into the *Bgl*II site downstream of the *ndhB* gene did not appear to be deleterious, so that this site can be used as a neutral integration site for exogenous DNA.

Assay of the bioluminescence rhythm. *Synechocystis* cells were plated on BG-11 agar in LL (46 μmol m^{-2} s^{-1} from white fluorescent lamps) at 30°C for 2 days and then maintained for 12 h in the dark to synchronize the circadian clocks in the various cells of each colony. After the dark period, an agar block (15 by 15 by 7 mm) with a patch of cells was cut out and transferred into a 20-ml vial, and an open, sterile microcentrifuge tube containing *n*-decanal dissolved in vacuum pump oil at a concentration of 1.5% (vol/vol) was placed upright in the

vial. Then, the vial was maintained in LL which was interrupted every 30 min for assay of the bioluminescence (25-min light and 5-min dark periods). We monitored the bioluminescence from cells on agar with a photomultiplier tube apparatus, as described previously (13, 14). Briefly, the vial was subjected to darkness for 100 s to allow chlorophyll fluorescence to decay, and the bioluminescence signal was accumulated for the next 100 s in the dark. After an additional exposure to darkness for 100 s, the vial was returned to light conditions. Measurements were repeated every 30 min for 5 days. The light fluence rate was 46 μmol m^{-2} s^{-1} during illuminated periods, and the temperature was maintained at 30°C unless otherwise specified. Brief, repetitive dark pulses during the assay protocol can have minor effects on circadian clocks but do not cause major interference (3).

The average level of bioluminescence of the *dnaK* reporter strains CFC2 and CFC3 was about 1/20 of that expressed by the *psbAI* reporter *Synechococcus* strain (AMC149 [13]). Thus, it was necessary to enhance the sensitivity of our apparatus by using a low-noise photomultiplier tube (R2693P; Hamamatsu Photonics Co., Hamamatsu, Japan) at 900 V, whose output was coupled to a photon-counting unit (C3866; Hamamatsu Photonics) to reduce thermal noise. The output pulses were counted by an electronic counter (model 7201; Iwatsu Co., Tokyo, Japan), and data were collected by data acquisition computers (PC-E200, Sharp Co., Osaka, Japan; Macintosh Plus, Apple Computer Co., Cupertino, Calif.). This modified apparatus was about 100 times more sensitive to light than the previously reported apparatus (14).

Mapping of the transcriptional initiation site of the *dnaK* gene by the primer extension method. Wild-type *Synechocystis* cells were grown in liquid BG-11 medium and harvested at the logarithmic growth phase. RNA was extracted from cells as described by Mohamed and Jansson (22). Primer extension experiments were carried out by standard procedures (29), with SuperScript II RNase H$^-$ reverse transcriptase (Gibco BRL) and nine primers: A (5′-CAGGAACGGT-GATGACGGCTTGGGTAACGGTCTCC-3′, +430 to +396; the first nucleotide A of the translational initiation codon of the *dnaK* gene is numbered +1), B (5′-GTACCGAGGTCAATCCCAACAACTTTTCCCATAAT-3′, +32 to −3), C (5′-AGTGACGGTAGGGGCTTAGTTCTCGGAAGTGCAGG-3′, −107 to −141), D (5′-GTCATCTTTGGTGGCGGAGCCCTTCTGCTGTT GAT-3′, −287 to −321), E (5′-ACTACCATCTGACCCACTTGCTGATT GAAGTTGGG-3′, −573 to −607), F (5′-GGAAAACATCTAGGGGGTCT TCTAGCAACCCACCT-3′, −669 to −704), G (5′-AGGGGATCGCCAGTAC GATTGATAATTAGGGCAGT-3′, −705 to −739), H (5′-GAATTGTCAGT GGTGAAGGAATCCGGCTCTAGTTG-3′, −1131 to −1165), and I (5′-GGATTATTGAAATAGGCCTTGGGGGAAAGGGTCTC-3′, −1188 to −1222). The 5′ ends of these primers were labeled with [γ-^{32}P]ATP (>5,000 Ci/mmol) by the T4 polynucleotide kinase reaction (T4 polynucleotide kinase from Promega Co.). Ten micrograms of *Synechocystis* RNA and 4 ng of ^{32}P-labeled primer (~10^5 cpm) in 30 μl of hybridization buffer were heated at 85°C for 10 min in a heat block for denaturation and then held overnight in the heat block without heating for annealing. RNA annealed with primer was recovered by ethanol precipitation and dried. The RNA and 5 μl (1,000 U) of reverse transcriptase (Gibco BRL) in a 50-μl reaction mixture for reverse transcriptase was incubated at 37°C for 2 h for the primer extension reaction. The reaction product was recovered by ethanol precipitation and applied to sequencing gels.

Northern (RNA) blotting analysis. Cells of the *dnaK* reporter *Synechocystis* sp. strain CFC2 were spread on BG-11 agar in 9-cm plastic dishes to yield about 5,000 colonies per dish. The dishes were incubated at 27°C in LL (38 μmol m^{-2} s^{-1}). When the colonies had grown to 0.3 mm in diameter (growth for about 10 days), the dishes were subjected to darkness for 12 h to synchronize the circadian clocks among cells in the colonies and then returned to LL. The cells were harvested every 4 h in LL from four dishes by washing each dish with 2 ml of BG-11 and collecting cell pellets by centrifugation, and then the pellets were immediately frozen in liquid nitrogen. RNA was extracted from each frozen sample as described by Mohamed and Jansson (22). RNA was subjected to electrophoresis on 1.0% formaldehyde-containing agarose gels (0.5 μg of total RNA was loaded per lane for *dnaK* mRNA gels and 4 μg of total RNA was loaded per lane for *luxAB* mRNA gels), blotted onto positively charged nylon membranes (Boehringer Mannheim GmbH, Mannheim, Germany), and hybridized with a ^{32}P-labeled probe specific for *dnaK* mRNA or *luxAB* mRNA by standard procedures (29). A 1.2-kb *Eco*RI fragment of the *dnaK* gene (the second nucleotide of codon 238 to the second nucleotide of codon 631) derived from pDNAK and a 1.2-kb *Pst*I-*Xcm*I fragment of the *luxAB* genes (the second nucleotide of codon 185 of the *luxA* gene to the first nucleotide of codon 233 of the *luxB* gene) derived from pLAV1 were labeled with [α-^{32}P]dCTP (3,000 Ci/mmol) by the nick translation reaction (random primer DNA labeling kit; Takara Shuzo) and used as hybridization probes. Hybridized bands were visualized and quantified with a Bio-Image Analyzer BAS2000 (Fuji Film Co., Kanagawa, Japan). The detection of radioactive bands with the BAS2000 is at least 20 times more sensitive than the usual X-ray film radioautographic detection method. The bioluminescence of the colonies on an agar block cut out from the dishes used for RNA extraction was also monitored for comparison by the procedure described above.
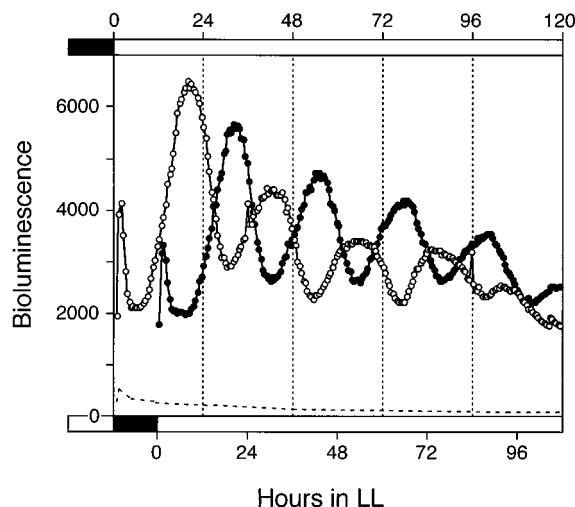
FIG. 2. Bioluminescence rhythms of the *dnaK* reporter strain CFC2 in LL. Cells of the *dnaK* reporter strain CFC2 were cultured at 30°C in LL (46 μmol m⁻² s⁻¹) on BG-11 agar for 2 days and subjected to darkness for 12 h to synchronize the circadian clock. Then, they were returned to LL for monitoring of bioluminescence. Expression of bioluminescence from two CFC2 cultures that had been subjected to 12-h dark periods which were 180° out of phase are shown. The 12-h dark periods (solid bars) and the LL (open bars) are shown on the upper (for open circles) and lower (for solid circles) abscissae. The bioluminescence of CFC1 cells, which carried the promoterless *luxAB* genes without the *dnaK* promoter segment, is also shown as a control (dashed line). The light regimen for the CFC1 culture is as shown on the upper abscissa. The vertical axis indicates bioluminescence intensity as photon counts per second. The first transient peaks of the bioluminescence should be ignored because they were artifacts of the saturation and penetration into cells of *n*-decanal vapor, which was added at the ends of the dark periods.

RESULTS AND DISCUSSION

Bioluminescence rhythms of the *dnaK* reporter *Synechocystis* strains. To monitor the expression of the *dnaK* gene (6) in *Synechocystis* sp. strain PCC 6803 as bioluminescence, we used the same procedure used for monitoring the expression of the *psbAI* gene in another unicellular cyanobacterium, *Synechococcus* sp. strain PCC 7942 (13, 14) except that our current apparatus is more sensitive to light (see Materials and Methods).

We first constructed a *dnaK* reporter plasmid, pCF4, which carries a USR_{dnaK}::*luxAB* gene fusion, namely, a 2.16-kb upstream region segment of the *dnaK* gene fused to a promoterless segment of the luciferase *luxAB* gene set from *V. harveyi* (Fig. 1). In this construct, the expression of the *luxAB* genes is under the control of the upstream region of the *dnaK* gene including the *dnaK* promoter, since activity from the upstream flanking region of the USR_{dnaK} in the *dnaK* reporter plasmid is blocked by a transcriptional terminator located in the Ω fragment. To construct a *dnaK* reporter *Synechocystis* strain (CFC2), we transformed wild-type *Synechocystis* cells with the reporter plasmid and integrated the reporter gene without vector sequences into a specific integration site of the chromosome, a *Bgl*II site downstream of the *ndhB* gene (23), via homologous recombination. Growth of the *dnaK* reporter strain was normal and thus was apparently unaffected by the insertion of the reporter gene into this integration site in the chromosome.

Figure 2 shows the time course of bioluminescence measured from the *dnaK* reporter strain maintained in LL at 30°C. To synchronize a presumed circadian clock, we exposed two cultures to dark intervals (12 h in duration) that were 180° out of phase before the onset of LL and then returned them to LL

to monitor their bioluminescence. The bioluminescence from both cultures oscillated with a period of 22.4 ± 0.4 h (data from four separate experiments) but with opposite phases, which depended upon the timing of the prior 12-h dark interval. The peak of bioluminescence occurred at about 19.1 h after the onset of LL. The peak-to-trough ratio of the rhythms was about 3 on the first day of LL, but it decreased gradually. These results indicate that the bioluminescence rhythm was the expression of an endogenous circadian clock, because the rhythms continued under constant conditions with a period of about 1 day, and also indicate that the phase of the rhythm was entrained by the 12-h dark intervals which preceded LL.

As a control, we also monitored the bioluminescence from a control reporter strain (CFC1) which had only a promoterless segment of the *luxAB* genes and lacked an exogenous promoter segment (Fig. 2). The level of the bioluminescence was about 1/40 of the average level of the bioluminescence from the *dnaK* reporter strain CFC2, and as predicted, the bioluminescence showed no obvious oscillation. This result indicates that the bioluminescence rhythm of the *dnaK* reporter strain was mainly controlled by the upstream region of the *dnaK* gene.

The 2.16-kb upstream region of the *dnaK* gene used here as the USR_{dnaK} also carried an uncharacterized upstream open reading frame, *ORF70-5* (6), which is adjacent to the 5′ region of the *dnaK* gene and may also carry additional upstream genes. Thus, CFC2 reported the *dnaK* gene expression from an additional upstream promoter(s) as well as that controlled by the major *dnaK* promoter. To monitor the activity of the major *dnaK* promoter, we constructed another *dnaK* reporter strain, CFC3, which was the same as *dnaK* reporter strain CFC2 except that strain CFC3 carried an 846-bp upstream region segment of the *dnaK* gene as a promoter. This segment carries the 3′ portion of the *ORF70-5* but lacks the 5′ portion of the *ORF70-5*, including its promoter region. We monitored the bioluminescence from strain CFC3 (Fig. 3b). The level, amplitude, and waveform of the bioluminescence rhythm from strain CFC3 were very similar to those of the bioluminescence rhythm from strain CFC2 (Fig. 3a and b). This result indicates that the bioluminescence rhythm of the *dnaK* reporter strains
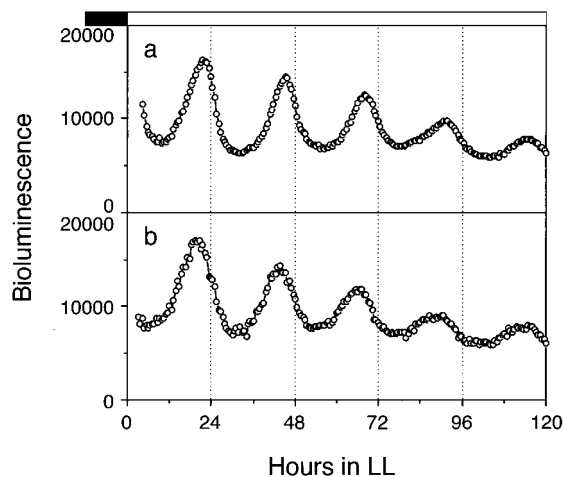

FIG. 3. Bioluminescence rhythms of the *dnaK* reporter strains CFC2 and CFC3 in LL. Cells of the *dnaK* reporter strains CFC2 (a) and CFC3 (b) were subjected to darkness for 12 h to synchronize the circadian clock. Then, they were returned to LL for monitoring of bioluminescence. The light regimen is shown on the upper abscissa. Other conditions were the same as described in the legend to Fig. 2 except that the light intensity was 23 μmol m⁻² s⁻¹.

was mainly controlled by a major *dnaK* promoter which should be located in the 846-bp upstream region of the *dnaK* gene.

Mapping of the transcriptional initiation site of the *dnaK* gene by primer extension. Northern blotting experiments demonstrated two *dnaK* transcripts, a 2.2-kb major transcript and a minor longer transcript (see Fig. 5d; the minor band is not visible in Fig. 5d), as described previously (6). We mapped the transcriptional initiation site of the *dnaK* gene by the primer extension method with nine primers (A to I). We found a single primer extension band which corresponds to an initiation site at T which is located at base 1278 upstream from the translational initiation codon of the *dnaK* gene by primer H (-1131 to -1165) (the nucleotide number of the T is -1278 when the first nucleotide A of the translational initiation codon of the *dnaK* gene is numbered $+1$). This transcriptional initiation site probably corresponds to a minor longer *dnaK* transcript. We also mapped a major *dnaK* transcriptional initiation site which should be located on the 846-bp upstream region of the *dnaK* gene by using primers A ($+430$ to $+396$), B ($+32$ to -3), C (-107 to -141), D (-287 to -321), E (-573 to -607), F (-669 to -704), and G (-705 to -739), but we could not find any clear primer extension band. When we used primer E, we detected a doublet band in primer extension and stop bands in all lanes in a sequencing gel which corresponded to the double band in primer extension. Thus, the primer extension reaction might be hindered by a possible secondary structure of a major shorter *dnaK* transcript or for unknown reasons.

Temperature compensation of the bioluminescence rhythm. Another key characteristic of circadian rhythms is the temperature compensation of the period. We examined the effects of temperature on the period length of the bioluminescence rhythm. After synchronization of the circadian clock by exposure to a 12-h dark period, we maintained cells of the *dnaK* reporter strain CFC2 at 25, 30, or 35°C in LL and monitored their bioluminescence. Figure 4a shows the time course of the bioluminescence at the three different temperatures in LL. The period lengths of the rhythm were almost constant and were found to be 22.6 ± 0.1, 22.4 ± 0.4, and 21.2 ± 0.4 h at 25, 30, and 35°C, respectively. The rhythms were more obvious at lower temperatures, especially for the first 3 days. Even at 35°C, however, clear peaks and troughs of the rhythm were observed, although the amplitude of the rhythm was low. We plotted the period length against temperature (Fig. 4b) and calculated the Q_{10} value for frequency (1/period) to be 1.1 (the period decreased by 1.4 h when the temperature increased from 25 to 35°C). This indicates that the period length of the bioluminescence rhythm was well compensated for against changes in ambient temperature.

Therefore, the bioluminescence rhythm of the *dnaK* reporter strain CFC2 satisfied the three criteria for circadian rhythms: persistence in constant conditions (Fig. 2), phase resetting by light-dark signals (Fig. 2), and temperature compensation of the period (Fig. 4). Thus, we can conclude that both cyanobacterial species *Synechocystis* sp. strain PCC 6803 and *Synechococcus* sp. strain PCC 7942 (14) have circadian pacemakers that exhibit the same basic properties as those of eucaryotic organisms.

Rhythmic changes in the abundance of *dnaK* mRNA in LL. The data described above suggest that the expression of the *dnaK* gene was controlled by the circadian clock. To test this possibility more directly, we examined the changes in the abundance of *dnaK* mRNA and that of *luxAB* mRNA of the *dnaK* reporter strain CFC2 during LL. After synchronization of the circadian clock by a 12-h dark interval, we maintained cells of the *dnaK* reporter strain at 27°C in LL and extracted RNA from cells harvested at 4-h intervals. We estimated the relative
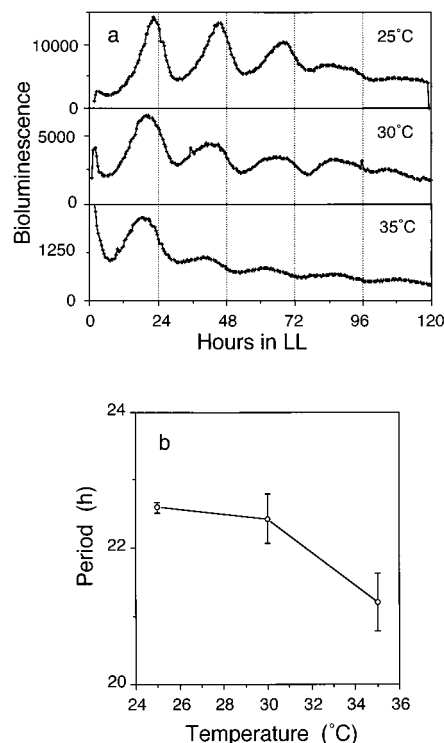

FIG. 4. Temperature compensation of the period of the bioluminescence rhythms. After the 12-h dark period to synchronize the circadian clock, CFC2 cells were maintained in LL (46 μmol m^{-2} s^{-1}) at three different temperatures (25, 30, and 35°C) to have their bioluminescence monitored. (a) Rhythms of bioluminescence at the three different temperatures. (b) Periods of the rhythms plotted as a function of temperature. The bars indicate the standard deviation of at least three replicate experiments. Other conditions were the same as described in the legend to Fig. 2.

abundance of *dnaK* mRNA and of *luxAB* mRNA at various times in LL by Northern blotting analysis with ^{32}P-labeled probes.

The *dnaK* mRNA bands were two bands, a longer discrete band with a smear, which corresponds to *dnaK* mRNA and its degradation products, and a shorter uncharacterized band (Fig. 5d). The shorter band was a discrete band and might be a processed *dnaK* mRNA or an antisense transcript of the *dnaK* gene. The *luxAB* mRNA bands were smears (Fig. 5e).

Figures 5a and b show the rhythmic changes in the abundance of *dnaK* mRNA and *luxAB* mRNA during LL. We also included a profile of the bioluminescence rhythm for comparison (Fig. 5c). We found that both mRNAs showed daily rhythmic changes in their abundance. The peak-to-trough amplitude (the mean ratio of the first peak value to the second trough value obtained from two or three separate experiments) of the rhythms in *dnaK* mRNA and *luxAB* mRNA was about 1.8 and 3.3, respectively. The peaks of the rhythm in the *luxAB* mRNA abundance occurred at approximately the same times as those of the bioluminescence rhythm (Fig. 5c), that is, at 20 and 44 h. However, the peaks of the rhythm in the *dnaK* mRNA abundance were delayed by 4 to 8 h compared with those of the rhythm in *luxAB* mRNA abundance. The abundance differed greatly between *dnaK* mRNA and *luxAB* mRNA. The average abundance of *luxAB* mRNA was about 10-fold less than that of *dnaK* mRNA. This difference in phase of the mRNA rhythms and the low abundance of *luxAB* mRNA may reflect differences in the decay rates of the two mRNAs.
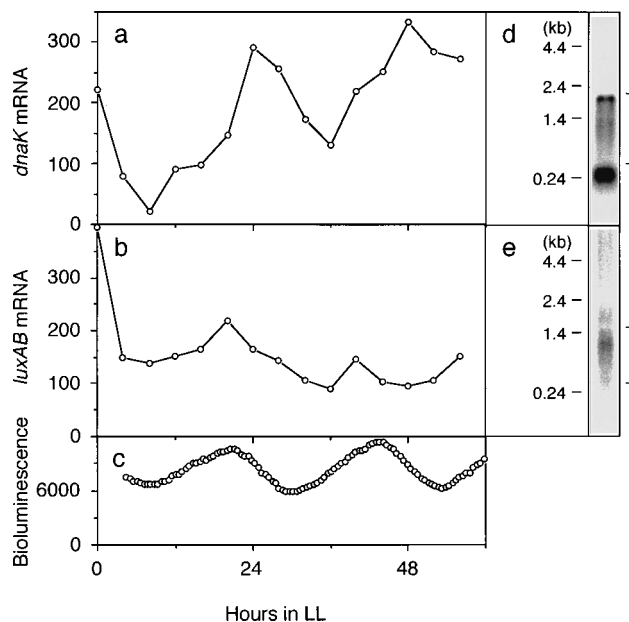
FIG. 5. Rhythmic changes in the abundance of *dnaK* mRNA in LL. CFC2 cells were grown on BG-11 agar in plastic dishes at 27°C in LL (38 μmol m^{-2} s^{-1}) until the colonies grew to 0.3 mm in diameter. Then, the cells were subjected to darkness for 12 h to synchronize the circadian clock and thereafter maintained in LL. The cells from aliquots of dishes were harvested every 4 h in LL, and RNA was extracted from each sample. The abundance of *dnaK* mRNA and *luxAB* mRNA was estimated by Northern blotting analyses with ^{32}P-labeled probes specific for *dnaK* mRNA and *luxAB* mRNA, respectively. Hybridized bands were visualized by a Bio-Image Analyzer BAS2000 (d, *dnaK* mRNA; e, *luxAB* mRNA), and the relative radioactivity of the bands was also quantified by BAS2000 and plotted against time in LL. Estimated regions of the bands with smear are also shown by the lines in panels d and e. Bioluminescence from the colonies on a BG-11 agar block is also shown for comparison. (a) *dnaK* mRNA; (b) *luxAB* mRNA; (c) bioluminescence.

These data indicate that a circadian clock controls the activity of the *dnaK* promoter, resulting in daily rhythmic changes in both *dnaK* mRNA and *luxAB* mRNA levels. Although we have not yet determined the intracellular level of bacterial luciferase, the bioluminescence rhythm exhibited by the *dnaK* reporter strain probably results from rhythmic changes in the intracellular level of luciferase. The *psbAI* reporter derivative of *Synechococcus* sp. strain PCC 7942 (AMC149) showed daily changes in the intracellular level of luciferase (14, 19). As discussed previously (14), the bioluminescence reaction catalyzed by bacterial luciferase can be influenced not only by changes in the intracellular level of the luciferase but also by changes in the levels of its substrates, a reduced form of flavin mononucleotide (FMNH$_2$), *n*-decanal, and O$_2$. The effective level of *n*-decanal is expected to be constant because it was supplied exogenously, but the levels of FMNH$_2$ and O$_2$ are unknown. Therefore, other steps for generating bioluminescence could also be possibly controlled by the circadian clock, e.g., degradation of *luxAB* mRNA, translation of *luxAB* mRNA (de novo synthesis of luciferase), degradation of luciferase, and metabolic control of the intracellular levels of FMNH$_2$ and/or O$_2$. However, because the phase of bioluminescence rhythms reported for various genes in *Synechocystis* sp. strain PCC 6803 (as well as in *Synechococcus* sp. strain PCC 7942 [20]) differs from gene to gene (unpublished data; see also below), it is not easy to determine at which step(s) the circadian clock is regulating bioluminescence.

We demonstrate here that the circadian clock controls the expression of the *dnaK* gene in this *Synechocystis* strain. At present, we do not know how the clock controls the activity of the *dnaK* promoter. Our monitoring system with the luciferase *luxAB* genes as a reporter should be useful in solving this problem at the molecular level. Because DnaK has a key role in controlling the expression of other heat shock (or stress) genes in *E. coli* (34, 36), such downstream genes are also likely to be controlled by the clock via DnaK. We want to verify this circadian control cascade by the same method as described here. Circadian control of the expression of the *dnaK* gene may be involved in the adaptation to 24-h high-temperature/low-temperature cycles resulting from the day-night cycles on the earth. Consistent with that idea, the circadian clock in the *Synechocystis* strain can be reset not only by light-dark cycles but also by low-temperature/high-temperature cycles (unpublished data). An interesting question is whether heat shock proteins, including DnaK, are involved in the temperature-sensing mechanism of the circadian clock.

There are several differences between the bioluminescence rhythms of the reporter strains in *Synechocystis* sp. strain CFC2 versus *Synechococcus* sp. strain AMC149 (13, 14). The period of CFC2 (22.4 h) is shorter than that of AMC149 (24 h) by 1.6 h at 30°C. We know that this difference in period is not due to the difference between using promoters from the *dnaK* and *psbAI* genes. For example, we have examined many other genes in *Synechococcus* sp. strain PCC 7942 (20) and several other genes in *Synechocystis* sp. strain PCC 6803 (unpublished data) and have found that although the peak positions of bioluminescence reported for the promoters of different genes are different, the period of the bioluminescence rhythms is specific to each species: 22.4 h in the *Synechocystis* strain and 24 h in the *Synechococcus* strain. The peak of the rhythm of CFC2 occurred 19.1 h after the onset of LL, which corresponds to 3 h before subjective dawn ("dawn" in continuous conditions) when cells have been entrained under 24-h light-dark cycles, while the peak of the rhythm expressed by AMC149 occurs 12 h after the onset of LL, which corresponds to subjective dusk ("dusk" in continuous conditions). The rhythm of CFC2 persisted for only 5 days, whereas that of AMC149 continued for more than 2 weeks. Thus, the optimal conditions for persistence of the rhythms may be different between these two species, or desynchronization of the clocks of individual cells in CFC2 might occur earlier than in AMC149. Furthermore, the average level of bioluminescent signals obtained from CFC2 was only 1/20 of that from AMC149. At present, it is not feasible to monitor bioluminescence rhythms of individual colonies of CFC2 on agar plates by an automated monitoring system which has recently been developed for screening for clock mutants of AMC149 (15).

It has become evident that a circadian clock exists in various kinds of cyanobacteria, whether they are unicellular or filamentous, nitrogen fixing or nonfixing. The two species characterized by us, *Synechocystis* sp. strain PCC 6803 and *Synechococcus* sp. strain PCC 7942, are unicellular cyanobacteria that do not fix nitrogen. Cell division of the unicellular cyanobacterium *Synechococcus* sp. strain WH7803, which does not fix nitrogen, is controlled by a circadian clock (35). In the nitrogen-fixing unicellular cyanobacteria *Synechococcus* sp. strain Miami BG 043511 (21), *Synechococcus* sp. strain RF-1 (5, 10), and *Cyanothece* sp. strain ATCC 51142 (31), metabolic activities such as nitrogen fixation, amino acid uptake, photosynthetic oxygen evolution, and carbohydrate granule formation or cell division are also under the control of the clock. A circadian clock also exists in nitrogen-fixing filamentous cyanobacteria, such as *Oscillatoria* sp. strain 23 (33), *Trichodesmium thiebautii* (28), and *Anabaena* sp. strain PCC 7120, as we recently discovered by using the luciferase reporter system

described here (10a). In relation to the evolution of the circadian clock, it will be interesting to determine whether organisms that have a lower or higher organization than that of cyanobacteria but are not too distantly related to cyanobacteria have a similar circadian clock. We should be able to address this question by using our luciferase reporter system.

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