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# Bacterial Luciferase as a Reporter of Circadian Gene Expression in Cyanobacteria

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**To allow continuous monitoring of the circadian clock in cyanobacteria, we previously created a reporter strain (AMC149) of *Synechococcus* sp. strain PCC 7942 in which the promoter of the *psbAI* gene was fused to *Vibrio harveyi* luciferase structural genes (*luxAB*) and integrated into the chromosome. Northern (RNA) hybridization and immunoblot analyses were performed to examine changes in abundance of the *luxAB* mRNA, the native *psbAI* mRNA, and the luciferase protein to determine whether bioluminescence is an accurate reporter of *psbAI* promoter activity in AMC149. Under constant light conditions, the mRNA abundances of both *luxAB* and *psbAI* oscillated with a period of approximately 24 h for at least 2 days. The expression of these two genes followed the same pattern: both mRNAs peaked in the subjective morning, and their troughs occurred near the end of the subjective night. The amount of luciferase protein also oscillated with a period of approximately 24 h, and the protein rhythm is in phase with the bioluminescence rhythm. The rhythm of the luciferase mRNA phase-leads the rhythms of luciferase protein and in vivo bioluminescence by several hours. Comparable results were obtained with a short-period mutant of AMC149. Together, these results indicate that the bioluminescence rhythm in AMC149 is due primarily to circadian oscillation of *psbAI* promoter activity in this cyanobacterium.**

Despite many years of study, the molecular mechanisms of circadian rhythms are essentially unknown. Although a wide variety of organisms manifest circadian rhythms (2), most researchers believed until recently that circadian clocks required eukaryotic cell organization (5, 14, 31, 33, 35). A few years ago, however, circadian rhythms in the prokaryotic cyanobacteria were reported (9, 25, 34). Those unexpected results have required a reevaluation of the level of cellular complexity required for circadian rhythmicity.

Our research is directed towards understanding the molecular basis of circadian gene expression in cyanobacteria. Although rhythms of mRNA and protein abundances have been used as molecular markers for circadian rhythms (11, 15), these measurements are time-consuming, technically difficult, and practically impossible to use for continuous monitoring of circadian rhythms in vivo for many cycles. A method of circumventing these difficulties is to construct a reporter strain which expresses an automatically assayable phenotype, as has been done with a firefly luciferase reporter in *Arabidopsis thaliana* (13, 24). In our previous study (17), we reported the development of a circadian-reporter strain of cyanobacteria (named AMC149). AMC149 was made by transforming wild-type *Synechococcus* sp. strain PCC 7942 with a genetic construct in which the promoter for the *psbAI* gene, which encodes the D1 protein of photosystem II, drives the bacterial luciferase genes. This reporter strain expresses a robust bioluminescence rhythm in vivo that can be continuously assayed by systems which automatically monitor the luminescence from liquid cultures (17) and from colonies on agar (16, 18). The bioluminescence rhythm of AMC149 satisfies all the criteria of circadian rhythms (17, 28): a period of approximately 24 h in constant conditions, phase resetting by light-dark (LD) signals, and temperature compensation of the period.

So that subsequent research using this strain can be interpreted properly, it is important to characterize the molecular events that lead to the rhythm of bioluminescence. In particular, we want to confirm our expectation that the rhythm of bioluminescence in AMC149 is due to circadian control of the activity of the *psbAI* promoter. Because bacterial luciferase catalyzes the reaction $FMNH_2 + n$-decanal $+ O_2 \rightarrow$ FMN + $n$-decanoic acid $+ H_2O$ + light (where FMN is flavin mononucleotide), the level of bioluminescence could be changed not only by changes in the level of luciferase protein but also by changes in the level of one or more of the substrates. We need to know at what level the bioluminescence rhythm is regulated and how well this *luxAB* reporter reflects the expression patterns of the native *psbAI* gene.

In this study, we measured the levels of luciferase mRNA and protein and of *psbAI* mRNA throughout the circadian cycle. After comparing the expression patterns of *luxAB* mRNA, luciferase protein, and bioluminescence, we conclude that the bioluminescence rhythm expressed by AMC149 is due to rhythmic changes in abundance of reporter gene transcript. Comparable experiments using a short-period mutant of AMC149 (named SP22) further support our conclusions. The correspondence of the expression pattern of *luxAB* mRNA with that of the native *psbAI* mRNA suggests that the bioluminescence rhythm of AMC149 is an accurate reporter of the activity of the native *psbAI* promoter.

## MATERIALS AND METHODS

**Strains.** The strains used in this study were AMC149, SP22, and wild-type *Synechococcus* sp. strain PCC 7942. Strain AMC149, described by Kondo et al. (17), contains a cassette in which the *psbAI* promoter was transcriptionally fused to promoterless *Vibrio harveyi* luciferase structural genes (*luxAB*). This reporter construct was recombined and integrated into a neutral site of the wild-type *Synechococcus* sp. genome. Strain SP22 was isolated after chemical mutagenesis

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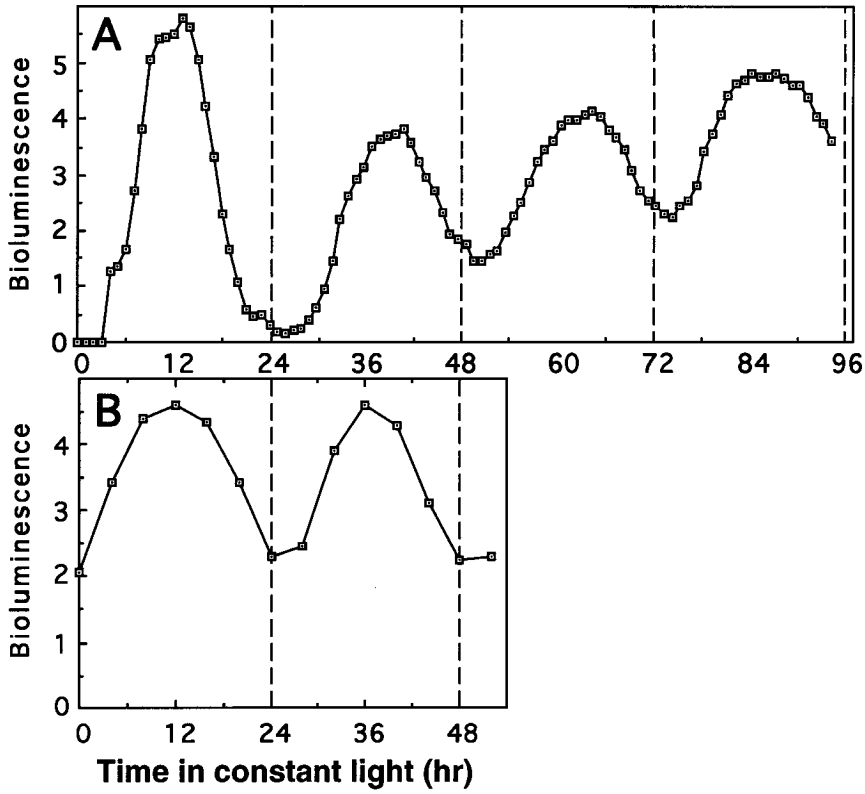


FIG. 1. Bioluminescence rhythms of AMC149 cultures. (A) Continuous bioluminescence monitoring. At the beginning of the sample collection, 1 ml of culture was placed in a 20-ml vial, and 0.3 ml of decanal was added to a microcentrifuge tube which was placed inside the vial. Then the vial was sealed and placed in the continuous bioluminescence monitoring apparatus under constant light conditions (40 to 50 microeinsteins $m^{-2}$ $s^{-1}$) at 30°C. The bioluminescence level of the sample was automatically measured every hour for several days. Relative bioluminescence levels are shown. (B) Single-point bioluminescence measured at the time of sampling. At each time point, 1 ml of culture was removed, placed in a 20-ml vial, and treated with decanal as described above. Then the sealed vial was maintained in light for 1 h to allow the decanal to saturate the culture. After 1 h of incubation, the bioluminescence level of the sample at this time point was measured with a luminometer apparatus.

of AMC149, and it has a shorter period (~22 h) of the circadian bioluminescence rhythm (18).

**Growth conditions and harvesting protocol.** All strains were grown in BG-11 medium (1) modified as described by Bustos and Golden (3). The wild-type strain was grown without any antibiotics, whereas AMC149 and SP22 were cultured with spectinomycin sulfate (40 μg/ml). Cultures were grown at 30°C with continuous aeration and illumination (~40 to 50 microeinsteins $m^{-2}$ $s^{-1}$). When the cell density reached an optical density at 750 nm ($OD_{750}$) of approximately 0.15 to 0.20, the cell cultures were placed in a cycle of 12 h of light and 12 h of darkness for 2 days and then returned to constant light (aeration and 30°C maintained throughout). Sample collection was started at the beginning of the constant light conditions which followed the LD cycle (cell density, $OD_{750}$ of ~0.3). Samples were collected every 4 h for up to 52 h. Cultures were diluted with fresh spectinomycin-containing BG-11 medium to an $OD_{750}$ of 0.3 at each time point to maintain an approximately constant cell concentration (dilution was done after the sample for bioluminescence measurement had been collected). Culture samples (40 ml for RNA isolation and 5 ml for protein assay) were collected by centrifugation at each time point. The cell pellet for RNA isolation was quickly frozen in liquid nitrogen and kept at −70°C prior to extraction. The cell pellet for protein assay was washed once with 50 mM Tris-HCl (pH 6.8), resuspended in 0.5 ml of 50 mM Tris-HCl (pH 6.8), quickly frozen in liquid nitrogen, and kept at −70°C before the assay.

For the growth curve experiment (see Fig. 6), both wild-type *Synechococcus* sp. and AMC149 cells were shaken in BG-11 medium without spectinomycin in constant light at 30°C. Two equal samples of both strains were inoculated at time zero and grown under 40 to 50 and 25 to 30 microeinsteins $m^{-2}$ $s^{-1}$. At each time point, cell density was measured as $OD_{750}$.

**Measurement of in vivo bioluminescence.** Two different techniques for measuring in vivo bioluminescence were used. For single-point measurement of bioluminescence at the time of the sampling, 1 ml of cell culture was transferred to a 20-ml vial at each time point. Then, 0.3 ml of 10% *n*-decanal was added to an open microcentrifuge tube which was placed upright in the vial. The vials were sealed and placed under constant light (40 to 50 microeinsteins $m^{-2}$ $s^{-1}$) at 30°C for 1 h. This 1-h incubation allowed the decanal vapor to saturate the vapor phase of the culture. The bioluminescence of the culture was then measured with a luminometer apparatus (10). For continuous measurement, vials were prepared as described above at the beginning of the experiment and placed under the same light conditions in an automated bioluminescence monitoring apparatus for continuous measurement for several days as described previously (17).

**RNA extraction and Northern (RNA) blot analysis.** Total RNA was prepared from frozen pellets as described previously (20). Equal amounts of total RNA (about 5 μg per well) were separated by electrophoresis in formaldehyde-containing RNA gels and transferred onto nylon membranes (Hybond-N; Amersham, Arlington Heights, Ill.) as described by Sambrook et al. (29). The membranes were then probed with radiolabeled nucleic acid probes for the bacterial luciferase structural genes (*luxAB*), *psbAI*, and rRNA genes. The *luxAB* probe was a *Sal*I and *Eco*RI fragment which included the whole *luxA* gene and part of *luxB* (4, 12). It was labeled with [α-$^{32}$P]dCTP by the random prime method (DECAprime DNA labeling kit from Ambion Inc., Austin, Tex.). The *psbAI* probe was an antisense RNA probe which specifically recognizes the *psbAI* message (19); it was labeled with [α-$^{32}$P]UTP by using an in vitro transcription kit (Ambion Inc.). The rRNA probe recognizes all three rRNA messages, 23S, 16S, and 5S rRNAs (36), and it was labeled by the nick translation method (Nick Translation System; Bethesda Research Laboratories, Inc., Gaithersburg, Md.). A computerized densitometer was used to quantify the autoradiograms of the RNA gel blots (Technology Resources model MC1000). rRNA signals were used as a control to normalize equal loading of RNA in each lane.

**Protein assays.** The cell suspension was thawed and mixed with 0.1-mm-diameter glass beads, and the cells were broken by using a Minibead Beater (Biospec Products, Bartlesville, Okla.) at high speed for two cycles of shaking, each cycle lasting 80 s (4°C). After a brief centrifugation, the supernatant fraction was collected for determination of total protein by using Spector's modification of the Bradford method (32).

Equal amounts of total protein (~1 mg) from each sample were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for immunoblot analyses. Proteins were transferred to nitrocellulose membranes (pore size, 0.22 μm; Schleicher & Schuell) with a Genie electrophoretic blotter (Idea Scientific Co., Minneapolis, Minn.) and incubated with a polyclonal rabbit anti-

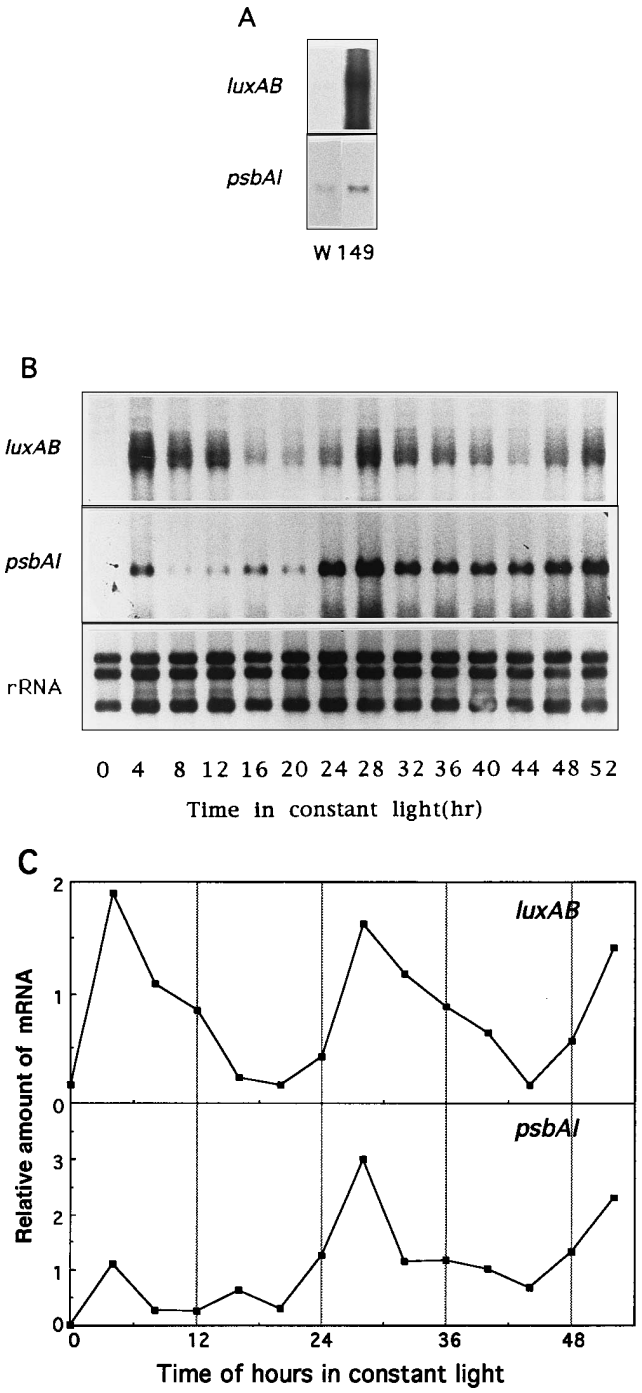


FIG. 2. Northern blot analyses of *luxAB* and *psbAI* mRNAs and rRNAs. After about 4 days of growth in constant light (30°C) with aeration, cells were entrained by 2 days of LD cycles. At the end of the second dark interval, the cultures were transferred to constant light (time zero). Samples were thereafter collected every 4 h for 52 h. (A) Northern blot analysis of wild-type *Synechococcus* sp. strain PCC 7942 versus AMC149 mRNAs. The two samples were collected at the same time (12 h in constant light) and probed with the *luxAB* and *psbAI* probes, respectively. Lane W, wild-type *Synechococcus* sp.; lane 149, AMC149. (B) At each of the indicated times, total RNA of AMC149 was extracted from the cells. Total RNA from each time point was separated by electrophoresis, blotted, and hybridized with the *luxAB* and *psbAI* probes. The same blots were stripped and reprobed with the probe for rRNA (to normalize for equal loading of all lanes). (C) Densitometric measurements of the band densities for *luxAB* and *psbAI* bands in panel B. The values were normalized to the rRNA band densities. Abscissa, time in hours in constant light. In these and all subsequent experiments, the cell concentration of the original culture was maintained at a relatively constant level by diluting to an $OD_{750}$ of ~0.3 at each time point.

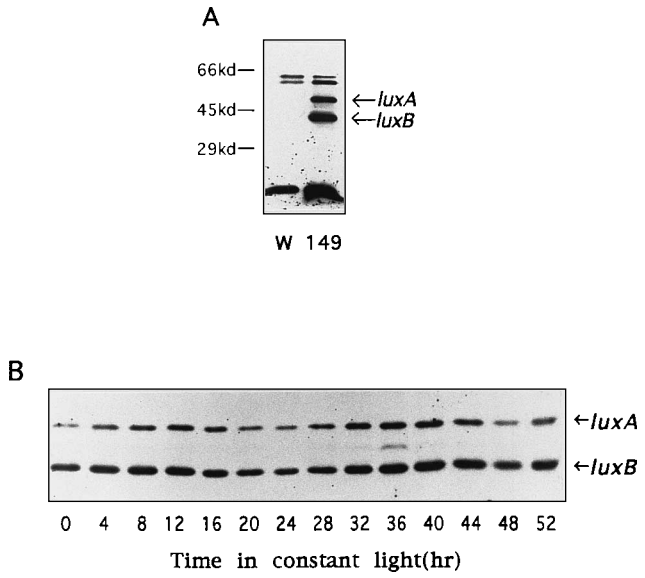


FIG. 3. Immunoblot analysis. (A) Western blot analysis of wild-type *Synechococcus* sp. and AMC149. Protein samples were collected at the same time (after 12 h in constant light) and probed with the antibody to luciferase. Molecular mass standards (in kilodaltons) are indicated on the left. Lane W, wild-type *Synechococcus* sp.; lane 149, AMC149. (B) Total protein prepared from the same time course cultures used for RNA extraction, separated by SDS-PAGE, blotted, and probed with the antiluciferase antibody.

serum to bacterial luciferase (37). Antibody binding to the blots was visualized with the ECL Western blotting (immunoblotting) analysis system (Amersham) for chemiluminescence assay, using the protocol recommended by the manufacturer. The resulting autoradiograms of the blots were analyzed by the computerized densitometer.

## RESULTS

**Bioluminescence rhythms in the reporter strain AMC149.** Previously, we showed that AMC149, which has a *PpsbAI*::*luxAB* fusion construct, expresses a robust bioluminescence rhythm under constant light conditions (17). Figure 1A shows comparable results from continuous bioluminescence monitoring of the AMC149 cells used in this study under standard conditions of constant light (intensity, ~40 to 50 microeinsteins $m^{-2}$ $s^{-1}$) at 30°C. The in vivo activity of luciferase in a culture of AMC149 can also be measured at single time points by withdrawing samples, incubating them with decanal for an hour, and then measuring the bioluminescence with a luminometer (Fig. 1B). This method of measurement also shows a robust daily cycle under constant illumination which is in phase with that measured by continuous bioluminescence monitoring (Fig. 1A). The peaks of the rhythm occurred at the end of the subjective day (at 12 and 36 h), and the troughs occurred at the end of the subjective night (at 24 and 48 h).

**Rhythms of mRNA and protein levels.** In order to determine how accurately this bacterial luciferase construct reports *psbAI* promoter activity, we measured the levels of *psbAI* mRNA, *luxAB* mRNA, and luciferase protein. Figure 2A depicts the results of a Northern blot analysis of *psbAI* and *luxAB* mRNAs in AMC149 and wild-type cells. As expected, no signal was detected in the wild-type extracts, whereas a strong signal was obvious in AMC149 cell extracts (Fig. 2A). Also as expected, *psbAI* message was detected in both strains (Fig. 2A). Note that the *luxAB* signal from AMC149 is not a distinct band, suggesting a short half-life for the message, as previously reported for a *lacZ* reporter transcript (30).

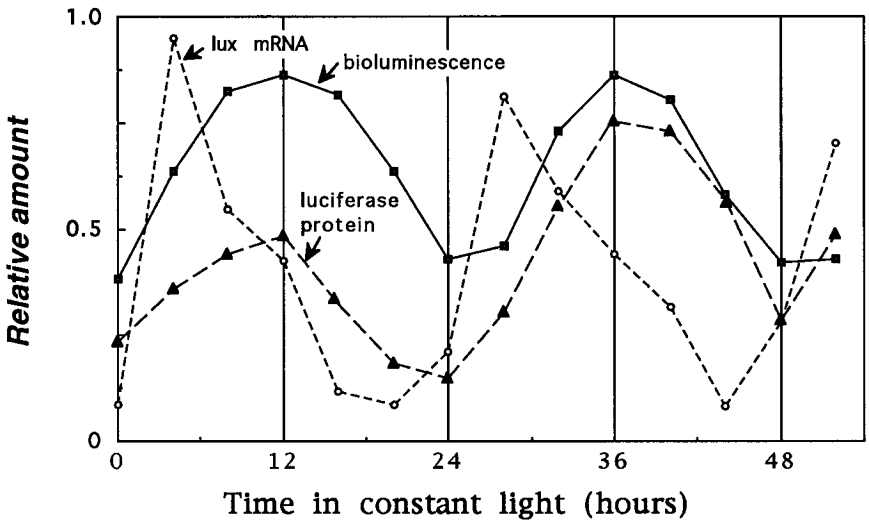


FIG. 4. Comparison of phase relationships of bioluminescence, *luxAB* mRNA, and luciferase protein rhythms. Bioluminescence data are from Fig. 1, mRNA data are from Fig. 2, and protein data are from a densitometric analysis of the luciferase protein band (LuxA) in Fig. 3. Relative levels of bioluminescence or of mRNA or protein abundance are shown.

We investigated the expression pattern of luciferase mRNA under constant light conditions. AMC149 cells were harvested at 4-h intervals under constant illumination, starting from the end of the second dark period of the LD cycle. Figure 2B shows typical results of a Northern blot analysis of total RNA extracted from these samples. The relative amounts of *luxAB* and native *psbAI* mRNAs are rhythmic, with a period close to 24 h, and they are also in phase with each other. The peaks occurred near the middle of the subjective day (at 4 to 8 and at 28 to 32 h), and the troughs occurred at the end of the subjective night (at 20 to 24 and at 44 to 48 h). rRNA levels were used as a control, and they were generally constant throughout the time course of the experiment. These experiments were repeated five times with similar results. The rhythm of *luxAB* mRNA levels generally showed a higher amplitude than that of *psbAI* mRNA. In two experiments, the *psbAI* mRNA levels showed a discernible cycle for only 1 day, whereas the *luxAB* mRNA levels continued to oscillate with a high-amplitude rhythm (data not shown). The waveforms of the *luxAB* and *psbAI* mRNA rhythms are asymmetric; the amount of message increases rapidly in the early subjective day and decreases gradually throughout the rest of the day (Fig. 2C). The fact that the unstable *luxAB* mRNA is rhythmically abundant suggests that the bioluminescence rhythm is regulated primarily at the transcriptional level.

The mRNA rhythms (Fig. 2C) seem to phase-lead the bioluminescence rhythm (Fig. 1). Especially in the case of the peak values, the mRNA rhythms are maximal about 4 to 8 h earlier than the bioluminescence rhythm, while the troughs of the mRNA and bioluminescence rhythms differ by only about 2 to 3 h. Dilution of the cells did not have any effect on gene expression; in some experiments, samples were collected without dilution of the original culture so that the culture became denser during the course of the experiment, and a similar pattern of mRNA expression was found (data not shown).

We then determined whether the amount of luciferase protein changes under the same experimental conditions. Figure 3A shows an immunoblot which compares the wild-type *Synechococcus* sp. with AMC149. The antiserum specifically recognized two bands which correspond to *luxA* and *luxB* proteins in the AMC149 sample (Fig. 3A). There is some nonspecific binding to other bands (at 60 and 62 kDa) in the wild-type sample as well as in AMC149. The immunoblot analysis of total proteins from the same set of samples that was used in the RNA time course analysis (Fig. 2C) is illustrated in Fig. 3B. The relative abundance of the specific luciferase protein bands is also rhythmic. The luciferase protein rhythm is in phase with the bioluminescence rhythm; as shown in Fig. 4, the peaks occurred at the end of the subjective day (at 12 and 36 h) and the troughs occurred at the end of the subjective night (at 24 and 48 h).

**Molecular rhythms in a short-period mutant.** If rhythmic transcription and translation are integral to the rhythmic expression of AMC149, a similar expression pattern should occur in some other reporter strains. SP22, a mutant with a short (~22-h) period, was isolated by chemical mutagenesis of AMC149 (18). The genomic site of this mutation does not occur within the *psbAI*::*luxAB* construct (18). Figure 5 shows the results of measurements of relative bioluminescence, *luxAB* mRNA, and luciferase protein levels for SP22 compared with those of AMC149. As predicted, both *luxAB* mRNA and luciferase protein levels cycled in SP22, and the luciferase protein rhythm is in phase with the bioluminescence rhythm. The peaks of mRNA rhythm were earlier than those of the protein and bioluminescence rhythms, in the same basic pattern as described for AMC149, except that the phase difference between the mRNA and the protein and bioluminescence rhythms was smaller in SP22 than in AMC149. By comparing the times at which the peaks and troughs occurred, the *luxAB* mRNA and luciferase protein rhythms appear to oscillate with a shorter period in SP22 than in AMC149; after 2 days under constant illumination, the troughs of the SP22 rhythms were a few hours earlier than those of AMC149. These results suggest that there is a shorter period of the molecular rhythms in SP22, although a longer-term experiment would be needed to pinpoint the phase and period differences, since the period difference between the bioluminescence rhythms is only about 2 h (18).

**Growth is not impaired in the reporter strain.** An ideal reporter construct should have no deleterious effects on the growth of the host organism. A recent study by Gonzalez-Flecha and Demple (7) found significantly increased amounts

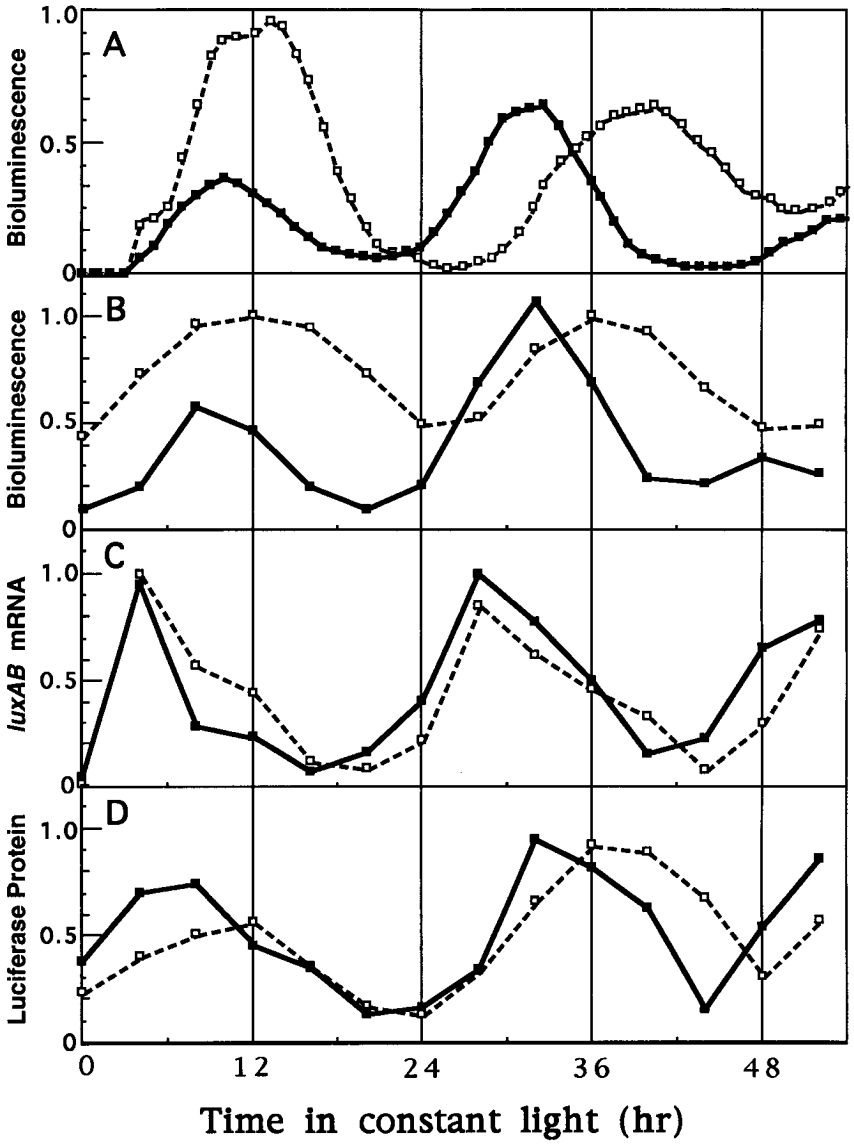


FIG. 5. Comparisons of rhythms of SP22 (solid lines) and AMC149 (dashed lines). Protocols are the same as described for AMC149 experiments. (A) Continuous bioluminescence rhythms of the two strains. (B) Bioluminescence levels at the time of sampling (single-point measurement). (C) *luxAB* mRNA levels. (D) Luciferase protein levels (LuxA).

of intracellular superoxide in one *Escherichia coli* strain that had been transformed with a *luxAB* construct. The results of that study suggested that the bacterial luciferase generated superoxide ions, which could be toxic to the cells. In order to know if our reporter construct has a similar effect, the growth rates of the wild-type *Synechococcus* sp. and AMC149 were compared (Fig. 6). There is no significant difference in growth rate between the wild-type strain and the reporter strain under normal culture conditions, which suggests that the *luxAB* construct does not adversely affect the cells. Either luciferase does not generate increased levels of superoxide in *Synechococcus* spp., or this cyanobacterium has efficient mechanisms to ensure that superoxide does not accumulate to toxic levels.

## DISCUSSION

***PpsbAI*::*luxAB* as a reporter of *psbAI* promoter activity.** The *PpsbAI*::*luxAB* construct appears to be an excellent reporter of *psbAI* promoter activity. First, the expression of luciferase in AMC149 does not affect the growth rate of the cells (Fig. 6). Moreover, our experiments show that the mRNA abundances of the *psbAI* and *luxAB* transcripts oscillate synchronously. The rhythm of *luxAB* mRNA always showed a high amplitude (8- to 10-fold). The amplitude of the *psbAI* mRNA rhythm is much more variable, ranging higher or lower than the amplitude shown in Fig. 2 in some experiments (data not shown). Even when the amplitude of the *psbAI* mRNA rhythm is low, however, its phase corresponds to that of the *luxAB* mRNA rhythm.

Why are the amplitudes of these two mRNA rhythms sometimes different? Since the expression of *luxAB* genes is directly under the control of the *psbAI* promoter, its transcription rate would be predicted to reflect the transcription rate of the *psbAI* promoter. The diffuse signals in the Northern blots (Fig. 2) suggest that *luxAB* mRNA has a short half-life. The mRNA of another foreign gene in the *Synechococcus* sp. is also rapidly

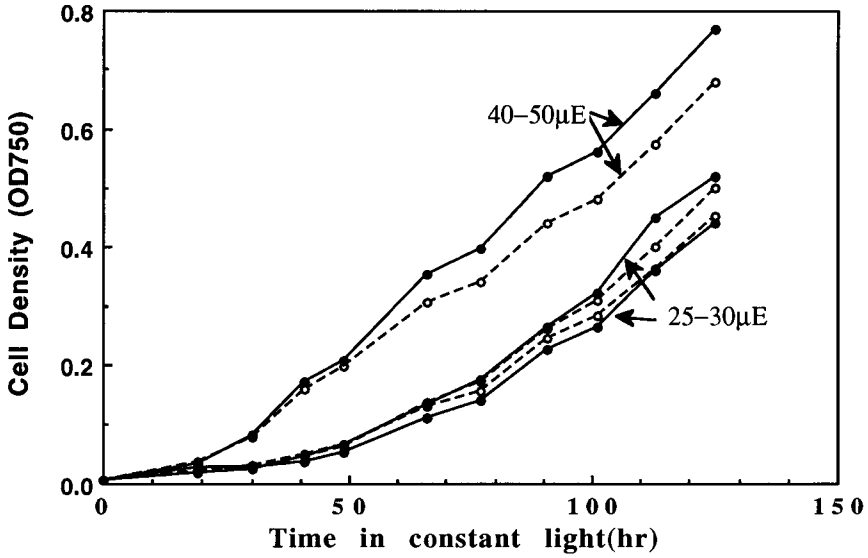


FIG. 6. Comparison of growth curves for the wild type and AMC149. The two strains were grown under two different light intensities, as indicated on the figure (in microeinsteins). Each curve is the growth curve for one batch of cells. Open circles, wild type; filled circles, AMC149. Abscissa, time (in hours) in constant light after inoculation.

degraded (30). These and other (21) experiments have shown that *psbAI* message oscillations often show a lower amplitude and damp in fewer cycles than the bioluminescence rhythm from the reporter gene. Our observations suggest that rhythms of message degradation are not always entrained in synchrony with the rhythms of transcription (21). The instability of the reporter transcript probably uncouples it from posttranscriptional effects and provides a clearer picture of what is happening at the level of transcription than does the abundance of the *psbAI* message, which is known to be under posttranscriptional control (20).

In *A. thaliana*, firefly luciferase has been used previously as a bioluminescent reporter of circadian promoter activity (24). In that paper, the researchers reported that plants needed to be continuously exposed to the substrate (firefly luciferin) in order for the reporter system to accurately track the promoter activity. With bacterial luciferase in *Synechococcus* sp. strain PCC 7942, on the other hand, the bioluminescence rhythms measured by continuous and single-point assays are equivalent (Fig. 1). Moreover, the presence or absence of the *n*-decanal substrate did not affect the rhythms of *luxAB* mRNA or luciferase abundance (Fig. 2 and 3 and other data not shown). We conclude that the presence of the substrate does not influence the regulation of bacterial luciferase in AMC149.

**Phase relationship of *lux* mRNA and protein rhythms.** The rhythm of luciferase protein mirrors that of bioluminescence in AMC149. This result suggests that the bioluminescence rhythm is not due to the fluctuation of substrates (oxygen or $FMNH_2$) for the luciferase reaction. On the other hand, although their troughs are close to each other, the peak of the luciferase protein rhythm lags behind that of the *luxAB* mRNA rhythm by about 4 to 8 h (Fig. 4 and 5). This time lag can be explained if the half-life of the luciferase protein is longer than that of *luxAB* mRNA. In experiments which continuously monitored in vivo bioluminescence levels of cell cultures, significant amounts of bioluminescence could be observed even 24 h after a transition from constant light to constant darkness (data not shown), while *luxAB* mRNA could not be detected after 12 h of darkness (Fig. 2B, h 0 [end of 12-h dark interval]). These observations suggest that luciferase protein has a significantly longer half-life than its mRNA. If so, luciferase protein could easily accumulate during the subjective day and reach its peak 4 to 8 h later than *luxAB* mRNA peaks. Our data do not determine precise half-lives of either the *luxAB* mRNA or the luciferase protein in *Synechococcus* sp. strain PCC 7942. Even if the luciferase protein has a longer half-life than the *luxAB* mRNA, the high-amplitude rhythms of bioluminescence (Fig. 1 and reference 17) demonstrate that luciferase protein is degraded rapidly enough to report the circadian rhythmicity of *psbAI* promoter activation.

What is the adaptive significance of circadian modulation of the *psbAI* gene? The answer may lie in the central role in photosynthesis of its product, the D1 protein of photosystem II. D1 and D2, the product of the *psbD* gene family, form a heterodimer at the heart of the photosystem II reaction center that coordinates the cofactors involved in primary photochemistry (8, 22, 26). The D1 protein undergoes light-dependent turnover that is thought to accompany specific damage to the protein which results from photochemical reactions (6, 23, 27). Because a rate of D1 synthesis in keeping with its damage and removal from the membrane is important for preserving photosystem II activity, and because the cells are normally subjected to a daily LD cycle, circadian control of D1 synthesis and degradation is not unexpected. Although the *psbAI* gene and the two other members of its gene family are differentially regulated at transcriptional and posttranscriptional levels by changes in light intensity (6), these regulatory mechanisms appear to work within the larger framework of circadian control.

## ACKNOWLEDGMENTS

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