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A sigma factor that modifies the circadian expression of a subset of genes in cyanobacteria

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We isolated mutants affected in the circadian expression of the *psbAI* gene in *Synechococcus* sp. strain PCC 7942 using a strategy that tags the genomic locus responsible for the mutant phenotype. The search identified one short period (22 h) mutant (M2) and two low amplitude mutants, one of which showed apparent arhythmia (M11) and one that was still clearly rhythmic (M16). We characterized the disrupted locus of the low amplitude but still rhythmic mutant (M16) as the *rpoD2* gene, a member of a gene family that encodes sigma^{70}-like transcription factors in *Synechococcus*. We also inactivated *rpoD2* in a number of reporter strains and showed that the circadian expression of some genes is not modified by the loss of this sigma factor. Therefore, we conclude that *rpoD2* is a component of an output pathway of the biological clock that affects the circadian expression of a subset of genes in *Synechococcus*. This work demonstrates a direct link between a transcription factor and the manifestation of circadian gene expression.

Keywords: circadian rhythm/cyanobacteria/*psbA*/sigma factor/*Synechococcus*

Introduction

Circadian (daily) rhythms of a wide variety of biological phenomena, ranging from behavioral and physiological parameters to biochemical processes and gene expression, have been documented in organisms as diverse as cyanobacteria and mammals (Pittendrigh, 1981; Sweeney, 1987; Edmunds, 1988; Dunlap, 1993; Kondo *et al.*, 1993). As a common feature, it appears that circadian pacemakers help to anticipate day/night changes of environmental illumination and temperature, allowing organisms to adapt more efficiently to such cyclic changes. Circadian systems consist of at least three major components: oscillator, photoreceptor and output(s) (Eskin, 1979; Johnson and Hastings, 1986). The central oscillator is entrained to the daily light/dark cycle by a photoreceptive system, and it controls output pathways which result in the rhythms that are directly observed. These output rhythms thus provide a monitor of the oscillator's phase and period, but are not themselves considered to be a part of the central time-keeping mechanism (Johnson and Hastings, 1986).

Despite many decades of study, the molecular mechanism(s) by which the central oscillator keeps time remains unknown. In vertebrates and higher invertebrates, information about the molecular basis of the circadian oscillator has been obtained using primarily biochemical and pharmacological approaches. In fruit flies and fungi, an additional successful strategy has been the use of genetics (Rosbash and Hall, 1989; Dunlap, 1993; Myers *et al.*, 1995). The *period* (*per*) and *timeless* (*tim*) genes of *Drosophila* and the *frequency* (*frq*) gene of *Neurospora* are the only clock genes that have been cloned and characterized to date; they have revealed only limited information concerning the oscillator itself, although the interaction of PER and TIM may constitute a component of a time-keeping feedback loop in *Drosophila* (Gekakis *et al.*, 1995).

Several studies have shown that the biological clock can operate within a single cell (Edmunds, 1988; Dunlap, 1990; Takahashi, 1991). Therefore, simple unicellular organisms can serve as models to assemble a comprehensive view of the molecular basis of the biological clock. The simplest group of unicellular organisms shown to exhibit circadian rhythms to date is the cyanobacteria (Sweeney and Borgese, 1989; Huang *et al.*, 1990; Chen *et al.*, 1991; Kondo *et al.*, 1993). Using a bioluminescent reporter strain that contains the *Vibrio harveyi luxAB* genes fused to a photosystem II gene promoter (P_{psbAI}), we have shown previously that the unicellular cyanobacterium *Synechococcus* sp. strain PCC 7942 exhibits an endogenous rhythm of gene expression (Kondo *et al.*, 1993, 1994; Liu *et al.*, 1995a,b) that fulfils the criteria of circadian oscillators, as determined for eukaryotic organisms: the persistence of the rhythms in constant conditions, phase resetting by light/dark signals and temperature compensation (Kondo *et al.*, 1993). Thus, this transgenic strain reports the output pathway of *psbAI* gene expression via bioluminescence. To identify other genes in *Synechococcus* that are controlled by the circadian clock, we inserted a *luxAB* gene set randomly throughout the *Synechococcus* chromosome to assay a large number of promoter activities, and monitored the resulting bioluminescence (Liu *et al.*, 1995b). Surprisingly, the expression patterns of all bioluminescent colonies monitored showed clear rhythmicity, suggesting that the cyanobacterial circadian clock controls the expression of not only a specific subset of genes, as has been assumed for eukaryotes, but the entire genome (Liu *et al.*, 1995b).

A powerful approach towards elucidating the molecular mechanism of the circadian oscillator is the isolation of clock-impaired mutants and the characterization of clock-related genes. The circadian oscillator is likely to be composed of more than one component, and the function

of several genes will need to be characterized to reveal clues as to its operation. In most systems, the isolation of clock mutants is a difficult task because thousands of individuals or clones have to be analyzed repeatedly for an extended length of time to detect abnormal patterns of rhythmicity. Clock mutants have been reported from eukaryotic organisms such as *Chlamydomonas*, *Neurospora*, *Arabidopsis*, *Drosophila*, mouse and hamster (Rosbash and Hall, 1989; Dunlap, 1993; Millar *et al.*, 1994; Vitaterna *et al.*, 1994). Recently we used chemical mutagenesis to isolate a large and diverse group of circadian clock mutants of *Synechococcus* that exhibit a broad range of periods and unusual patterns of *luxAB* expression, including arhythmia (Kondo *et al.*, 1994). We also demonstrated the ability to rescue mutant phenotypes by introducing wild-type DNA (Kondo *et al.*, 1994). Thus, the unicellular cyanobacterium *Synechococcus* provides an ideal model to dissect the molecular mechanism(s) of the biological clock using genetic approaches that have improved in recent years (Li and Golden, 1993; Tsinoremas *et al.*, 1994; Liu *et al.*, 1995b).

Here we report the isolation of a circadian clock-related mutant of *Synechococcus* by a genetic strategy that allows easy molecular cloning and identification of the affected gene. A screen for abnormal rhythms of bioluminescence from the reporter strain identified three mutant phenotypes: one short period (22 h) and two low amplitude phenotypes, one of which is apparently arhythmic. A molecular characterization of the gene responsible for the clearly rhythmic, but low amplitude, phenotype revealed an integration event at the *rpoD2* locus. This gene encodes a sigma^{70}-like transcription factor and is a member of a family of four principal sigma factor genes in *Synechococcus*. We show that the *rpoD2* gene is a component of an output pathway of the circadian oscillator that affects the rhythmic expression of a subset of clock-controlled genes in *Synechococcus*.

Results

Isolation of mutants that show altered circadian characteristics in AMC149

Previously we used a reporter strain of *Synechococcus* (AMC149) that carries a bacterial luciferase gene set (*luxAB*) fused to a photosystem II gene promoter (P_{psbAI}) to monitor the circadian control of gene expression via bioluminescence (Kondo *et al.*, 1993, 1994). We used the following strategy to obtain clock-affected mutants. Mutations were generated by inserting foreign DNA into the chromosome at random sites. We prepared a recombinant library of 0.3–0.9 kb *Sau*3AI fragments of *Synechococcus* genomic DNA ('mutagenic library') and introduced them into AMC149 via conjugative transfer from *Escherichia coli* (Tsinoremas *et al.*, 1994). Recombination between the chromosome and the identical fragment of DNA cloned into a non-replicating vector resulted in the integration of the plasmid at this locus (Golden *et al.*, 1987). Such a recombination event may disrupt the expression of a specific locus and therefore create a mutant phenotype (Dolganov and Grossman, 1993). The integrated plasmid plus flanking DNA can be recovered easily from the genome of the mutant strain, providing a powerful tool for correlating phenotypes with the loss of function of

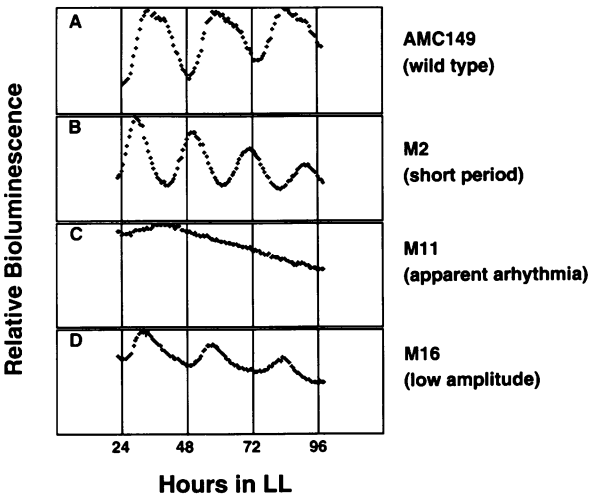

Fig. 1. Bioluminescence traces from putative insertional mutants of *Synechococcus* AMC149. (**A**) AMC149, wild type; (**B**) M2, short period mutant strain that exhibits a 22 h period; (**C**) M11, apparently arhythmic strain; and (**D**) M16, low amplitude rhythm strain. LL, constant light. Each graph is plotted such that the *y*-axis represents the full scale from zero to maximal bioluminescence for the indicated clone. Because the size of the measured cell patch varied from clone to clone, absolute values of bioluminescence are not comparable between strains.

specific genes (Dolganov and Grossman, 1993; Tsinoremas *et al.*, 1994; Liu *et al.*, 1996). AMC149 transconjugants screened for abnormal circadian characteristics yielded three mutant phenotypes: a 22 h period (M2; Figure 1B) instead of the 24 h period characteristic of the wild type (Figure 1A), apparent arhythmia (M11; Figure 1C) and a clearly rhythmic, but low amplitude, phenotype (M16; Figure 1D).

Analysis of the gene responsible for the low amplitude phenotype

The duplicated regions of the *Synechococcus* genome into which plasmids had integrated in M2, M11 and M16 were recovered in *E.coli* (Tsinoremas *et al.*, 1994). We determined that the mutant phenotypes of M2 and M11 resulted from second site mutations that are not linked to the inserted library DNA. We concluded this from the following data (not shown): (i) inactivation in the wild type of the open reading frames around the site of insertion in each mutant did not reproduce the altered clock phenotype; (ii) the plasmid from the mutant, recovered by *in vivo* homologous recombination as described in Materials and methods, did not reproduce the mutant phenotype when recombined into AMC149; and (iii) M2 and M11 derivatives from which the inserted library DNA was purged, restoring the native chromosomal locus, showed the same mutant phenotypes as the original isolates.

However, the following experiments showed that the low amplitude phenotype of M16 was the result of insertion of the library plasmid. First we isolated DNA from the mutant and digested it with restriction enzymes that cut outside the integrated vector but within the flanking *Synechococcus* DNA (Tsinoremas *et al.*, 1994; Liu *et al.*, 1996). After the intramolecular ligation and transformation of *E.coli*, plasmids of ~9 and 13 kb were recovered from *Bgl*II- and *Bam*HI-digested DNA respectively. Primers

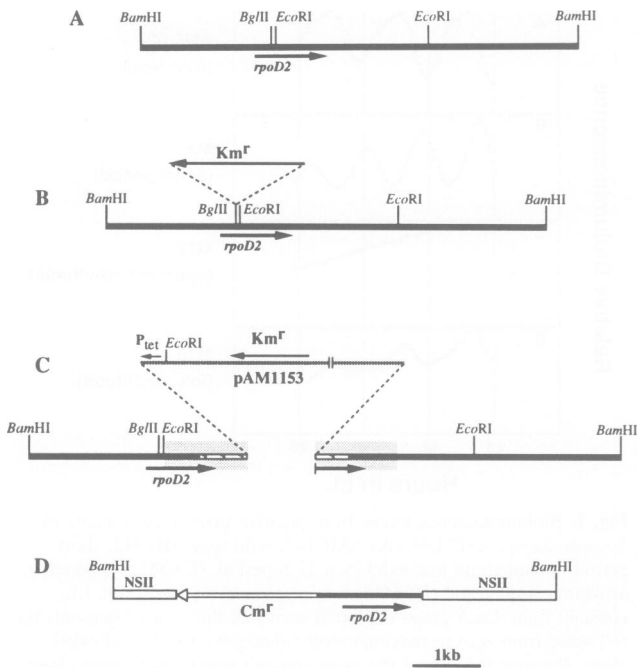

Fig. 2. Chromosomal maps of specific loci from wild-type and recombinant cyanobacterial strains. Physical map of (**A**) the *rpoD2* locus in wild-type *Synechococcus*, (**B**) the inactivated *rpoD2* locus in AMC149*rpoD2*$^-$, (**C**) the *rpoD2* locus in the low amplitude mutant, M16, and (**D**) the neutral site II (NSII) locus (see Materials and methods) in the strain AMC149*rpoD2*$^-$+*rpoD2*, where the *rpoD2* gene is provided *in trans* in the AMC149*rpoD2*$^-$ genetic background. Km^r, kanamycin resistance gene; Cm^r, chloramphenicol resistance gene; P_{tet}, promoter of the interrupted tetracycline resistance gene that is part of the pAM1153 vector; pAM1153, vector in which the mutagenic library was constructed (see Materials and methods); hatched open boxes, homologous DNA that is part of the library plasmid. Gray boxes highlight the region that is duplicated in M16. Arrows mark the location and direction of transcription of the indicated genes.

which corresponded to vector DNA allowed us to sequence into the attached *Synechococcus* DNA. A partial nucleotide sequence showed high similarity to genes for bacterial sigma^{70}-type RNA polymerase subunits. It also showed 100% identity to a previously characterized fragment (144 bp) of *Synechococcus* DNA which contains a small part of the *rpoD2* gene (database accession No. S44854). Restriction patterns of the inserts from the plasmids that were rescued by *Bgl*II or *Bam*HI digestion were consistent with integration of the vector into the *rpoD2* region (data not shown). This was confirmed by a Southern blot analysis using a DNA fragment that contains the *Synechococcus rpoD2* gene as a probe (data not shown). The physical map of the *rpoD2* region of the *Synechococcus* chromosome is shown in Figure 2A. The *rpoD2* gene is 963 bp long and is not preceded by a Shine–Dalgarno ribosomal binding site. The predicted amino acid sequence of the gene product showed extremely high similarity to bacterial sigma^{70}-type transcription factors (Figure 3).

Integration of mutagenic plasmid DNA near the *rpoD2* locus did not block the transcription of the P_{psbAI}*::luxAB* reporter gene because the overall bioluminescence emission from M16 was at least as high as that from equivalent-sized colonies of AMC149 (data not shown). The reduction in amplitude of the rhythm could be interpreted as a suppression of the daily trough of *psbAI* transcription. M16 colonies showed no noticeable growth defects other than the bleaching of cell pigment sooner than the wild type after prolonged incubation at the stationary phase.

To confirm that the lack of function of the *rpoD2* gene caused the low amplitude phenotype in the M16 mutant, we constructed a derivative of AMC149 which contains an interposon mutation in the *rpoD2* gene (Figure 2B). Integration of the kanamycin resistance interposon into the chromosome by an apparent double crossover event results in the inactivation of the *rpoD2* gene, as confirmed by a Southern blot analysis (data not shown). Several AMC149*rpoD2*$^-$ transformants were then analyzed for their circadian characteristics. All transformants showed the same bioluminescence pattern as in Figure 4C. Therefore, we conclude that it is likely that loss of function of the *rpoD2* locus in *Synechococcus* gives rise to the low amplitude phenotype.

Restriction analysis of the rescued *Bgl*II plasmid from M16 and sequence of the junctions of the vector and *Synechococcus* DNA revealed the map shown in Figure 2C for the M16 mutant chromosome at the *rpoD2* locus. It is apparent from these data that integration of the vector into the *rpoD2* locus did not physically interrupt the open reading frame, but rather resulted in a duplication of ~1.2 kb of *Synechococcus* DNA. This recombination event resulted in an intact copy of the *rpoD2* gene as well as a truncated one (Figure 2C), indicating that the C-terminal region of the *rpoD2* gene was present in the transforming plasmid. Thus, the apparent loss of function of the *rpoD2* gene in M16 may be caused by transcription interference from the tetracycline or the kanamycin resistance gene promoter reading out from the vector (Figure 2C). Alternatively, *rpoD2* could be part of an operon including additional open reading frames. In this case, it could be the disruption of a downstream open reading frame, rather than *rpoD2* itself, that causes the phenotype in both M16 and AMC149*rpoD2*$^-$. Because we were unable to detect any *rpoD2* mRNA using a Northern blot analysis, we could not determine whether *rpoD2* is part of a polycistronic message. A sequence analysis of the immediate downstream region did not reveal an obvious open reading frame. To establish whether loss of function of the *rpoD2* gene alone caused the low amplitude phenotype, we complemented the AMC149*rpoD2*$^-$ strain by providing *in trans* an intact copy of *rpoD2*. A 1.5 kb fragment containing the *rpoD2* gene and ~350 bp of the upstream region was amplified from the *Synechococcus* chromosome using PCR. This fragment was then inserted into a vector that can recombine at a specific non-essential part ('neutral site II') of the *Synechococcus* chromosome (Figure 2D). Proper integration of the *rpoD2* gene into neutral site II of the AMC149*rpoD2*$^-$ chromosome was confirmed by a Southern blot analysis (data not shown). The bioluminescence pattern of this strain was compared with those of wild type, the M16 mutant and the AMC149*rpoD2*$^-$ strain (Figure 4). As shown in Figure 4D, providing the *rpoD2* gene *in trans* rescued the low amplitude phenotype in the AMC149*rpoD2*$^-$ background (Figure 4C). Thus, we conclude that loss of *rpoD2* function itself in M16 and in AMC149*rpoD2*$^-$ caused the low amplitude circadian expression of the *psbAI* gene.

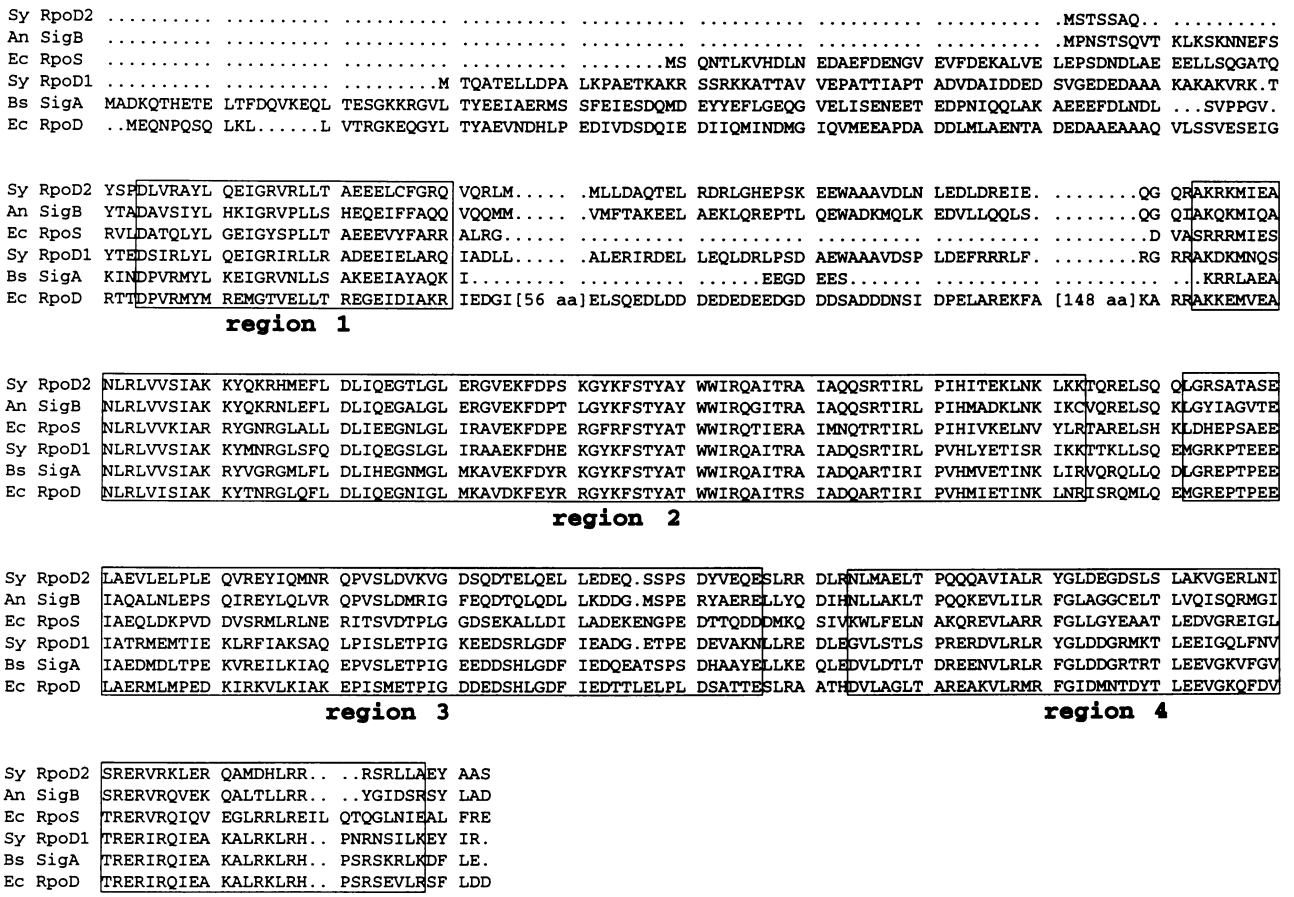

Fig. 3. Amino acid sequence comparison of *Synechococcus* RpoD2 with other members of the bacterial sigma^{70} family. Database accession numbers are indicated after each sequence identification: Sy RpoD2, *Synechococcus rpoD2* gene product (D78583); An SigB, *Anabaena* sp. strain PCC 7120 *sigB* gene product (Q03065); Ec RpoS, *E.coli rpoS* (*katF*) gene product (P13445); Sy RpoD1, *Synechococcus rpoD1* gene product (D10973); Bs SigA, *Bacillus subtilis sigA* gene product, σ^{43} (P06224); Ec RpoD, *E.coli rpoD* gene product, σ^{70} (P00579). Dots represent spaces (no amino acid present in the indicated sequence) added to facilitate alignment. Two blocks of amino acids that occur in Ec RpoD, but not in the other sequences, were omitted from the alignment and are indicated by [56 aa] and [148 aa] in the second row of aligned sequences. Boxes mark the four regions of high amino acid sequence identity that are characteristic of most members of the sigma^{70} family of transcription factors; regions 2 and 4 are involved in recognition of the −10 and −35 promoter elements, respectively.

The rpoD2 gene product is a component of an output pathway of the biological clock that affects the circadian expression of a subset of genes in Synechococcus

The inactivation of *rpoD2* appeared to affect only the amplitude of circadian oscillation in the AMC149 (wild-type) background. Amplitude theoretically could be affected by alteration of the central clock mechanism, a global output pathway that affects all downstream processes, or a specific output pathway that affects a distinct subset of processes. If RpoD2 is a component of the clock or of a general global output pathway, we would expect that all behaviors which receive information from the clock would be affected by its loss. Therefore, we tested whether loss of function of the *rpoD2* gene affected the circadian expression of several other *Synechococcus* promoters. Recently, we identified other genes controlled by the circadian clock in *Synechococcus* whose expression patterns exhibited a range of waveforms, amplitudes and phase relationships (Liu *et al.*, 1995b). We classified these rhythms into five categories. Classes 1 and 2 exhibited a symmetrical curve of bioluminescence rhythm. The distinction between these two classes is based on their phase, with class 1 showing the same phase as AMC149, and class 2 an almost reverse phase in constant light (Liu *et al.*, 1995b).

First we inactivated the *rpoD2* gene in a reporter strain that exhibited a reversed phase of bioluminescence relative to that of AMC149. Figure 5A shows the bioluminescence pattern of the class 2 strain, AMC287, which contains a P_{purF}*::luxAB* fusion (Liu *et al.*, 1995b). The inactivation of *rpoD2* did not affect the bioluminescence rhythm in the AMC287 background (Figure 5A and B). Next we wanted to see whether *rpoD2* inactivation affected rhythmic gene expression in other class 1 strains which exhibit a waveform, amplitude and phase of bioluminescence similar to AMC149. Inactivation of the gene in four class 1 strains was carried out as for AMC287. Three strains were unaffected by the loss of *rpoD2* function, one of which is shown in Figure 5C and D. However, one strain, LUX6*rpoD2*$^{-}$, exhibited a low amplitude rhythm, as observed originally for the M16 mutant (Figure 5E and F). We identified the gene to which the *luxAB* genes are fused in strain LUX6, whose circadian amplitude expression is RpoD2 dependent (see Materials and methods; Liu *et al.*, 1995b). The gene is *ndhD*, which encodes subunit D (chain 4) of NADH–plastoquinone oxidoreductase (determined by match to accession

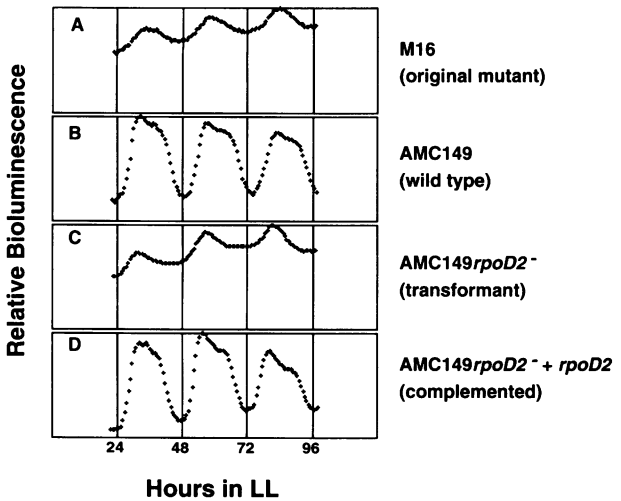

Fig. 4. Loss of *rpoD2* function causes the low amplitude bioluminescence rhythm in *Synechococcus*. Bioluminescence traces from (**A**) M16, original low amplitude mutant, (**B**) AMC149, wild-type strain, (**C**) AMC149*rpoD2*⁻, interposon mutant of AMC149 in which the *rpoD2* gene has been inactivated (see text and Figure 2B), and (**D**) AMC149*rpoD2*⁻+*rpoD2*, complemented AMC149*rpoD2*⁻ strain which carries an *rpoD2* gene *in trans* in neutral site II (see text and Figure 2D). Axes are as described in the legend to Figure 1.

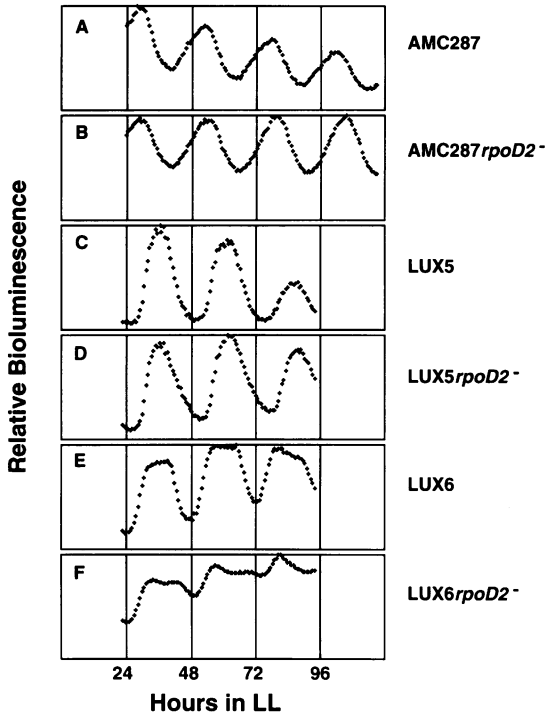

Fig. 5. Effect of loss of *rpoD2* function on bioluminescence rhythms of different *Synechococcus* reporter strains. Bioluminescence traces from (**A**) AMC287, a reporter strain that exhibits a rhythm of opposite phase to that of AMC149, (**B**) AMC287*rpoD2*⁻, in which *rpoD2* is inactivated in the AMC287 genetic background, (**C**) LUX5, a reporter strain that exhibits the same phase of bioluminescence rhythm as AMC149, (**D**) LUX5*rpoD2*⁻, in which *rpoD2* is inactivated in the LUX5 genetic background, (**E**) LUX6, a different reporter strain than LUX5 which also exhibits the same circadian characteristics as AMC149, and (**F**) LUX6*rpoD2*⁻, in which *rpoD2* is inactivated in the LUX6 genetic background. Axes are as described in the legend to Figure 1.

P32421). The *ndh* genes in higher plants are chloroplast encoded and are homologous to nuclear genes that encode the mitochondrial complex I (Schantz and Bogorad, 1988).

These data show that the low amplitude defect is specific for a subset of promoters, suggesting that RpoD2 is a component of an output pathway which affects the circadian expression of a subset of genes in *Synechococcus*. Mutants affected in the circadian period that carry the same P_{psbAI}::*luxAB* reporter gene as AMC149 (Kondo *et al.*, 1994) also show a decreased amplitude phenotype when the *rpoD2* gene is disrupted (Figure 6). Although the bioluminescence rhythm of AMC287, which carries a P_{purF}::*luxAB* reporter gene fusion, is unaffected by *rpoD2* disruption, its period is affected when this reporter gene is expressed in the SP22 (short period of 22 h) and LP27 (long period of 27 h) mutant backgrounds (data not shown). These data suggest that the *purF* and *psbAI* genes receive information from the same clock (or coupled clocks), as indicated by the period mutations, but that the output from the clock to the expression of these two genes has distinct intermediates.

Discussion

In our search for genes that affect circadian rhythms of bioluminescence in the AMC149 reporter strain, we identified a gene whose loss of function causes a low amplitude phenotype: *rpoD2*. It encodes a sigma^{70}-like protein, which is the subunit that confers promoter specificity to the RNA polymerase (Helmann and Chamberlin, 1988; Lonetto *et al.*, 1992). This establishes a direct link between a transcription factor and the manifestation of circadian gene expression.

A sequence analysis and the biochemical characterization of sigma factors reveal that they can be categorized into two large families. One is similar to the originally identified *E.coli* sigma^{70} (RpoD) polypeptide; the other is similar to the *E.coli* sigma^{54} (RpoN) subunit (Kustu *et al.*, 1989; Lonetto *et al.*, 1992). Cyanobacterial genomes encode multiple sigma^{70}-type factors, as is also observed in *Streptomyces coelicolor* (Brahamsha and Haselkorn, 1991, 1992; Tanaka *et al.*, 1991, 1992a,b). *Synechococcus* sp. PCC 7942 has at least four genes that encode sigma^{70}-type factors, designated *rpoD1–4* (Tanaka *et al.*, 1992b). The sigma^{70} family is subdivided further into three categories (Lonetto *et al.*, 1992), in which group 1 sigma factors are the 'housekeeping' (primary) sigma subunits. They share extensive sequence similarity, are responsible for most of the transcription in exponentially growing cells and are essential for cell growth. The *rpoD1* gene is essential in *Synechococcus*; based on this fact and its primary sequence, it represents the group 1 sigma factor of this organism (data not shown). Group 2 sigma factors, although very similar in sequence to the group 1 proteins, are not required for growth. The products of the *Synechococcus rpoD2*, *rpoD3* and *rpoD4* genes fall into this category. All of the sigma factors in the sigma^{70} family share at least two, and in several cases four, regions of similarity (Figure 3; Helmann and Chamberlin, 1988; Lonetto *et al.*, 1992). Group 3 consists of alternative sigma factors; they differ significantly from the primary sigma subunits and are responsible for the transcription of specific genes (Lonetto *et al.*, 1992). To date, no

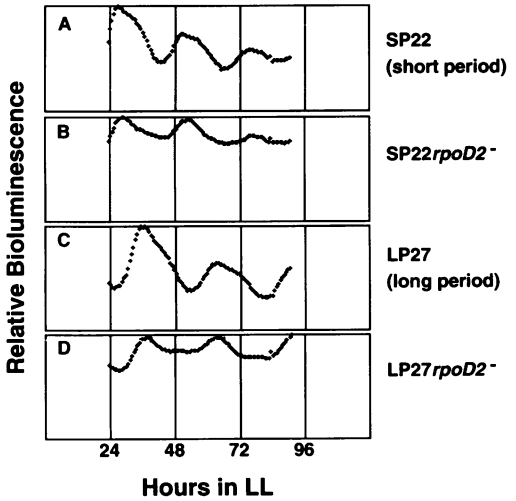

Fig. 6. Effect of loss of *rpoD2* function in *Synechococcus* period mutants that carry the P_{psbAI}::*luxAB* reporter. Bioluminescence traces from (**A**) SP22, short period mutant, 22 h period, (**B**) SP22*rpoD2*⁻, in which *rpoD2* is inactivated in the SP22 genetic background, (**C**) LP27, long period mutant, 27 h period, and (**D**) LP27*rpoD2*⁻, in which *rpoD2* is inactivated in the LP27 genetic background. Axes are labeled as in the legend to Figure 1.

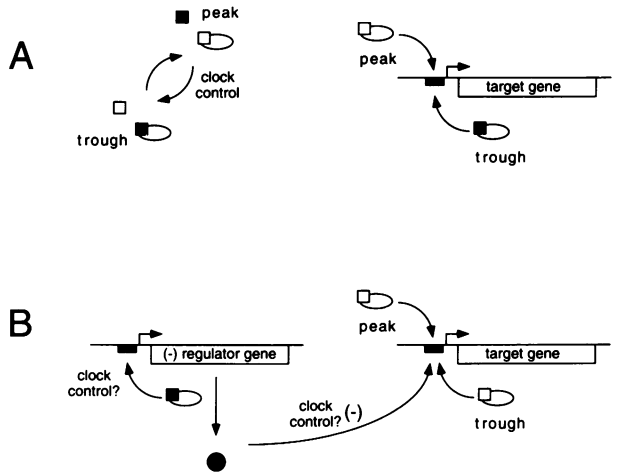

Fig. 7. Suggested pathways for RpoD2 involvement in generating high amplitude oscillations of gene expression. Oval, RNA polymerase core; filled square, RpoD2; open square, unspecified sigma subunit; bent arrows, transcription start sites; filled circle, product of regulatory gene. (**A**) The clock controls the association of alternative sigma factors with RNA polymerase. At trough times, RpoD2 associated with RNA polymerase recognizes the target gene promoter and initiates low level transcription; a holoenzyme with a different sigma factor catalyzes more robust transcription at peak times. (**B**) RNA polymerase associated with RpoD2 recognizes the promoter of a regulatory gene whose product negatively affects transcription of the target gene at trough times. The clock may control the transcription of the regulatory gene or the activity of its product.

group 3 sigma factor gene has been identified in a cyanobacterium.

With the exception of the RpoS (KatF) factor of *E.coli* (Loewen and Hengge-Aronis, 1994) and the SigE protein of *Synechococcus* sp. strain PCC 7002 (T.Gruber and D.A.Bryant, personal communication), which are involved in the expression of specific genes during the stationary phase, no function has been shown previously for the numerous group 2 proteins such as those encoded by cyanobacterial and *Streptomyces* genomes. We have shown that the *rpoD2* gene affects the circadian expression of the *psbAI* gene and at least one other *Synechococcus* gene. This phenotype is subtle, and was detected only because we used a reporter system specifically designed to look for this type of alteration. The difficulty in assigning functions to other group 2 sigma factors is probably based on similarly specific but subtle phenotypes associated with those mutants. Although inactivation of the gene does not cause noticeable growth defects, the *rpoD2*⁻ strains of *Synechococcus* show bleaching faster than the wild type after prolonged incubation at the stationary phase, suggesting that the *rpoD2* gene may also be involved in a stationary phase adaptation of the cells.

Our results indicate that RpoD2 is part of an output pathway of the clock that modulates the expression of a subset of genes in *Synechococcus*. Loss of function of RpoD2 affects the circadian oscillation of bioluminescence in the P_{psbAI}::*luxAB* reporter strain and in one other *promoter*::*luxAB* reporter strain (LUX6) out of the five tested (Figure 5). The *ndhD* gene, to which *luxAB* is fused in LUX6, encodes a protein of a type-1 NADH dehydrogenase complex that may be associated with the thylakoid membranes in cyanobacteria (Schmetterer, 1994). The mutation of *ndhD* is also associated with perturbation of the CO_2-concentrating mechanism in *Synechococcus* sp. strain PCC 7002 (D.Sültemeyer, B.Klughammer, M.Badger and D.Price, personal communication). Because of these suggested links with photosynthesis, it is not unexpected that *ndhD* might be coregulated with *psbAI*, whose product is the D1 protein of photosystem II.

We report progress towards the identification of genes that are part of the output pathways from the clock. Work in several eukaryotic systems has identified clock-controlled genes (Loros *et al.*, 1989; Stehle *et al.*, 1993; Heintzen *et al.*, 1994); however, the genes whose products transduce clock information to target genes remain largely unknown. The *rpoD2* gene encodes a product that contains all four essential domains of bacterial principal transcription factors and shows very high similarity in regions 2 and 4, which are responsible for binding the promoter –10 and –35 DNA sequences (Lonetto *et al.*, 1992). An attractive hypothesis is that RpoD2 acts directly on P_{psbAI} and other specific promoters as a sigma factor that recognizes their –10 and –35 regions (Figure 7A); in this scenario, the clock may exert control through a regulated competition between alternative sigma factors for association with RNA polymerase at different times of the cycle. If the RpoD2-containing holoenzyme predominates at the time of the trough and directs weaker transcription, then loss of RpoD2 function would elevate the trough of the circadian rhythm by a lack of competition for association with RNA polymerase. RpoD2 could also act directly by binding to the promoter independently of RNA polymerase, and therefore inhibit binding of the holoenzyme at trough times. However, the striking sequence similarity between RpoD1 and RpoD2 strongly suggests that RpoD2 is a genuine sigma factor, and we expect that it functions as a subunit of RNA polymerase. Alternatively, RpoD2 may act indirectly as the sigma factor needed to recognize the promoter of another gene, such as one encoding a *trans* factor that controls the amplitude of circadian expression of specific genes (Figure 7B).

Materials and methods

Strains and culture conditions

Synechococcus sp. strain PCC 7942 was grown photoautotrophically in modified (Bustos and Golden, 1992) BG11 medium (Allen, 1968) under constant illumination (100–150 $\mu Em^{-2}s^{-1}$) at 30°C. When appropriate, kanamycin was added to a final concentration of 5–20 μg/ml, spectinomycin to a final concentration of 20–40 μg/ml and chloramphenicol to a final concentration of 7.5 μg/ml.

Construction of the 'mutagenic' DNA library and transfer to Synechococcus

Genomic DNA from *Synechococcus* was isolated as described previously (Bustos *et al.*, 1990), partially digested with *Sau*3AI and separated by electrophoresis on a 1.2% agarose gel (Sambrook *et al.*, 1989). DNA fragments of 0.3–0.9 kb were isolated from the gel and purified. These fragments were then ligated into the plasmid vector pAM1153, as described (Tsinoremas *et al.*, 1994). Aliquots of the ligation mix were used to transform *E.coli* strain AM179, which contains the conjugation helper plasmid pRL528 and is chloramphenicol resistant (Elhai and Wolk, 1988). More than 40 000 chloramphenicol (17 μg/ml)- and kanamycin (50 μg/ml)-resistant transformants were collected by washing the plates with 5 ml LB medium (Maniatis *et al.*, 1982). The library contained >90% recombinant clones, as determined by the interruption of the tetracycline resistance gene, with most inserts in the expected size range based on an analysis of plasmids from randomly selected clones. Conjugal transfer of this library to *Synechococcus* cells was performed by triparental mating according to Tsinoremas *et al.* (1994). Surprisingly, all three circadian mutants obtained by this strategy contained larger plasmid inserts than predicted, based on the composition of the library (1.2–2.2 rather than 0.3–0.9 kb).

DNA manipulations

All DNA manipulations used standard procedures (Ausubel *et al.*, 1987; Sambrook *et al.*, 1989). PCR amplification of genomic DNA from *Synechococcus* with specific primers was performed according to Ausubel *et al.* (1987). DNA sequencing by the dideoxy chain termination method used the SequiTherm kit (Epicenter Technologies, Madison, WI) according to the manufacturer's instructions. Nucleotide sequences were determined either by visualization of the ^{32}P-labeled bands from autoradiograms or by using a Li-COR model 4000 automated DNA sequencer. A variety of programs were used for the sequence analysis, including a software package from the Genetics Computer Group (1994), DNA Strider (Marck, 1992) and the NCBI GenBank Blast email server (Altschul *et al.*, 1990).

Transformation, interposon mutagenesis and complementation of Synechococcus strains

Two segments of the *Synechococcus* genome containing all or part of the *rpoD2* gene were amplified by PCR using specific primers; in both cases base substitutions and/or deletions from the original sequence were used in each primer to engineer a *Bam*HI restriction site at either end of the PCR product. A 1137 bp fragment extending from ~200 bp upstream (5′-CTTAAGAGGATCCCCTGC-3′) to 925 bp inside the open reading frame (5′-GTAGGGATCCATGGCTTG-3′) was generated to facilitate inactivation of the *rpoD2* gene. This PCR product was cleaved with *Bam*HI and cloned into pUC18 (Yanisch-Perron *et al.*, 1985). The *rpoD2* gene was interrupted in this plasmid by inserting a spectinomycin or kanamycin resistance gene into a unique *Bgl*II site ~225 bp within the *rpoD2* open reading frame. The kanamycin resistance plasmid was used to inactivate *rpoD2* in AMC149 (which carries spectinomycin resistance as a marker for the P_{psbAI}::*luxAB* reporter gene) and its period mutant derivatives (SP22 and LP27). The spectinomycin resistance plasmid was used to inactivate the gene in reporter strains that had *luxAB* fused to other promoters, such as AMC287, LUX5 and LUX6, which have a kanamycin resistance marker associated with the reporter (Liu *et al.*, 1995b). Transformation of the *Synechococcus* strains was performed as in Golden *et al.* (1987). Spectinomycin- and kanamycin-resistant *Synechococcus* colonies were analyzed further by a Southern blot analysis (Ausubel *et al.*, 1987) to ensure replacement of the wild-type *rpoD2* gene by an apparent double homologous recombination event (data not shown). The complementation of AMC149*rpoD2*$^-$ was achieved as follows. A 1.5 kb fragment containing the entire *rpoD2* open reading frame, as well as 346 bp upstream (5′-AGTTGGATCCA-GTTGCAC-3′) and 168 bp downstream (5′-TAAAGGATCCCCA-GTTTG-3′), was amplified from *Synechococcus* DNA. This fragment, along with a chloramphenicol resistance gene (Shapira *et al.*, 1983) and a multiple cloning site from pIC19R (Marsh *et al.*, 1984), was cloned into the *Bst*EII site of pAM1385, a 'neutral site II' vector that facilitates recombination into a non-essential locus of the *Synechococcus* genome (database accession No. U44761). After the transformation of AMC149*rpoD2*$^-$ with this plasmid, spectinomycin-, kanamycin- and chloramphenicol-resistant colonies were analyzed by a Southern blot analysis (data not shown) to confirm the expected insertion of the *rpoD2* gene into the neutral site II locus (Figure 2D).

Recovery and analysis of the integrated plasmids from the M2 and M11 mutants

Previous observations (A.K.Kutach and S.S.Golden, unpublished data) suggested that when a plasmid integrates into the *Synechococcus* genome by single recombination, creating a duplication at the site of insertion, the plasmid will spontaneously loop out by homologous recombination (the reverse of the integration event) at a low frequency. These circular plasmids are present at very low levels in undigested total DNA preparations from strains that carry the integrated plasmid. They can be recovered by the transformation of *E.coli*. Genomic DNA from the M2 and M11 mutants was isolated as described previously (Bustos *et al.*, 1990). Undigested genomic DNA was introduced into *E.coli* DH10B cells by electroporation. Plasmid DNA was isolated from kanamycin-resistant (50 μg/ml) transformants. Six independent *E.coli* transformants showed the same restriction pattern, suggesting that the loopout event maintains the precise junctions of the repeat sequence. When this rescued plasmid was introduced into the AMC149 reporter strain, the resulting transformants retained a wild-type circadian rhythm.

In the converse experiment, derivatives of the M2 and M11 mutants, from which the plasmid insertion had been purged, were obtained. M2 and M11 were repeatedly subcultured (approximately seven subcultures of 2–3 days each) in the absence of kanamycin selection and plated to obtain single colonies; colonies were then tested for kanamycin sensitivity. A Southern blot analysis of three kanamycin-sensitive strains confirmed that they had lost the plasmid insert: both the vector and the genomic repeat. For both M2 and M11, these three plasmid-cured strains all maintained the mutant circadian phenotype, confirming that the phenotype is the result of a second site mutation. The plasmid-cured mutant strains were designated M2-A and M11-A.

Bioluminescence imaging and screening methods

Approximately 100 000 kanamycin-resistant colonies of *Synechococcus* which had received the mutagenic library by conjugation from *E.coli* were grown on modified BG11 agar plates supplemented with 5% LB (v/v) in constant light (white fluorescent lamp; 50 $\mu Em^{-2}s^{-1}$) at 26°C. Colonies were washed from the agar plates, resuspended in modified BG11 liquid medium and then plated again on modified BG11 agar containing kanamycin (20 μg/ml) and spectinomycin (40 μg/ml) but lacking LB supplementation to remove *E.coli* cells that interfere with the detection of bioluminescence from the *Synechococcus* colonies. Plates were incubated for 5–7 days under the same conditions, and then exposed to 12 h darkness to synchronize the circadian clocks of the colonies. An automated screening device was used to monitor the bioluminescence rhythms from the colonies, as described previously (Kondo *et al.*, 1994). Mutant clones were identified by their altered rhythm phenotypes and were used for DNA isolation to analyze the affected loci. Colonies of selected clones and subsequent transformants were transferred to fresh plates with sterile toothpicks and assayed by the same apparatus in parallel with wild-type controls to confirm circadian phenotypes.

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