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Transcriptional Analysis and Mutation of a *dnaA*-Like Gene in *Synechocystis* sp. Strain PCC 6803

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Transcription of the *dnaA* gene of the cyanobacterium *Synechocystis* sp. strain PCC 6803 is light dependent and yields a monocistronic mRNA, as determined by Northern analysis. Surprisingly, mutants with inactivated *dnaA* were viable. In batch cultures under standard conditions, the mutants grew like the wild type and did not show an aberrant phenotype. We conclude that, unlike the situation in other bacteria, *dnaA* of *Synechocystis* sp. cannot have an essential function, such as initiation of DNA replication.

Cyanobacteria are photosynthetic microorganisms with a light-dependent cell cycle. Cell division and initiation of DNA replication are blocked when an exponentially growing culture is transferred to the dark (2, 3, 11). Culture growth starts immediately upon a return to light. Molecular mechanisms that activate or repress the cell cycle under light or dark conditions are unknown.

In *Escherichia coli*, under a variety of growth conditions, DNA replication and cell division are coordinated. The initiation of DNA replication occurs simultaneously at all chromosomal origins, *oriC*, and the initiation frequency is correlated with the growth rate. One protein, DnaA, initializes chromosomal replication by specific binding to conserved nonamer sequences, DnaA boxes, which are organized as a cluster at *oriC*. Subsequently, DnaA promotes local DNA unwinding at *oriC* and organizes assembly of the replisome (for a review, see reference 12). DnaA is thought to be involved in the timing of replication initiation (6), is subject to an autoregulation mechanism (12), and operates in concert with other proteins that may modulate the initiation frequency (7).

The *dnaA* gene is highly conserved among eubacteria and was found to be present in cyanobacteria as well (15, 16), e.g., the identity between the deduced DnaA amino acid sequences of *E. coli* and the cyanobacterium *Synechocystis* sp. strain PCC 6803 is 38.8%. It is assumed that DnaA functions as an initiator protein in all eubacteria (21). The assumption is supported for cyanobacteria by demonstration of specific binding of two cyanobacterial DnaA proteins to isolated *oriC* fragments from *E. coli* and *Bacillus subtilis* in an in vitro binding assay (15). Functional analysis of the *dnaA* gene of a cyanobacterium, *Synechocystis* sp., might be helpful to elucidate the light-dependent regulation of replication initiation in cyanobacteria. However, a potential target sequence of DnaA, a DnaA box cluster with properties of a chromosomal origin, has not been isolated from *Synechocystis* sp. and is not evident from the complete nucleotide sequence (8).

In this study, examination of *dnaA* mRNA levels in light- and dark-incubated cells of *Synechocystis* sp. demonstrates light-dependent transcription of the gene. However, analysis of *dnaA* mutants revealed that the gene is not essential for growth under standard conditions.

Synechocystis sp. strain PCC 6803 was used in all experiments and cultured on agar plates or in batch cultures as described previously (5). *E. coli* TG1 (17) was used for routine DNA manipulations and cultured in Luria broth at 37°C. The plasmids used in this work are listed in Table 1.

Transcription of *dnaA* and the adjacent genes in *Synechocystis* sp. cells cultured under light and dark conditions. The *dnaA* gene and the adjacent reading frames have the same transcriptional orientation (16). Upstream of *dnaA*, there is an open reading frame, *orf134*, with an unknown function. The *psbDC* operon, which encodes photosystem II reaction center proteins D2 and CP43, lies downstream of *dnaA*. Transcription of *dnaA* was studied under light and dark conditions and compared with the transcription of adjacent genes.

An exponentially growing culture was transferred to the dark, and after 12 h, the cells were incubated under standard conditions with light. RNA of *Synechocystis* sp. cells was isolated as described previously (5). RNA samples were separated, and the relative content of 16S rRNA (size, 1.5 kb; see Fig. 1A) of each sample was quantified and used as the internal standard. RNA was transferred from gels onto nylon membranes (Hybond-N; Amersham) and hybridized with ^{32}P-labeled antisense transcripts which were synthesized by in vitro transcription in accordance with the manufacturer's protocols (MAXIscript kit; Ambion). Different DNA templates for in vitro transcription were generated as PCR fragments of coding regions (*Synechocystis* sp. genome sequence, http://www.kazusa.or.jp/cyano/cyano.html; full-length *dnaA*, nucleotide positions 1,351,579 to 1,350,236; 5′-end of *dnaA*, 1,351,579 to 1,351,179; 3′-end of *dnaA*, 1,350,648 to 1,350,236; *orf134*, 1,352,030 to 1,351,634; partial *psbC* sequence, 1,348,463 to 1,347,474) with an appended T7 phage promoter which was introduced by the antisense primers containing a 23-base T7 promoter sequence at the 5′ end (19). In vitro transcription controls were checked by electrophoresis. Relative content of in vitro transcripts was quantified by using ImageQuant software (Molecular Dynamics) and normalized to a standard length of 1 kb. Based on the normalized values, one specific factor for each in vitro antisense transcript was calculated to equalize the slightly different yields observed in control reactions. The specific factors were used to standardize signals detected by ^{32}P-labeled antisense transcripts on dot blots or Northern blots. Labeling reactions and hybridizations were done simultaneously to allow direct comparisons.

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TABLE 1. Plasmids used in this study

Plasmid	Relevant features	Reference
pSYN411	3.5-kb *Xba*I fragment of *Synechocystis* sp. *dnaA* region (positions 314–3848)[a] cloned into pSU2718; encodes chloramphenicol resistance	16
pSYNK−	Derived from pSYN411; *dnaA* partially replaced (positions 2286–2770)[a] by 1.3-kb *Hinc*II fragment of *aph* from pUC4K; *dnaA* and *aph* in opposite orientations; encodes chloramphenicol and kanamycin resistance	This study
pSYNK+	Same as pSYNK−, except that *aph* and *dnaA* are in the same orientation; encodes chloramphenicol and kanamycin resistance	This study
pUC4K	Contains *aph* gene cassette, which encodes aminoglycoside 3′-phosphotransferase; confers kanamycin resistance (Km^r cassette); another marker gene confers ampicillin resistance	20

[a] GenBank nucleotide sequence accession no. L336958.

Transcripts of *dnaA*, *orf134*, and *psbDC* were barely detectable after 12 h in the dark. They were strongly induced by light and reached steady-state levels after 4 h. Changes of transcript levels were less than 20% between h 4 and h 12 of the light period (Fig. 1A).

The proximity of *orf134* and *dnaA* suggested transcription of a dicistronic mRNA (16). However, only monocistronic transcripts were detected with either the *dnaA*-specific or the *orf134*-specific probe. The transcript which hybridized with the *dnaA* probe was found to be very unstable (Fig. 1A). The largest transcripts detected by the probe were estimated to be 1.6 kb, which corresponds to the predicted size of a monocistronic message. In another experiment, total RNA was probed by using a 5′- and a 3′-end antisense transcript of *dnaA*. A ratio of 4:1 was found for the relative signal intensities of the 5′ probe compared to the 3′ probe, suggesting that degradation occurs mainly from the 3′ end (data not shown). In the light-dark experiment, the *orf134* probe hybridized with a 0.4-kb transcript which did not show detectable degradation products and whose level was about 100-fold higher than the *dnaA* transcript level (Fig. 1B). The *psbC* probe detected a 2.5-kb transcript of the *psbDC* operon and a smaller monocistronic *psbC* transcript (Fig. 1B). About 10% of the transcripts detected by the *psbC* probe were monocistronic. The level of all

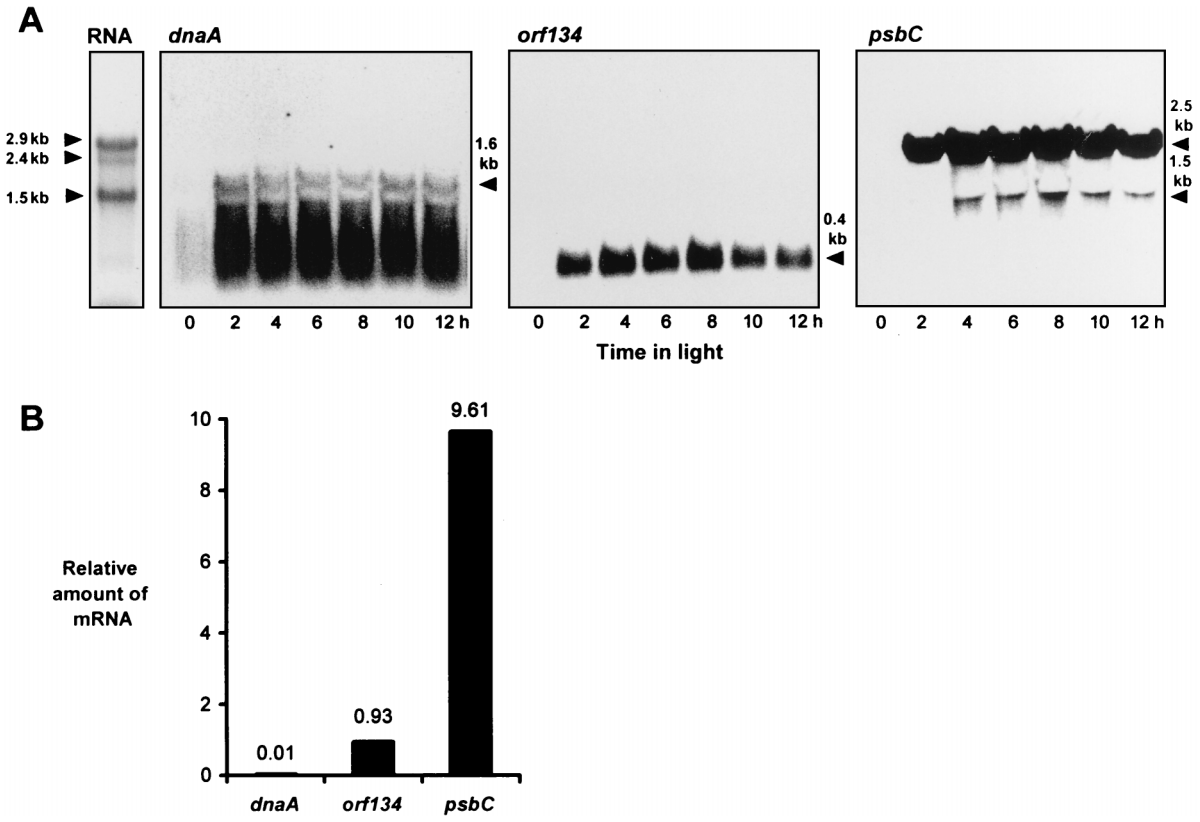

FIG. 1. Northern analysis of *dnaA*, *orf134*, and *psbDC* from *Synechocystis* sp. under light and dark conditions. Samples for RNA isolation were collected at the end of a 12-h dark period and at 2-h intervals during a following 12-h light period. (A) RNA is a negative image of total RNA stained with SYBRgreen; sizes of rRNA fragments are indicated (14). *dnaA*, *orf134*, and *psbC* are images of Northern blots with total RNA of the samples that were probed by ^{32}P-labeled antisense transcripts of *dnaA*, *orf134*, and *psbC*. Arrowheads indicate the locations of full-length transcripts. Images were generated by ImageQuant software. (B) Comparison of the relative mRNA levels from dot blots in which total RNA of a 6-h light sample was hybridized by *dnaA*, *orf134*, and *psbC* antisense transcripts.

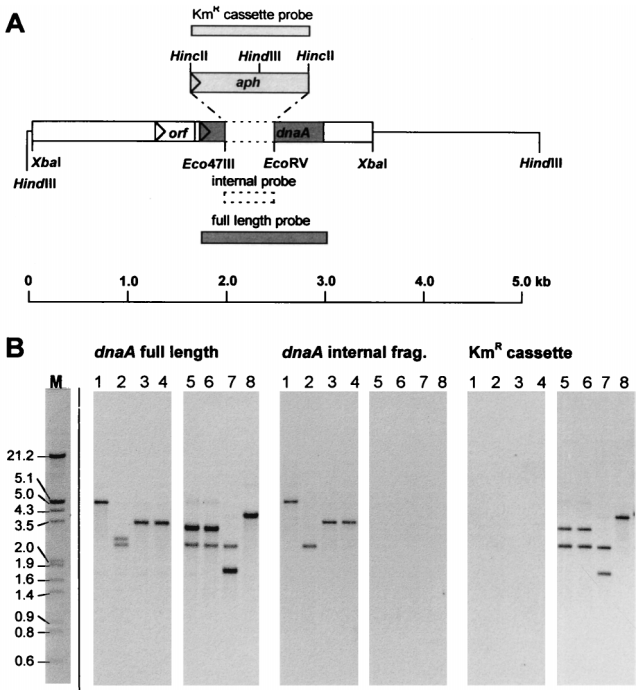

FIG. 2. Physical map and Southern analysis of a *dnaA* knockout mutant of *Synechocystis* sp. (A) Restriction map of the *dnaA* region and the Km^r cassette (*aph* gene) insertion site. *dnaA* is a dark gray box, *aph* is a light gray box, and *orf134* (upstream of *dnaA*) is a blank box. The fragment which is replaced in the mutant is shown as a blank box with dotted borders. Fragments used as probes in Southern analysis are represented by bars with the same shading. (B) Southern analysis of the *dnaA* region. Southern blots with cleaved chromosomal DNAs from the wild type and the mutant were hybridized with the following probes: full-length *dnaA* fragment, internal *dnaA* fragment and Km^r cassette. Lanes: 1 to 4, wild-type DNA cleaved with *Hin*dIII (lane 1), *Hin*dIII/*Eco*RV (lane 2), *Hin*dIII/*Xba*I (lane 3), or *Xba*I (lane 4); 5 to 8, mutant DNA cleaved with *Hin*dIII (lane 5), *Hin*dIII/*Eco*RV (lane 6), *Hin*dIII/*Xba*I (lane 7), or *Xba*I (lane 8). M, digoxigenin-labeled DNA molecular weight marker III (Boehringer). Fragment (frag.) sizes in kilobases are indicated on the left.

psbC transcripts is about 1,000-fold higher than the *dnaA* transcript level. The transcriptional structure of *psbDC* was described previously (22).

The Northern analysis demonstrates that in *Synechocystis* sp. cells, transcription of *dnaA* and adjacent genes is light dependent. Under standard conditions, *dnaA* mRNA was present, albeit at a low level compared with the other two transcripts. The *dnaA* transcript was completely absent after a 12-h dark period. Furthermore, only a monocistronic transcript could be detected which seems to be quickly degraded, starting from its 3′ end.

Construction and characterization of a *dnaA* knockout mutant of *Synechocystis* sp. strain PCC 6803. If DnaA has an essential function in *Synechocystis* sp., a knockout mutant of the *dnaA* gene should not be viable. To test this assumption, we undertook an experiment to replace part of the *dnaA* gene with a kanamycin resistance (Km^r) cassette. Plasmid pSYN411 (Table 1), containing a 3.5-kb fragment encoding *Synechocystis* sp. DnaA, was digested by *Eco*47III and *Eco*RV to generate blunt ends and to cut out a fragment coding for an essential part of DnaA (Fig. 2A). The fragment was replaced by ligation of a Km^r cassette isolated from a *Hin*cII digest of pUC4K (Table 1). Constructs with both orientations of the Km^r insert, i.e., Km^r encoded on the strand complementary to *dnaA* (pSYNK−; Table 1) and Km^r encoded on the same strand as *dnaA* (pSYNK+; Table 1), were obtained. Transformation into *Synechocystis* sp. and selection of Km^r clones were carried out as described previously (5). To our surprise, Km^r clones were easily obtainable. Selected clones were transferred into medium with kanamycin and cultured for several generations to complete segregation of wild-type *dnaA*.

Ten clones, five with each orientation (clones K1− to K5− and K1+ to K5+, respectively), were examined by Southern analysis. DNA templates for synthesis of specific probes were generated by digestion of plasmids pSYN411 (Table 1) and pUC4K (Table 1) with appropriate restriction enzymes, followed by DNA fragment isolation. Synthesis of digoxigenin-labeled DNA probes, Southern transfer, and hybridization were performed in accordance with the manufacturer's instructions (nonradioactive DNA labeling and detection kit; Boehringer, Mannheim) and standard protocols (17). The result for one clone, K1−, which was analyzed and compared with the wild type, is shown in Fig. 2B. A probe covering the *dnaA* coding region detected only a 5.2-kb band in *Hin*dIII-digested wild-type DNA. The Km^r cassette has an internal *Hin*dIII site which resulted in two fragments (2.6 and 3.4 kb) detected by the *dnaA* probe in the *Hin*dIII-digested mutant DNA (Fig. 2B). Another probe made from the internal *dnaA* fragment, which was replaced by the Km^r cassette in the pSYNK− and pSYNK+ constructs, did not show any signal with different digests of mutant DNA (Fig. 2B). The correct chromosomal integration of the Km^r cassette was finally confirmed by the signals detected after probing of digested mutant DNA with the labeled Km^r fragment (Fig. 2B). In all of the other mutants tested, we found replacement of the internal *dnaA* fragment by the Km^r cassette as well (data not shown).

In a culturing experiment done under standard conditions, the growth of the wild-type strain was compared with that of some selected mutants. No significant differences between the mutant strains and the wild type were found. The average growth rates (± the standard deviations) of wild-type *Synechocystis* sp. and *dnaA* mutants K1− (*dnaA*::*aph*) and K5+ (*dnaA*::*aph*) were 0.0167 ± 0.0024, 0.0165 ± 0.0017, and 0.0169 ± 0.0016 h^{-1}, respectively. (Clones K1− and K5+ show different transcriptional orientations of the *aph* gene relative to *dnaA*. Batch cultures were incubated under standard conditions. These data were calculated from three independent measurements of optical density at 750 nm.) The mutagenesis experiment clearly demonstrates that *dnaA* is not essential for the viability of *Synechocystis* sp. cells under standard conditions.

Conclusions. Molecular components which are involved in a light-regulated signal pathway that activate or repress the cell cycle in cyanobacteria are not known. The *dnaA*-like gene recently found in the cyanobacterium *Synechocystis* sp. could be a target of such a signal pathway.

We studied *dnaA* transcription under light and dark conditions in *Synechocystis* sp. cells and mutagenized the gene. Transcripts of *dnaA* and adjacent genes, *orf134* and *psbDC*, were almost absent after a 12-h dark period and were strongly induced by light. Similar results were obtained previously by examination of transcript levels of several photosynthetic genes in *Synechocystis* sp. cells under light and dark conditions (13). Here we show that the mRNA of a nonphotosynthetic gene, *dnaA*, behaves like photosynthetic transcripts. *orf134* is probably a nonphotosynthetic gene as well, since similar open reading frames were found in the heterotrophic organisms *E. coli* (4) and *Mycobacterium tuberculosis* (GenBank accession no. Z77163). In another study, the amount of mRNA of the *dnaK* gene encoding a chaperone was found to be relatively large in *Synechocystis* sp. cells after a 12-h dark period (1). Comparison

of this result with our data suggests that mRNA stability in *Synechocystis* sp. cells is transcript specific under darkness.

Surprisingly, our mutagenesis analysis revealed that *Synechocystis* sp. cells are viable without a functional *dnaA* gene. The *dnaA* mutants examined grew like the wild type and did not show an aberrant phenotype on agar plates or in batch cultures. In *E. coli*, the *dnaA* gene was originally defined by isolation of temperature-sensitive mutants blocked in the initiation of DNA replication at 42°C (10). The *dnaA* gene cannot be knocked out directly in this organism. There are two ways to explain our observation; either (i) *Synechocystis* sp. has at least two modes of replication, a DnaA-dependent one and a DnaA-independent one which is active in the mutant, or (ii) *Synechocystis* sp., unlike *E. coli*, has only DnaA-independent replication. In *E. coli*, there are two other modes of DNA replication that circumvent the DnaA requirement under certain conditions (for a review, see reference 9). One mode, inducible stable DNA replication, is induced under circumstances that activate an SOS response. The other mode, constitutive stable DNA replication, takes place in mutants of the *rnhA* gene encoding RNase HI with specificity for RNA in RNA-DNA hybrids. It is conceivable that *Synechocystis* sp. also possesses DnaA-independent replication that allows some growth under unfavorable conditions and which was activated in the *dnaA* mutants, whereas in the wild type, the DnaA-dependent replication is normally active under optimized growth conditions. One would expect that the growth of such *dnaA* mutants is impaired under optimized conditions. However, *Synechocystis* sp. *dnaA* mutants behaved like the wild type, suggesting that this *dnaA*-like gene does not have an essential function like initiation of replication. In addition, DnaA amino acid sequences of different species (including *Synechocystis* sp.) used in a basic local alignment search tool search did not match another open reading frame from *Synechocystis* sp. that exhibits the homology pattern typically found for DnaA proteins. Furthermore, there is no indication for a DnaA-dependent *oriC* in *Synechocystis* sp. which is characterized by a DnaA box cluster(s) with adjacent AT-rich regions. A search of the DNA sequence of the entire *Synechocystis* genome (8) did not reveal DnaA box clusters. This negative finding is supported by our unsuccessful attempts to isolate potential *oriC* fragments from chromosomal DNA of *Synechocystis* sp. by using the DNA-binding domain of authentic DnaA in an in vitro assay described previously (15, 16a). Thus, *Synechocystis* sp. is the only eubacterium known that carries a nonessential *dnaA*-like gene. The mechanism of initiation of DNA replication in *Synechocystis* sp. remains to be elucidated. However, characterization of *Synechocystis* sp. DnaA as a specific DNA-binding protein (15) and its light-dependent expression suggest that this protein could have another function which is important under certain conditions. Statistical analysis of the entire genome of *Synechocystis* sp. revealed a relatively high frequency of some DnaA boxes; e.g., a total of 36 copies of the nonamer sequence 5′-TTATCCACA-3′, which is characterized as an efficient DnaA box (18), were found to be present, whereas an average of 13.6 copies of a nonamer would occur on a random sequence with the same length as the *Synechocystis* genome. Nonrandom occurrence of some DnaA boxes suggests the involvement of DnaA in regulatory processes.

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