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A novel small stable RNA, 6Sa RNA, from the cyanobacterium *Synechococcus* sp. strain PCC6301

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Abstract We isolated a novel RNA species from the unicellular cyanobacterium *Synechococcus* PCC6301 and determined its gene sequence. This novel RNA was termed 6Sa RNA from its length (185 nt). Cross-hybridization of 6Sa RNA to other related microorganisms suggests that its existence is restricted to the *Synechococcus* genus or related organisms. A high level of accumulation of this RNA was observed by Northern analysis, indicating that 6Sa RNA is stable in cells. Computer-aided prediction of the 6Sa RNA secondary structure also supports its stability.

Key words: Small stable RNA; 6Sa RNA; Gene structure; *Synechococcus* sp. strain PCC6301

1. Introduction

A number of stable RNA species other than rRNA and tRNA have been found in prokaryotes. In *Escherichia coli*, several small RNA species and their genes have been reported, such as 4.5S RNA [1], 10Sa RNA (tmRNA) [2], 6S RNA [3], M1 RNA [4] and spot 42 RNA [5]. M1 RNA is a part of ribonuclease P which catalyzes the processing of pre-tRNA (reviewed in [6]) and 4.5S RNA is a part of the signal recognition particle which is involved in translocation of secretory proteins (reviewed in [7]). tmRNA has recently been reported to be involved in rapid degradation of proteins derived from damaged mRNA [8], though it is known to process the binding property for several DNA-binding proteins [9]. On the other hand, the function of 6S RNA and spot 42 RNA remains unclear.

In this report, we describe a novel 185 nt RNA (termed 6Sa RNA) and its gene from the unicellular cyanobacterium *Synechococcus* PCC6301.

2. Materials and methods

2.1. Growth of cyanobacteria

Synechococcus PCC6301 was grown under constant illumination (3000 lux) in a modified C medium of Kratz and Myers [10] at 30°C. Cells were harvested at late log phase and stored at −70°C.

2.2. Preparation and sequencing of RNA

Frozen cells (24 g, wet weight) were homogenized with quartz sand in a mortar and pestle. Total RNA was isolated from the frozen cells essentially as described [11]. Total RNA was loaded on a 5–20% (w/v) sucrose gradient in 20 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$ and 0.1 M NaCl, and centrifuged for 22 h at 63000×*g* in a Hitachi SRP28SA rotor at 4°C. Fractions between 16S rRNA and 4S RNAs were collected. The 5′ end of RNA was labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP after treatment with bacterial alkaline phosphatase [12]. Alternatively, the 3′ end of RNA was labeled with T4 RNA ligase and [^{32}P]pCp. End labeled RNAs were loaded on a 10% denaturing polyacrylamide gel (40 cm height) in 7 M urea, electrophoresed for 2.5 h at 4000 V) and recovered from the gel using elution buffer (0.1% SDS, 0.5 M ammonium acetate and 1 mM EDTA). RNA sequences were determined enzymatically [13], using an RNA sequencing enzyme kit (Pharmacia Biotech). Reactions were carried out according to the manufacturer's manual.

2.3. Cloning and DNA sequencing

A *Synechococcus* PCC6301 genomic DNA library constructed in λDASH II [14] was screened by plaque hybridization with a ^{32}P-labeled oligonucleotide (5′-AGATCAAAAAACATTGTAGGCAGTCACCAACATCACAGCCAGATC-3′) which was synthesized according to the determined RNA sequence. Plaque hybridization was performed at 40°C in 5×SSC, 5×Denhardt's solution, 0.5% SDS and 20 μg/ml denatured salmon sperm DNA. Membranes were washed at 40°C in 0.5×SSC containing 0.1% SDS. A genomic fragment was subcloned into pBluescript II KS+ (Stratagene) and the clone containing a 6Sa RNA gene (1.47 kbp *Pst*I fragment) was designated pPP1.5. Both strands of the DNA fragment were sequenced with the chain termination method using the Dye Primer Cycle Sequencing kit (Applied Biosystems).

2.4. Ribonuclease protection assay

The 3′ end of RNA was determined using the RPAII Kit (Ambion). The T3 promoter of pPP1.5 linearized with *Nsp*V or *Acc*I was utilized to generate antisense transcripts of 6Sa RNA (458 nt or 357 nt, respectively). Reactions were carried out according to the manufacturer's manual.

2.5. Northern and Southern hybridization

RNA electrophoresis and Northern blotting were done as described [15]. Genomic DNAs of *Synechococcus* PCC6301, *Synechococcus* PCC7942, *Synechocystis* PCC6803, *Anabaena variabilis* IAM M-3, *Rhodospirillum rubrum* S1 and *Chromatium vinosum* D were subjected to Southern blotting and hybridization was done as described [16]. Washing of Northern membrane was done at 65°C in 0.5×SSC containing 0.1% SDS and washing of Southern membrane was done at 65°C in 5×SSC. The 183 bp *Bgl*II DNA fragment (positions 1028–1207, Fig. 2) was labeled with [α-^{32}P]dCTP by the random primer labeling method [12] and used as a probe.

3. Results and discussion

3.1. RNA sequences

The RNA fractions sedimenting from 4S RNAs to 16S rRNA were labeled with ^{32}P at the 5′ end and electrophoresed on a long polyacrylamide gel. As shown in Fig. 1A (lane 5′), we could detect at least five strong bands (D, E, F, J and K) and over 10 faint bands. The 3′ end labeled RNA was also fractionated in parallel and bands corresponding to 5S rRNA and tRNAs could be detected (Fig. 1A, lane 3′). Due to low labeling efficiency of recessed 5′ ends in tRNA molecules, tRNA bands were hardly detectable in the [5′-^{32}P]RNA preparation. Among separated bands from the [5′-^{32}P]RNA, 11 bands (designated A–K) were excised and their partial RNA

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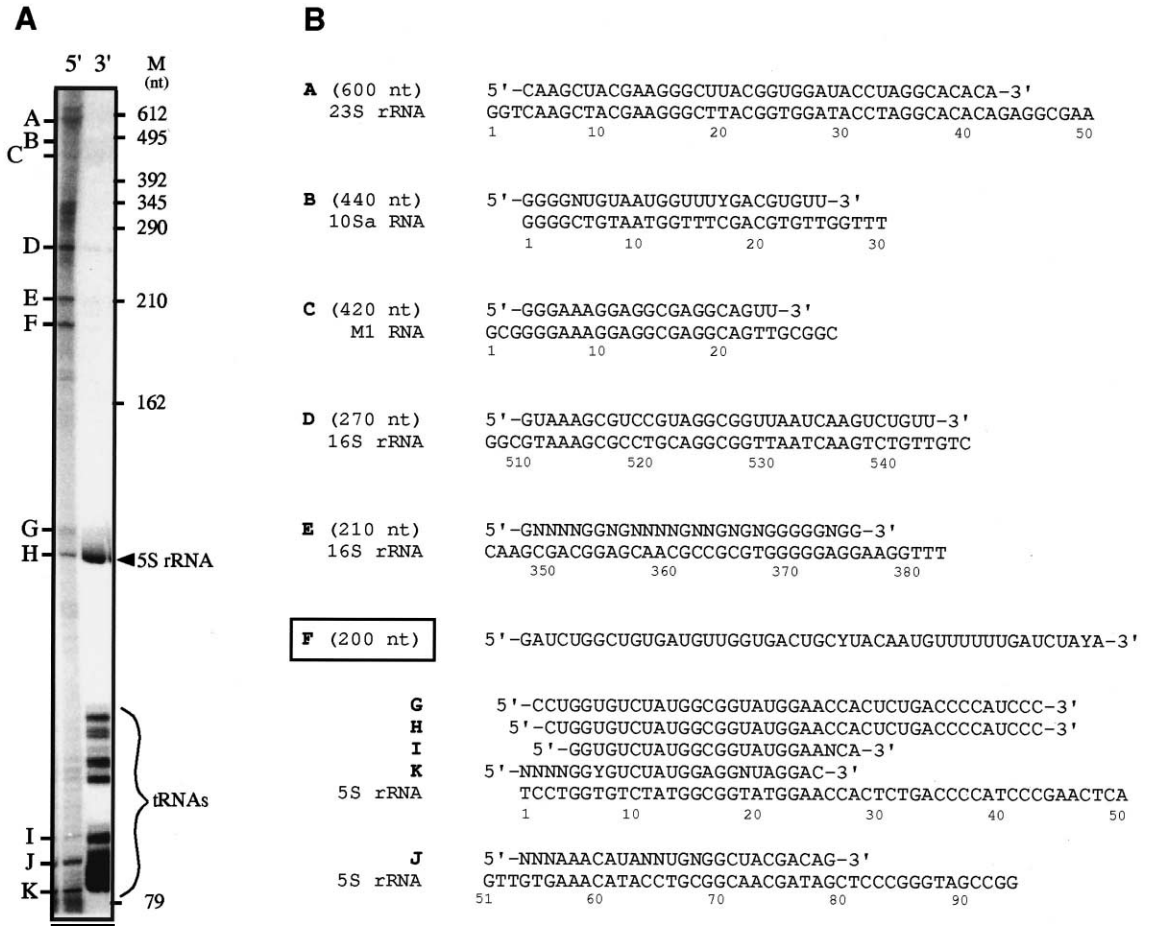


Fig. 1. Detection and sequences of small RNA species from *Synechococcus* PCC6301. A: The RNAs were labeled with ^{32}P at the 5′ end (lane 5′) or the 3′ end (lane 3′) and electrophoresed on a 10% denaturing polyacrylamide gel. M, DNA molecular markers (*Hin*cII digests of φX174 RF-DNA). B: Determined RNA sequences from 5′ end of isolated RNA fragments. A–K correspond to the bands in A and their approximate sizes from DNA markers are shown. The upper rows indicate the RNA sequences determined and the lower rows are the gene sequences that have been already reported and are homologous to respective RNA end sequences. Differences between the RNA sequence and gene sequence are considered due to the ambiguity of enzymatic activity during RNA sequencing reaction. N indicates A, U, C or G and Y indicates U or C.

sequences were determined from the 5′ end by the enzymatic method (Fig. 1B). Based on sequence alignment, band A was found to be a fragment of 23S rRNA [17], band B was identified as 10Sa RNA (tmRNA) [18], band C corresponded to the RNA component (M1 RNA) of RNase P [19] and bands D and E are from 16S rRNA [20]. Bands G–K were from 5S rRNA and its fragments [21]. The sequence of band F, however, showed no homology to any known RNA sequences.

3.2. A novel stable RNA

To characterize further the band F RNA, we isolated its gene from a genomic library using an oligonucleotide synthesized according to the band F RNA sequence. Five independent clones were obtained and their common 1.47 kbp *Pst*I fragment was sequenced (Fig. 2). Comparison to the 5′ sequence and length of the RNA revealed that the *Pst*I fragment contains the band F RNA coding region.

To determine the 3′ end of the RNA, a ribonuclease protection assay was carried out (Fig. 3). Based on the protected RNA fragments (132 nt from probe *Nsp*V and 98 nt from probe *Acc*I), the 3′ end corresponds to C at position 1213 (Fig. 2) and the length of this RNA molecule was calculated to be 185 nt. The sequence neither contains a significant reading frame nor shows similarity to any known RNA sequence, and we now designated this novel RNA 6Sa RNA from Svedverg unit estimated from its nucleotide length, and its gene was named *ssaA* from small stable RNA, Six Sa RNA A. Sequence comparison revealed that this RNA is different from *E. coli* 6S RNA species (188 nt, [3]), although the length of RNA is quite similar. Computer-aided analysis [22,23] predicted that 6Sa RNA can be folded into a tight double-helix structure as shown in Fig. 4.

A reading frame which is able to encode at least 335 amino acids was found at only 25 bp upstream of the 6Sa RNA coding region. This sequence was found to be identical to a part of ORF1 (395 codons) of *Synechococcus* PCC7942 [24], a strain closely related to *Synechococcus* PCC6301. ORF1 was suggested to be the cyanobacterial equivalent of *purK*, the gene encoding subunit II of phosphoribosyl aminoimidazole carboxylase (AIR carboxylase) and is located 100 bp downstream of *rbcL/S* encoding large and small subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase in *Synechococcus* PCC7942 [24].

3.3. Expression of the 6Sa RNA gene

Northern hybridization was carried out for the detection of

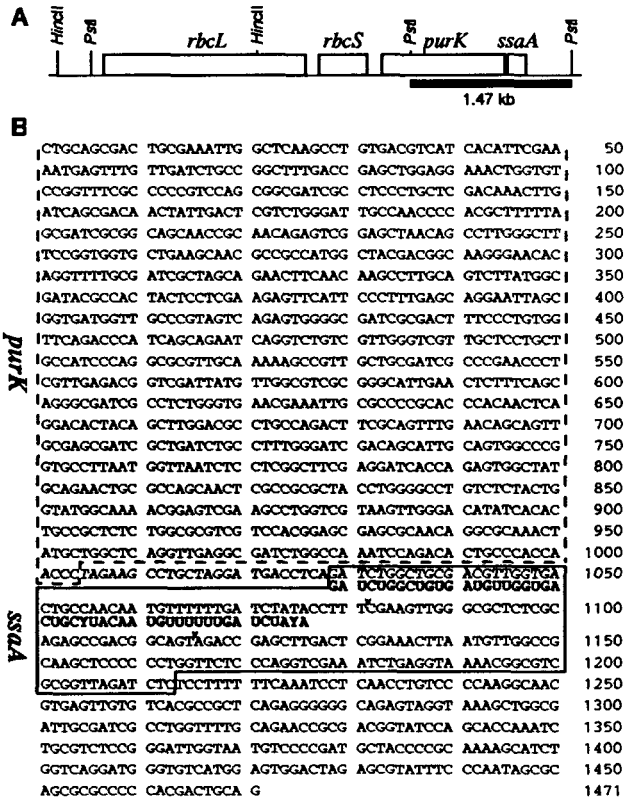


Fig. 2. Cloning and sequencing of *ssaA* (6Sa RNA gene). A: Restriction map of the genomic region of *ssaA*. A 1.47 kbp fragment of *Pst*I (black rectangle) was sequenced. B: Nucleotide sequence of the DNA between *Pst*I sites (DDBJ database accession number AB006759). The region encoding 6Sa RNA is enclosed with a solid line. A reading frame that is equivalent to a portion of *purK* is enclosed with a dotted line. The RNA sequence that was determined by enzymatic method is aligned below the corresponding DNA sequence by bold characters. The differences between DNA and RNA sequences are considered due to the ambiguity of RNA sequencing reaction. Restriction sites that were utilized in ribonuclease protection assays (*Nsp*V: TTCGAA, *Acc*I: GTAGAC) are shown by vertical arrowheads.

transcripts from the 6Sa RNA gene. As shown in Fig. 5A, 6Sa RNA is accumulated in all cell stages examined. However, a dramatic decrease in its level was observed in the stationary phase, suggesting that 6Sa RNA is functional when cells divide actively. No larger band was detected, suggesting that either this gene comprises a single transcription unit or its primary transcript is extremely unstable. No promoter motifs, such as '−10' and '−35' sequences, were found in the 25 bp spacer and the 3′ part of the *purK* coding region (see Fig. 3). If the 6Sa RNA gene is transcribed by a unit of its own, the promoter must be unique to it, e.g. internal promoters as in the case of nuclear RNA polymerase III-dependent genes. It is possible that 6Sa RNA is co-transcribed with the preceding *purK* gene. In that case, however, the primary transcript should be over 1.4 kbp and its cleavage should be rapid. Schwarz et al. [24] have reported that the *purK* transcript in *Synechococcus* PCC7942 is approximately 1.2 kbp and far less abundant than the small transcript which is derived from the downstream of *purK* and which we now regard as 6Sa RNA.

Kovacs et al. [25] have reported that small RNA species of 188 nt and 216 nt long are present in ribonucleoproteins that are immunoprecipitated with anti-ribonucleoprotein (RNP) and anti-Sm sera from *Synechococcus* PCC6301 extracts. Although we could not detect an RNA of 216 nt long, the 188 nt RNA may correspond to 6Sa RNA (185 nt). This report suggests that 6Sa RNA is associated with eukaryotic small nuclear RNP-like protein(s) in *Synechococcus* PCC6301 and hence is stable. Relevant to this, eukaryotic RNA-binding proteins have been isolated from cyanobacteria [4,26]. The observation that the 216 nt RNA but not 188 nt RNA was immunoprecipitated with anti-m_3G antibody [25] is consistent with the fact that we could detect 6Sa RNA but not 216 nt RNA by 5′ end labeling with [γ-^{32}P]ATP and T4 polynucleotide kinase after alkaline phosphatase treatment. If the 216 nt RNA has a m_3G cap at the 5′ end, it cannot be a substrate for alkaline phosphatase and not be labeled with [γ-^{32}P]ATP.

3.4. Organization and distribution of the 6Sa RNA gene

Genomic Southern hybridization was carried out for several related microorganisms: the unicellular cyanobacteria *Synechococcus* PCC6301, *Synechococcus* PCC7942, *Synechocystis* PCC6803, the filamentous cyanobacterium *A. variabilis*, the photosynthetic purple bacteria *C. vinosum* and *R. rubrum*. As shown in Fig. 5B, single positive bands of 1.47 kbp in *Pst*I digests and of 7.7 kbp in *Cla*I digests of *Synechococcus* PCC6301 DNA were detected. The gene probe hybridized only to the *Swa*I fragment 'W55a' in the genome [14]. These observations indicate that the 6Sa RNA gene is present as a single copy. The same length of band was detected in *Synechococcus* PCC7942. This observation supports the hypothesis

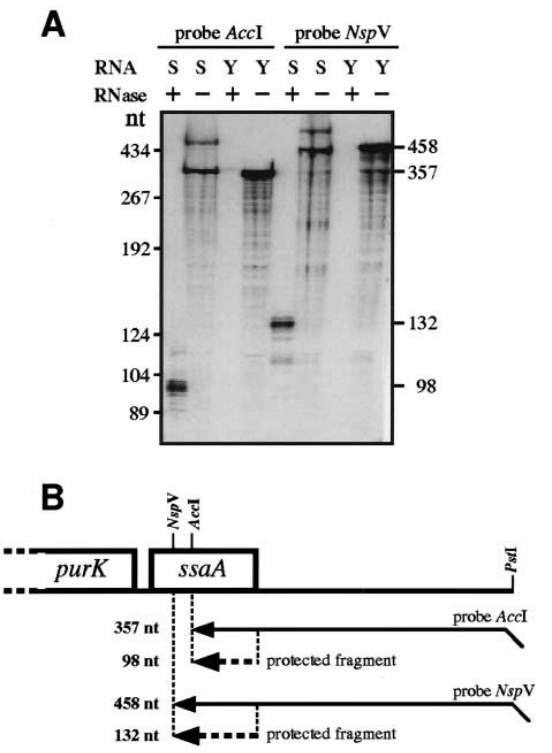
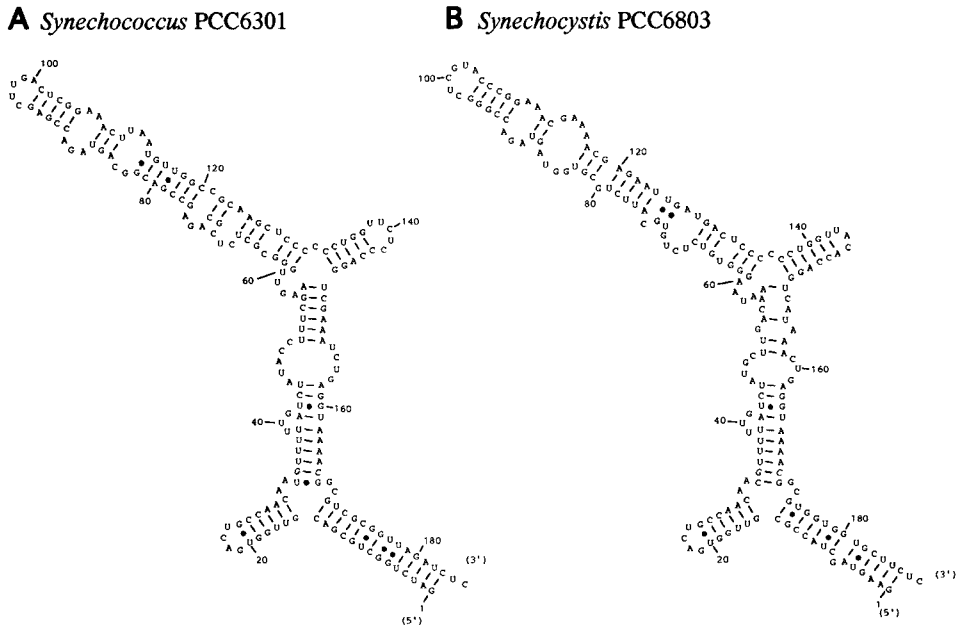


Fig. 3. Ribonuclease protection assay. A: Synthesized RNA probes from plasmid pPP1.5 linearized by *Acc*I or *Nsp*V were incubated with *Synechococcus* PCC6301 RNA (lanes S) or yeast total RNA (lanes Y) followed by digestion with (lanes +) or without (lanes −) RNases A and T_1. DNA molecular markers are *Hae*III digests of pBR322. B: The experimental design of the ribonuclease protection assays is shown. Solid arrows indicate RNA probes (458 nt, 357 nt) and dashed arrows indicate protected products.



C

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PCC6301     1  GATCTGGCTGCGACGTTGGTGACTGCCAACAATGTTTTTTGATCTATACCTTTCGAAGTT
               **  *  ** *  ********************* ************** * *    **
PCC6803     1  GAAGTAACTACCGCGTTGGTGACTGCCAACAACGTTTTTTGATCTATGCTTGAAAAATAA

PCC6301    61  GGGCG-CTCT-CGCAGAGCCGACGGCAGTAGACCGAGCTTG-ACTCGGAAACTTAATGTT
               *** * ****  ***         ** ********* *** * ** *******  **
PCC6803    61  GGGTGTCTCTGTGCATTCTGCGTGGTAGTAGACCGGGCTCGTACCCGGAAACGAAAACGA

PCC6301   118  GGCCGCAAG-CTCCCCCCTGGTTCTCCCAGGTCGAAATCTGAGGTAAAACGGCGTCGCGG
               *     * * *************   *******  ** ****************** * **
PCC6803   121  GAATTGATGACTCCCCCCTGGTTACACCAGGTCATAAACTGAGGTAAAACGGCGTGGTGG

PCC6301   177  TTAGATCTC
                *   ****
PCC6803   181  -TGCTTCTC
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Fig. 4. Predicted secondary structures of 6Sa RNA of *Synechococcus* PCC6301 (A) and *Synechocystis* PCC6803 (B). Watson-Crick type base pair and G●U wobble base pair are indicated by a dash (-) and a bullet (●), respectively. The structures were folded by the program MulFold version 2.0 [22] and plotted by LoopViewer version 1.0d59 [23]. C: Nucleotide sequence alignment of the *Synechococcus* PCC6301 6Sa RNA gene (PCC6301) with the *Synechocystis* PCC6803 homolog (PCC6803). Asterisks indicate identical nucleotides and dashes denote gaps.

that these two species are closely related [27]. No signal was detected in the genome of *Synechocystis* PCC6803, *A. variabilis*, *R. rubrum* and *C. vinosum* under the same hybridization conditions. This RNA may be present only in the genus *Synechococcus*, otherwise the sequence is so diverged that we could not detect the corresponding gene by heterologous hybridization. Recently the total genome sequence of *Synechocystis* PCC6803 has been reported [28]. Homology search revealed the region from positions 1 886 879 to 1 887 067 (38 638–38 826 of DNA database accession No. D90913), which is located between ORF slr1288 (hypothetical

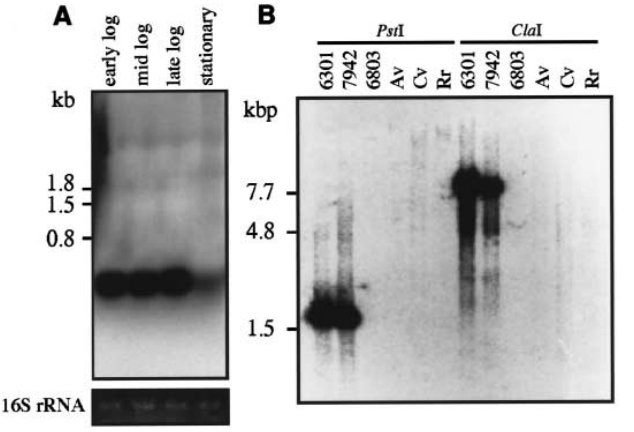

Fig. 5. A: Northern analysis of *Synechococcus* PCC6301 6Sa RNA. RNAs were isolated from cells of early-log phase, mid-log phase, late-log phase and stationary phase. B: Southern analysis by cross-hybridization of *Synechococcus* PCC6301 6Sa RNA gene. Genomic DNAs were prepared from *Synechococcus* PCC6301 (6301), *Synechococcus* PCC7942 (7942), *Synechocystis* PCC6803 (6803), *A. variabilis* (Av), *C. vinosum* (Cv) and *R. rubrum* (Rr) and digested with *Pst*I or *Cla*I.

protein, 37912–38496) and ORF sll1166 (hypothetical protein, 38918–40021), showed 69% of homology to *Synechococcus* PCC6301 (126 nt out of 185 nt are identical; Fig. 4). This suggests that a homologous gene is present in *Synechocystis* PCC6803.

Our preliminary targeted gene disruption experiment (unpublished) suggests that the 6Sa RNA is dispensable for cell viability under the growth condition for selection of transformants. It remains to be solved whether 6Sa RNA interacts with proteins or other RNA species and how expression of the 6Sa RNA gene is regulated.

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