Translation Initiation of Cyanobacterial rbcS mRNAs Requires the 38-kDa Ribosomal Protein S1 but Not the Shine-Dalgarno Sequence

**DEVELOPMENT OF A CYANOBACTERIAL IN VITRO TRANSLATION SYSTEM**

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Little is known about the biochemical mechanism of translation in cyanobacteria though substantial studies have been made on photosynthesis, nitrogen fixation, circadian rhythm, and genome structure. To analyze the mechanism of cyano-bacterial translation, we have developed an in vitro translation system from *Synechococcus* cells using a *psbA*1-lacZ fusion mRNA as a model template. This in vitro system supports accurate translation from the authentic initiation site of a variety of *Synechococcus* mRNAs. In *Synechococcus* cells, rbcL and rbcS encoding the large and small subunits, respectively, of ribulose-1,5-bisphosphate carboxylase/oxygenase are co-transcribed as a dicistronic mRNA, and the downstream *rbcS* mRNA possesses two possible initiation codons separated by three nucleotides. Using this in vitro system and mutated mRNAs, we demonstrated that translation starts exclusively from the upstream AUG codon. Although there are Shine-Dalgarno-like sequences in positions similar to those of the functional Shine-Dalgarno elements in *Escherichia coli*, mutation analysis indicated that these sequences are not required for translation. Assays with deletions within the 5′-untranslated region showed that a pyrimidine-rich sequence in the −46 to −15 region is necessary for efficient translation. *Synechococcus* cells contain two ribosomal protein S1 homologues of 38 and 33 kDa in size. UV cross-linking and immunoprecipitation experiments suggested that the 38-kDa S1 is involved in efficient translation via associating with the pyrimidine-rich sequence. The present in vitro translation system will be a powerful tool to analyze the basic mechanism of translation in cyanobacteria.

Cyanobacteria are prokaryotes that perform plant-type photosynthesis, and chloroplasts in plants are thought to be derived from an ancestral photosynthetic prokaryote related to cyanobacteria (1–4). Cyanobacterial genes are generally transcribed by an *Escherichia coli*-type RNA polymerase (reviewed in Ref. 5), and the regulation of transcription has been extensively analyzed using several representative cyanobacterial species (e.g. Refs. 6–8). However, little is known about their post-transcriptional events including RNA processing and translation. The molecular analysis of post-transcriptional processes in cyanobacteria is important to understand the evolutionary relationship between cyanobacteria and chloroplasts, as it is known that chloroplast gene expression is regulated mainly at the post-transcriptional level (reviewed in Ref. 9). Several RNA-binding proteins possessing eukaryotic-type RNA-binding motifs (CS-RBD or RRM) (10, 11) and their involvement in various physiological phenomena (12, 13) have been reported, suggesting that the gene expression of cyanobacteria is also regulated at the RNA level.

Prokaryotic mRNAs generally have the Shine-Dalgarno (SD) sequence in the 5′-untranslated region (5′-UTR), which participates in translation initiation (14, 15). The distance between a functional SD sequence and a start codon is critical with an optimal spacing of 7–9 nucleotides (nt) in *E. coli* (16, 17). Most mRNAs of the cyanobacterium *Synechocystis* sp. PCC 6803 also possess SD-like sequences in 5′-UTRs but their positions vary (18), and only 26% of the genes harbor SD-like sequences at the optimal position (19). In tobacco chloroplasts, about 40% of the protein-coding genes contain no SD-like sequences within 20 nt upstream from start codons and the remaining 60% have SD-like motifs but not always at a conserved position (20). Using an in vitro translation system from isolated chloroplasts, it was suggested that translation initiation of chloroplast *psbA*, *rps2* and *atpB* mRNAs does not depend on the SD-like sequence and requires *trans*-acting factors that interact with their 5′-UTRs (21–23). Cyanobacterial tRNAs and ribosomal proteins were assigned mainly based on the DNA sequence similarity with those of *E. coli* and of chloroplasts (20, 24). Recent proteomic analyses revealed that chloroplast ribosomes include several additional proteins not found in *E. coli* ribosomes (25–29). On the other hand, few biochemical studies have been made for cyanobacterial ribosomes. Ribosomal proteins from *Anabaena variabilis* have been analyzed by

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The abbreviations used are: SD, Shine-Dalgarno; UTR, untranslated region; nt, nucleotide; aa, amino acids; Tricine, *N*-[(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine; WT, wild-type.
two-dimensional gel electrophoresis, and 50 proteins were detected (30, 31). Ribosomal protein S1 is known to interact with the 5′-UTR and regulate translation initiation in *E. coli* (32–34). Two proteins related to *E. coli* S1 were isolated from *Synechococcus* sp. PCC6301 (35, 36). *E. coli* S1 is the largest ribosomal protein (61 kDa) and contains four repeats of the so-called S1 motif (37), whereas the cyanobacterial proteins are 33 and 38 kDa in size and possess three S1-like motifs. The presence of two S1 homologues has also been predicted from sequenced genomes in several other cyanobacteria, suggesting that these cyanobacteria have unique mechanisms of translation initiation. However, little is known about the mechanism of translation in cyanobacteria due probably to the lack of tools to analyze translation reactions. In this study, we have developed an *in vitro* translation system from *Synechococcus* cells and applied this system to investigate the translation mechanism of *Synechococcus* *rbcS* mRNA encoding the small subunit of ribulose-1,5-bisphosphate carboxylase/oxyg enase.

**EXPERIMENTAL PROCEDURES**

**Preparation of mRNA Templates**—Unless otherwise indicated, all techniques for manipulating DNA and RNA were as described (38). The 5′-UTR (99 bp) with the following 5′-coding part (329 bp) of *psbA1* (1,083 bp for the entire coding region; Ref. 39) was amplified by PCR using the genomic DNA from *Synechococcus elongatus* PCC 7942 and the primer combination of Al-f (5′-AAAGATCCGATGGAAGAGTTCGTTCAA-3′, restriction site underlined) and A-r (5′-AAAAAGCT-CCCTACGTTGTTACGACGACTCGT-3′). The PCR product was cut with BamHI and HindIII to fuse with the reading frame of ribulose-1,5-bisphosphate carboxylase/oxygenase.

The 5′-UTR (354 bp for the entire coding region) was synthesized from the plasmid for *Synechococcus rbcS* (40) fragment having SacI and HindIII sites, and L4-f (5′-GGGGAGCTGGAGTTCTCG-3′) and L4-r (5′-GGGGGAAGCTTCTCGATGGAAGAGTTCGTTCAA-3′, restriction site underlined) for an *rbcS* (40) fragment having Sacl and BamHI sites, L3-f (5′-GGGGAGCTGGAGTTCTCGATGGAAGAGTTCGTTCAA-3′) and L3-r (5′-GGGGGAAGCTTCTCGATGGAAGAGTTCGTTCAA-3′, restriction site underlined) and L1-f (5′-GGGGAGCTGGAGTTCTCGATGGAAGAGTTCGTTCAA-3′) and L1-r (5′-GGGGGAAGCTTCTCGATGGAAGAGTTCGTTCAA-3′, restriction site underlined) for an *rpl3* (41) fragment having Sacl and HindIII sites, and L4-f (5′-GGGGAGCTGGAGTTCTCGATGGAAGAGTTCGTTCAA-3′) and L4-r (5′-GGGGAGCTGGAGTTCTCGATGGAAGAGTTCGTTCAA-3′, restriction site underlined) for an *rpl4* (41) fragment having Sacl and BamHI sites. The calculated molecular masses of hybrid translation products were 20.9 kDa (187 amino acids (aa)) for *psbA1*, 21.6 kDa (187 aa) for *rbcS*, 20.3 kDa (187 aa) for *rpl3*, and 20.6 kDa (187 aa) for *rpl4*. Deleted and site-directed mutants of the 5′-UTR from *psbA1* and *rbcS* were constructed with the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instruction.

**Growth of Cyanobacterial Cells**—*Synechococcus* 7942 or 6301 cells were grown in the BG-11 liquid medium (42) containing 5 mM NaHCO₃ at 30 °C for 88 h under continuous light (50 μE/m²/s) with a sterile air of 4 liters/min in a 20-liter polycarbonate vessel. After dark adaptation for 12 h in the above condition except for light, the cells were harvested by continuous centrifugation using a rotor No. 15N (TOMY) at 4,000 × g at 4 °C and were stored at −70 °C.

**Preparation of S30 Fractions**—The cell pellet (~4 g) defrosted at room temperature was suspended by pipetting in 20 ml of EX buffer (30 mM HEPES-KOH, pH 7.7, 10 mM magnesium acetate, 30 mM potassium acetate, 2 mM dithiothreitol and was aliquoted to ~10 tubes of a Mini Bead-Beater™ (Bio-spec Products) with 400 mg/tube quartz sand (Katayama Chemical). After short vortex, the suspensions were frozen in liquid nitrogen and then immediately put into a 37 °C water bath for ~3 min to allow partial melting. The suspension was agitated by the Mini Bead-Beater™ for 30 s at the maximal speed. The supernatant (cell extract) was obtained by centrifugation at 25,000 × g for 1 min at 2 °C, and the precipitate was resuspended in 200–300 μl each of EX buffer followed by centrifugation. The extraction was repeated two to three times to raise the yield of cell extracts. The combined extract (~10 ml) was centrifuged twice at 30,000 × g for 30 min at 2 °C. The resulting blue supernatant (~8 ml) was incubated at 30 °C for 15 min with 40 μg each of 20 non-radioactive amino acids, followed by treatment with micrococcal nuclease (0.26 unit/ml, GE Healthcare) at 30 °C for 15 min in the presence of 1 mM CaCl₂. The nuclease reaction was stopped by adding 300 mM EGTA to the final concentration of 3 mM. The treated extract was dialyzed twice against 4 liters of EX buffer at 4 °C for 8 h using Spectra/Pore CE MWCO: 3,500 membrane (Spectrum). The dialyzed fraction (S30, ~15 mg/ml protein) was aliquoted (34 μl each) and stored at −70 °C. S30 was diluted with EX buffer to adjust its protein concentration to 3 mg/ml before use.

**In Vitro Translation Reaction**—The standard reaction was carried out at 37 °C for 40 min in a 25 μl solution containing 30 mM HEPES-KOH, pH 7.7, 10 mM magnesium acetate, 30 mM potassium acetate, 30 mM NH₄Cl, 1% polyethylene glycol 6000, 1 mM ATP, 0.1 mM GTP, 8 mM creatine phosphate, 0.4 mg/ml creatine phosphokinase (Type I, Sigma), 2 mM dithiothreitol, 133 units of RNase inhibitor (TaKaRa), 0.4 mM each of 19 amino acids, 0.37 MBq 1-[^35]S]-cysteine (>37 TBq/mmol, Amersham Biosciences) or 0.37 MBq 1-[^35]S]-methionine (>37 TBq/mmol, Amersham Biosciences), 10 pmol mRNA, and 12 μl of S30 (36 μg of protein). After reaction, proteins were precipitated with 75 μl of cold 100% acetone and were rinsed once with cold 50% acetone. The precipitated protein was suspended in the Lae-mml sample buffer (Bio-Rad) containing 8 μl urea and separated by 0.1% SDS/12.5% PAGE in Tris-glycine or Tris-Tricine running buffer after heat treatment at 95 °C for 3 min. Separated products were visualized and quantified by a Bio-imaging Analyzer (LAS2000, Fuji Photo Film Co.).

**UV Cross-linking and Immunoprecipitation**—A 32P-labeled *rbcS* mRNA portion (a 120-nt 5′-UTR with a 67-nt 5′-coding region) was synthesized from the plasmid for *rbcS-lacZ* fusion mRNA (linearized with PvuI) using the in *vitro* transcription kit (Stratagene) with [α-32P]UTP (>110 TBq/mmol, Amersham Biosciences) and purified by 4% PAGE containing 8 μl urea. The mRNA of 2.5 pmol was incubated for 25 min at 30 °C in the
25-μl translation mixture as above except 35S-labeled amino acids were replaced with the corresponding non-radioactive ones, and then the mixture was diluted with 75 μl of EX buffer. UV cross-linking of the mRNA and proteins was performed twice on ice using a UV illuminator (254 nm, 900 ml/cm², FS1500, Funakoshi), and then the mixture was treated with 0.01 unit of RNase A for 30 min at 37 °C. Cross-linked proteins were precipitated and rinsed with 80% acetone. 32P-Labeled proteins were separated by PAGE as above and visualized by the Bio-imaging Analyzer. For immunoprecipitation, protein A-Sepharose beads (25 μl wet volume each) were mixed with 250 μl each of antibodies against Synechococcus 38-kDa S1, EF-Tu, Rbp1, and Rbp2 for 1 h at room temperature and then centrifuged to remove excess antibodies. Anti-S1 and anti-EF-Tu were gifts of M. Sugita, and anti-Rbp1 and anti-Rbp2 were as described (13). The beads were washed seven times with 1 ml each of phosphate-buffered saline, pH 7.4, added to 100 μl of the reaction mixture containing UV cross-linked proteins, and the suspension was incubated for 3 h at 4 °C. After collecting beads by centrifugation and washing three times with 1 ml each of phosphate-buffered saline, the beads were suspended in 25 μl of the Laemmli Sample buffer and subjected to PAGE as above. 32P-Labeled proteins were visualized by the Bio-imaging Analyzer.

RESULTS

Development of an in Vitro Translation System—During the course of development and then optimization of an in vitro translation system from the cyanobacterial cells, a psbA1-lacZ fusion mRNA was used as a model template, and translation in vitro was monitored by its 32P-labeled translation products. The psbA1 mRNA of Synechococcus elongates PCC7942 (a strain closely related to Synechococcus sp. PCC6301) encodes the D1 protein (40 kDa) inserted into the photosystem II complex and is known as one of the actively translated transcripts in photosynthetic organisms (39). The fusion to lacZ mRNA makes the translation product soluble, normalizes efficiencies of translation elongation, and eliminates possible effects of the psbA1 3′-UTR on translation initiation. The hybrid mRNA template was synthesized with T3 RNA polymerase from a linearized construct including the 5′-psbA1 and the 3′-lacZ gene portions as shown in Fig. 1A.

Synechococcus 6301 or 7942 cells were grown to an early log phase and subjected to dark adaptation for 12 h to reduce endogenous mRNA levels. Cell extracts were preincubated with unlabeled amino acids and then treated with micrococcal nuclease to digest endogenous transcripts. These steps were critical to reduce nonspecific translation in vitro. Extensive dialysis was also required to remove EGTA added to inactivate micrococcal nuclease and to standardize reaction constituents. Using this cell extract (S30) and the model template, a major polypeptide of ~18 kDa was detected on PAGE with a relatively low background (Fig. 1B). This size is ~3 kDa smaller than the calculated mass of 20.9 kDa. This is probably due to the presence of a long hydrophobic region (21st to 60th amino acids) in the translated products.

In vitro reaction conditions were then optimized using the psbA1-lacZ mRNA (data not shown). Optimal buffer conditions were 10 mM magnesium acetate, 40 mM potassium chloride, and 20 mM ammonium chloride in 60 mM HEPES-KOH, pH 7.7. ATP was essential for translation and its optimal concentration was 2 mM. GTP (the optimum at 0.5 mM) and an ATP-generating system enhanced translation. An RNase inhibitor, leupeptin (thiol proteinase inhibitor), and E. coli tRNAs slightly affected translation, and hence, we did not add them. Translation was hardly detected with the mRNA of 1 pmol or less, but it increased linearly up to 10 pmol and then slowly up to 50 pmol (Fig. 1C). Using 10 pmol of mRNA template, translation increased linearly with S30 amounts (up to 8 mg of protein/ml) (Fig. 1C). Translation was observed after a time lag of ~10 min and reached a plateau at 60 min (Fig. 1C). We adopted S30 of 3 mg/ml protein, 10 pmol of mRNA template, and 40-min incubation as our standard in vitro reaction. As
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including the entire spacer region (93 nt) between rbcL and rbcS coding regions and the 5'-coding sequence (299 nt) of rbcS (see WT (wild-type) in Fig. 4A). An exchange of the 1st AUG for ACG arrested translation completely, while that of the 2nd AUG for ACG still supported translation though reduced activity by half (Fig. 3, A and C). The N-terminal amino acid sequence deduced from the 1st AUG matches that determined from the Synechococcus RbcS (SMKTLPE--; Ref. 43). This result indicates that the 1st AUG, but not the 2nd AUG, is essential for the rbcS mRNA translation.

In the 5'-UTR, there are SD-like sequences (AGGA, AGG, or GGA; Fig. 3A) and additional short sequences (UGA and AUC; Fig. 3B) complementary to the 3'-end region of Synechococcus 6301 16S rRNA (44). To examine the effect of these sequences on rbcS mRNA translation, these sequences (S1, S2, and S3) were mutagenized. As shown in Fig. 3D, none of the mutated mRNAs abolished translation. Mutation of S1 (AGGAU to ACCAU) or S3 (AUC to UAC) enhanced translation and that of S2 (UGA to ACA) retained 80% of the wild-type efficiency. It should be noted that mutation of S1, an SD-like sequence (GGA) at a proper position, resulted in 200–250% increase in translation. Thus, the interaction between an SD-like sequence and the 3'-16 S rRNA is unnecessary for rbcS mRNA translation.

**Sequences Necessary for rbcS mRNA Translation**—As described above, translation of the Synechococcus rbcS mRNA requires no SD-like elements, and therefore an additional mechanism should operate to recognize the genuine start site. To identify sequences necessary for the translation initiation of rbcS mRNAs, we first constructed a series of deleted mRNAs based on the rbcS-lacZ fusion mRNA (Fig. 4A) and assayed their translation. Deletion from the 5'-end (−120) to −85 did not significantly affect translation; however, surprisingly a 4-fold increase was observed by deletion up to −47 (Fig. 4B). This result suggests that the original mRNA construct does not possess a maximal activity and that the −84 to −47 region includes a negative element to regulate rbcS mRNA translation.

As a long pyrimidine-rich sequence lies in the −46 to −15 region (Fig. 4A), mRNAs with further deletion toward the initiation site were used to examine the role of this sequence. The translation efficiency of these mRNAs was dramatically reduced compared with Δ−66 and Δ−47 mRNAs, and deletion to −15 (removal of the entire pyrimidine-rich sequence) virtually abolished translation (Fig. 4B, Δ−15). We then examined mRNAs with internal deletions. As shown in Fig. 4C, deletion of the whole pyrimidine-rich sequence (Δ−46/−15) caused a drastic reduction (~10% of the wild-type), and translation decreased to ~20% by deleting half of the pyrimidine-rich sequence (Δ−27/−15). mRNA quantity was monitored during incubation using WT, Δ−66, and Δ−15 mRNAs. Despite the presence or absence of the pyrimidine-rich region, these mRNAs decayed in a similar manner (~70% for 40 min), suggesting that the observed difference is not due to the difference in mRNA stability (supplemental Fig. S1). These results confirmed that the pyrimidine-rich region is an important element for efficient translation of rbcS mRNAs. However, this region was not essential for translation because its complete removal still retained translation (~10% of the wild-type). Translation expected, translation was completely inhibited with 4 μg/ml chloramphenicol (data not shown).

To examine whether the optimized in vitro system translates faithfully cyanobacterial mRNAs, we replaced the authentic initiation codon AUG in the psbA-lacZ mRNA with UAC. The mutated mRNA lost completely its translation activity (Fig. 1B, lane 2). No difference was observed when the authentic UGA stop codon was replaced with UAA (Fig. 1B, lane 3). An exchange of the UGA for UGG produced a long polypeptide of 23 kDa as the 3'-terminal region (Fig. 1B, lane 4). This is consistent with that of the predicted product from the start codon to the 3'-end of mRNA (see Fig. 1A), that is, the calculated mass of 25.9 kDa subtracted by 3 kDa due to the hydrophobicity as mentioned above. We also carried out translation reactions in vitro using three additional mRNAs from Synechococcus 6301 rbcS, rpl3, and rpl4 (Fig. 2A; Refs. 40 and 41). All these mRNAs fused with the lacZ mRNA synthesized translation products of practically expected sizes at varied efficiencies (Fig. 2, B and C). Thus, our in vitro system supports correct translation from various Synechococcus mRNAs.

**Translation Initiation Site and SD-like Sequences of rbcS mRNA**—The rbcL and rbcS genes encoding the large and small subunits, respectively, of ribulose-1,5-bisphosphate carboxylase/oxygenase constitute an operon in Synechococcus 6301 (Fig. 3A; Ref. 40), and the rbcS mRNA has two possible initiation codons, both AUGs, separated by three nt, namely as “1st AUG” and “2nd AUG” in Fig. 3A. To define the actual translation initiation site, we constructed an rbcS-lacZ fusion mRNA...
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was also arrested by deleting the immediately upstream sequence from the start site (Δ−14/−1), suggesting that an additional sequence(s) is required for translation. We next examined whether the enhancer activity requires a specific sequence or a high pyrimidine content in the pyrimidine-rich region. The −27 to −15 region of rbcS mRNAs was replaced by random pyrimidine-rich sequences or random purine-rich sequences. Replaced constructs were cloned and 14 mRNAs derived from randomly selected clones were analyzed. As listed in Table 1, all the mRNAs with different purine-rich sequences showed lower translation efficiency than the original mRNA (42 to 5% of the wild-type). On the other hand, pyrimidine-rich constructs exhibited generally the opposite effect; 9 out of the 14 mRNAs showed higher efficiency than the wild-type mRNA (42 to 56% of the wild-type). The translation of rbcS mRNA fragments consisting of the 5′-UTR of 90 nt and its 5′-coding region of 66 nt (no lacZ mRNA) was incubated in the standard translation mixture. After RNase A treatment of UV cross-linked samples, a clear protein band of 38 kDa (p38) was detected on PAGE (Fig. 6A). The translation of rbcS mRNAs is strictly dependent on Mg²⁺ (the optimum at 10 mM) as described, and no translation was detected by 100-fold excess 30R. These results suggest that a depletable factor(s) interacts with the pyrimidine-rich region of rbcS mRNAs for efficient translation.

UV cross-linking experiments were then performed to detect a possible protein factor(s) interacting with the rbcS mRNA. The 32P-labeled rbcS mRNA fragments consisting of the 5′-UTR of 90 nt and its 5′-coding region of 66 nt (no lacZ mRNA) was incubated in the standard translation mixture. After RNase A treatment of UV cross-linked samples, a clear protein band of 38 kDa (p38) was detected on PAGE (Fig. 6A). The translation of rbcS mRNAs is strictly dependent on Mg²⁺ (the optimum at 10 mM) as described, and no translation was detected by 100-fold excess 30R. These results suggest that a depletable factor(s) interacts with the pyrimidine-rich region of rbcS mRNAs for efficient translation.

The 38-kDa Ribosomal Protein S1 Interacts with the Pyrimidine-rich Sequence—Our in vitro experiments indicated that translation of the rbcS mRNA starts exclusively from the 1st AUG, but not from the 2nd AUG, and requires the pyrimidine-rich region (Δ−46 to −15) but not SD-like sequences. It is therefore expected that a trans-acting factor(s) is involved in recognizing the correct initiation site. To examine the existence of a protein factor(s) functionally interacting with the pyrimidine-rich region of the rbcS mRNA, we carried out competition assays with 2.5 pmol of the mRNA template (¼ of the standard condition) to detect competition sharply. As mentioned above, not only the −46 to −15 region but also a variety of pyrimidine-rich sequences enhanced translation, and hence we used a mixture of 30-mer random deoxyxypuridine sequences (30Y) or of 30-mer random deoxyxypuridine sequences (30R) as competitors. Translation was reduced by adding 2.5 pmol of 30Y (an equivalent amount of the mRNA template) and arrested completely by 25 pmol or more (10-fold excess or more) of 30Y (Fig. 5). On the other hand, 2.5 pmol of 30R did not affect translation, and 25 pmol of 30R decreased translation, and no translation was detected by 100-fold excess 30R. These results suggest that a depletable factor(s) interacts with the pyrimidine-rich region of rbcS mRNAs for efficient translation.

FIGURE 3. Identification of the translation start site of rbcS mRNA and effect of SD-like sequences on translation. A, schematic representation of the rbcS locus and a partial sequence around the translation initiation site of rbcS-lacZ fusion mRNAs. Possible initiation codons are shown by bold type. Potential ribosome-binding sites are boxed in which underlined sequences can form base pairs with the 3′-end of 16 S rRNA. Arrows indicate mutated nucleotides with site names (S1, S2, and S3). B, the 3′-end sequence in Synechococcus 6301 16 S rRNA and its complementary sequence (gray characters). Underlined sequences are present in the 5′-UTR (shown in A). C, identification of the bona fide translation start codon from two AUG codons. Translation activities were calculated from band intensities and shown above as a bar graph (the wild-type mRNA (WT) as 100%, n = 3). Mutated nucleotides are indicated in A. D, effect of potential ribosome-binding sites on translation. Translation activities were shown as described for C.
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(Δ−47) up to the pyrimidine-rich region (−46 to −15) retained p38 interaction at a level comparable with the wild-type mRNA. Further deletion into the pyrimidine-rich region (Δ−28 and Δ−21) reduced cross-linked signals ~60% and ~10% of the wild-type, respectively. A residual signal was still detected by deleting the entire pyrimidine-rich region (Δ−15) and the whole 5′-UTR (Δ−1), probably due to nonspecific binding of p38. A similar result was obtained using internal deletion mRNAs (Δ−24/−15 and Δ−46/−15 in Fig. 6B). These results indicate that p38 preferentially binds to the pyrimidine-rich region of the rbcS mRNA.

As can be seen in Figs. 4 and 6, the level of translation correlates roughly with that of p38 cross-linking using a set of deleted mRNAs. No additional protein bands could be detected by the UV cross-linking assay, suggesting that p38 is a major trans-acting factor for efficient translation of rbcS mRNAs.

Previously, we reported that the ribosomal protein S1 from Synechococcus 6301 is 38 kDa in size (35). To examine whether p38 is S1, we carried out immunoprecipitation using the antibody against the cyanobacterial S1. The p38 protein cross-linked with the 32P-labeled rbcS mRNA was digested with RNase A, and then it was applied to immunoprecipitation followed by PAGE. As shown in Fig. 6C, p38 cross-reacted exclusively with the anti-S1, indicating that p38 is the 38-kDa ribosomal protein S1.

DISCUSSION

Our in vitro system supported faithful translation initiation from authentic initiation sites and accurate translation termination at stop codons (see Fig. 1). This system would be useful to define genuine protein-coding regions when multi-
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We showed that the pyrimidine-rich sequence (−45 to −15) in the 5′-UTR is required for efficient translation of the mRNA and that the SD-like sequence is not necessary for its translation initiation. Furthermore, mutation of the SD-like sequence (S1, from AGGAU to ACCAU, −14 to −10) significantly enhanced translation. A possible explanation is that the mutation generated a longer pyrimidine-rich sequence (the −46 to −6 region; pyrimidine:purine = 32:8) than that of the wild-type mRNA (the −46 to −15 region; pyrimidine:purine = 24:6). The region between the start codon and the pyrimidine-rich sequence (−14 to −1) is also important for translation probably not in a sequence-specific manner but as a distance because base changes did not affect translation but its deletion inhibited translation. Our UV cross-linking and immunoprecipitation experiments showed that the 38-kDa ribosomal protein S1 interacts with the pyrimidene-rich sequence under translation conditions. Pyrimidine-rich sequences are found in the 5′-UTR of other mRNAs from Synechococcus 7942 and 6301. For example, psba1 (39), nrtA (46), zwf (47), and sigA (Kyoto Encyclopedia of Genes and Genomes data base) contain over 75% pyrimidine residues in the −10 to −30 region. The 38 kDa-S1 protein is possibly involved in efficient translation of these mRNAs because E. coli S1 has no strict sequence specificity (33).

Previously, we reported that the S1 protein is present not only in ribosomal fractions but also in soluble fractions in Synechococcus 6301 (36). Based on the model proposed for efficient translation of rpsA mRNAs encoding S1 in E. coli (48), free S1 may also be inhibitory for translation in Synechococcus. E. coli S1 possesses a long N-terminal domain that is responsible for protein-protein interactions, whereas Synechococcus S1 has no such domain and consists mainly of three S1 motifs (four in E. coli S1). These two S1 proteins exhibit different nucleic acid binding specificities in vitro; E. coli S1 binds poly(C), poly(U), and poly(A), while Synechococcus S1 binds poly(G), poly(U), and poly(A) (36). The S1-binding target in 5′-UTRs is AU-rich in E. coli (49) but CU-rich in Synechococcus.

Synechococcus 6301 cells possess an additional S1 homologue of 33 kDa in size. It is not associated with ribosomes, and its nucleic acid binding specificity is distinct from that of the 38-kDa S1 protein: high affinity for both single- and double-stranded DNAs as well as poly(G) and poly(A) (36). This 33-kDa protein may participate in translation initiation of a different type of cyanobacterial mRNAs. As a summary, our in vitro translation system will provide powerful approach to analyze translation reactions in cyanobacterium and to disclose novel translation mechanisms unique to cyanobacteria. Comparison of the translation mechanism elucidated by using our in vitro systems from cyanobacteria and tobacco chloroplasts will provide basic data to understand an interesting evolutionary relationship.

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REFERENCES
1. McFadden, G. I. (1999) Curr. Opin. Plant Biol. 2, 513–519
2. Sato, N. (2002) Genome Inform. Ser. Workshop Genome Inform. 13, 173–182
3. Raven, J. A., and Allen, J. F. (2003) Genome Biol. 4, 209
4. Gruber, T. M., and Gross, C. A. (2003) Annu. Rev. Microbiol. 57, 441–466
5. Thornton, L. E., Ohkawa, H., Roose, J. L., Kashino, Y., Keren, N., and Pakrasi, H. B. (2004) Plant Cell 16, 2164–2175
6. Ogawa, T., Bao, D. H., Katoh, H., Shibata, M., Pakrasi, H. B., and Bhattacharyya-Pakrasi, M. (2002) J. Biol. Chem. 277, 28981–28986
7. Michel, K. P., and Pistorius, E. K. (2004) Physiol. Plant 120, 36–50
8. Wang, T., Shen, G., Balasubramanian, R., McIntosh, L., Bryant, D. A., and Golbeck, J. H. (2004) J. Bacteriol. 186, 956–967
9. Choquet, Y., and Wollman, F. A. (2002) FEBS Lett. 529, 39–42
10. Sugita, M., and Sugiyama, M. (1994) Nucleic Acids Res. 22, 25–31
Translation Initiation of Cyanobacterial rbcS mRNAs

11. Sato, N. (1995) Nucleic Acids Res. 23, 2161–2167
12. Sato, N., and Wada, A. (1996) Plant Cell Physiol. 37, 1150–1160
13. Matsuda, M., Sugiura, M., and Sugita, M. (1999) Plant Cell Physiol. 40, 1203–1209
14. McCarthy, J. E., and Brimacombe, R. (1994) Trends Genet. 10, 402–407
15. Green, R., and Noller, H. F. (1997) Annu. Rev. Biochem. 66, 679–716
16. Gold, L. (1988) Annu. Rev. Biochem. 57, 199–233
17. Ringquist, S., Shinedling, S., Barrick, D., Green, L., Binkley, J., Stormo, G. D., and Gold, L. (1992) Mol. Microbiol. 6, 1219–1229
18. Hirosawa, M., Sazuka, T., and Yada, T. (1997) DNA Res. 4, 179–184
19. Ma, J., Campbell, A., and Karlin, S. (2002) J. Bacteriol. 184, 5733–5745
20. Sugiura, M., Hirose, T., and Sugita, M. (1998) Annu. Rev. Genet. 32, 437–459
21. Hirose, T., and Sugiura, M. (1996) EMBO J. 15, 1687–1695
22. Hirose, T., and Sugiura, M. (2004) Nucleic Acids Res. 30, 3503–3510
23. Pledger, W., and Sugiura, M. (2003) Plant J. 34, 377–382
24. Subramanian, A. R. (1993) Trends Biochem. Sci. 18, 177–181
25. Yamaguchi, K., and Subramanian, A. R. (2000) J. Biol. Chem. 275, 28466–28482
26. Yamaguchi, K., Von Knooblauch, K., and Subramanian, A. R. (2000) J. Biol. Chem. 275, 28455–28465
27. Yamaguchi, K., Prieto, S., Beligni, M. V., Haynes, P. A., McDonald, W. H., Yates, J. R., III, and Mayfield, S. P. (2002) Plant Cell 14, 2957–2974
28. Yamaguchi, K., Beligni, M. V., Prieto, S., Haynes, P. A., McDonald, W. H., Yates, J. R., III, and Mayfield, S. P. (2003) J. Biol. Chem. 278, 33774–33785
29. Yamaguchi, K., and Subramanian, A. R. (2003) Eur. J. Biochem. 270, 190–205
30. Sato, N., Tachikawa, T., Wada, A., and Tanaka, A. (1997) J. Bacteriol. 179, 7063–7071
31. Sato, N., Wada, A., and Tanaka, A. (1998) Plant Cell Physiol. 39, 1367–1371
32. Zhang, J., and Deutscher, M. P. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 2605–2609
33. Boni, I. V., Isaeva, D. M., Muschenko, M. L., and Tzareva, N. V. (1991) Nucleic Acids Res. 19, 155–162
34. Sengupta, J., Agrawal, R. K., and Frank, J. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 11991–11996
35. Sugita, M., Sugita, C., and Sugiura, M. (1995) Mol. Gen. Genet. 246, 142–147
36. Sugita, C., Sugita, M., and Sugita, M. (2000) Mol. Gen. Genet. 263, 655–663
37. Subramanian, A. R. (1983) Prog. Nucleic Acids Res. Mol. Biol. 28, 101–142
38. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
39. Golden, S. S., Brusslan, J., and Haselkorn, R. (1986) EMBO J. 5, 2789–2798
40. Shinozaki, K., and Sugiura, M. (1985) Mol. Gen. Genet. 200, 27–32
41. Sugita, M., Sugishita, H., Fujishiro, T., Tsuboi, M., Sugita, C., Endo, T., and Sugiura, M. (1997) Gene (Amst.) 195, 73–79
42. Rippka, R., Deurreuelles, J., Waterbury, J. B., Herdman, M., and Stanier, R. Y. (1979) J. Gen. Microbiol. 111, 1–61
43. Sauer, J., Schreiber, U., Schmid, R., Volker, U., and Forchhammer, K. (2001) Plant Physiol. 126, 233–243
44. Tomioka, N., and Sugita, M. (1983) Mol. Gen. Genet. 191, 46–50
45. Watanabe, T., Sugita, M., and Sugita, M. (1998) Biochim. Biophys. Acta 1396, 97–104
46. Omata, T. (1991) Plant Cell Physiol. 32, 151–157
47. Scanlan, D. J., Newman, J., Sebaihia, M., Mann, N. H., and Carr, N. G. (1992) Plant Mol. Biol. 19, 877–880
48. Boni, I. V., Aronamonova, V. S., Tzareva, N. V., and Dreyfus, M. (2001) EMBO J. 20, 4222–4232
49. Komarova, A. V., Tchufistova, L. S., Supina, E. V., and Boni, I. V. (2002) RNA (N. Y.) 8, 1137–1147