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RNA Polyadenylation and Degradation in Cyanobacteria Are Similar to the Chloroplast but Different from *Escherichia coli**

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The mechanism of RNA degradation in *Escherichia coli* involves endonucleolytic cleavage, polyadenylation of the cleavage product by poly(A) polymerase, and exonucleolytic degradation by the exoribonucleases, polynucleotide phosphorylase (PNPase) and RNase II. The poly(A) tails are homogenous, containing only adenosines in most of the growth conditions. In the chloroplast, however, the same enzyme, PNPase, polyadenylates and degrades the RNA molecule; there is no equivalent for the *E. coli* poly(A) polymerase enzyme. Because cyanobacteria is a prokaryote believed to be related to the evolutionary ancestor of the chloroplast, we asked whether the molecular mechanism of RNA polyadenylation in the *Synechocystis* PCC6803 cyanobacteria is similar to that in *E. coli* or the chloroplast. We found that RNA polyadenylation in *Synechocystis* is similar to that in the chloroplast but different from *E. coli*. No poly(A) polymerase enzyme exists, and polyadenylation is performed by PNPase, resulting in heterogeneous poly(A)-rich tails. These heterogeneous tails were found in the amino acid coding region, the 5′ and 3′ untranslated regions of mRNAs, as well as in rRNA and the single intron located at the $tRNA^{fmet}$. Furthermore, unlike *E. coli*, the inactivation of PNPase or RNase II genes caused lethality. Together, our results show that the RNA polyadenylation and degradation mechanisms in cyanobacteria and chloroplast are very similar to each other but different from *E. coli*.

The molecular mechanism of RNA degradation in prokaryotes and organelles includes a series of sequential steps. The degradation starts with the initial endonucleolytic cleavage carried out primarily by the endoribonuclease E (RNase E). The cleavage products are then polyadenylated at their 3′ ends by the poly(A) polymerase (PAP)[1] enzyme in *Escherichia coli* and the polynucleotide phosphorylase (PNPase) in the chloroplast. The polyadenylated molecules are then rapidly degraded exonucleolytically by PNPase and ribonuclease II (RNase II) (1–3). Finally, the remaining short oligoribonucleotides are degraded by the oligoribonuclease enzyme (4). Although the inhibition of polyadenylation in the chloroplast inhibited exonucleolytic degradation, implying that this is the only mechanism in the RNA degradation process, two RNA degradation mechanisms, a polyadenylation-dependent and a polyadenylation-independent one, were suggested to take place in *E. coli* (5, 6). Polyadenylation in *E. coli* is carried out primarily by PAP (7). Also in this bacterium, the RNase E enzyme, part of the PNPase population, an RNA helicase, some RNA molecules, and the glycolytic enzyme enolase are associated in a high molecular weight complex called a degradosome (8).

In the chloroplast, the photosynthetic organelle of the plant cell is believed to have an evolutionary prokaryotic origin; many characteristics of the gene expression system resemble those of bacteria. When the RNA degradation mechanism was analyzed in the chloroplast, it was found to be very similar to that of *E. coli* (1–3). However, significant differences were observed in the two systems. The first is the lack of the degradosome, the high molecular weight complex described above (9). Second, whereas PAP is responsible for most of the polyadenylation activity in *E. coli*, PNPase performs both the polymerization and the degradation of the endonucleolytic cleavage products during the RNA degradation process in the chloroplast of higher plants. This is the reason why, unlike the situation in *E. coli* where the sequences of the poly(A) tails are mostly homogenous adenosines, these sequences in the chloroplast are heterogeneous and also include the three other nucleotides (10).

These observations raised the question as to why RNA polyadenylation is performed by PAP in *E. coli* whereas the same function is performed by PNPase in the chloroplast. Assuming a common, unique evolutionary ancestor for prokaryotes and the chloroplast organelle, one possibility is that the PAP and degradosome functions were acquired by *E. coli* late in its evolution. Another possibility is that they might have been lost in the chloroplast.

Cyanobacteria is a prokaryote believed to be evolutionarily closely related to the chloroplast ancestor. However, unlike the chloroplast of higher plants in which the mRNA half-life is in the order of hours, it is in the range of several minutes or less in cyanobacteria, similar to *E. coli* (11–13). The *Synechocystis* genome contains genes that are highly homologous to RNase E, PNPase, RNase II/R, and PAP, the major players in the RNA polyadenylation and degradation pathway. Here, we analyzed whether the polyadenylation of RNA molecules is performed in the *Synechocystis* cyanobacteria by the PAP enzyme, as in *E. coli*, or by the PNPase enzyme, as in the chloroplast of higher plants. Our results suggest that PNPase carries out this function in *Synechocystis*. We also confirmed a previous observation that RNase E in *Synechocystis* is not engaged in an *E. coli*-type degradosome multicomponent complex (14). Therefore, it is most likely that *E. coli* and some other related bacteria have acquired the PAP and degradosome complex functions late in evolution, possibly to enable rapid RNA turnover in these bacteria strains. We also analyzed the single intron located at the $tRNA^{fmet}$ gene and found it to be heavily polyadenylated at

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[1] The abbreviations used are: PAP, poly(A) polymerase; PNPase, polynucleotide phosphorylase; RT, reverse transcription.

internal sites, suggesting that introns are degraded by the same mechanism following splicing.

EXPERIMENTAL PROCEDURES

Determination of poly(A) Tail Length—*Synechocystis* PCC6803 was grown in a BG11 medium at 30 °C (15). A freshly grown logarithmic culture was divided into two parts; half was transferred to a medium without phosphate for 2 days, and half was maintained in a phosphate-containing medium. Total RNA was isolated using the "hot phenol" protocol (16), and the poly(A) RNA was isolated using the Qiagen kit (Qiagen Inc.). Total spinach leaf RNA (30 μg) and the poly(A)$^+$ RNA of *Synechocystis* (obtained from 200 μg of total RNA) were 3′ end-labeled with [^{32}P]pCp and T4 RNA ligase for 24 h at 4 °C before digestion with 25 μg of RNase A and 300 units of RNase T1 for 1 h at 37 °C (17). poly(A) tails were resolved in 12% polyacrylamide sequencing gels containing 7 M urea. To obtain an equal signal, the equivalent of 8 and 200 μg of total RNA from spinach and *Synehcocystis,* respectively, were loaded on the gel.

Determination of the poly(A) Sites and Sequences—RNA was isolated from a freshly grown logarithmic culture of *Synechocystis* PCC6803. The guanylation of the RNA was performed on 40 μg of RNA incubated for 60 min at 25 °C with 1 mM GTP and *E. coli* PNPase (Sigma). Following phenol extraction and ethanol precipitation, cDNA was synthesized using avian myeloblastosis virus-reverse transcriptase and d(C_9T_6) primer at 50 °C for 2 h (18, 19). Following reverse transcription, the reaction was stopped by adding 200 μl of H_2O, and 0.5 μl was used for PCR amplification using d(C_9T_6) and gene-specific primers (see Fig. 2). The PCR products were gel-purified and either directly sequenced or cloned into the pGEM-T vector (Promega Inc.). In some of the clones, the oligo(dT)-adaptor oligonucleotide was used to prime the cDNA synthesis, as described previously (17). The nucleotide sequences of the oligonucleotides used were as follows: oligonucleotide rbcLA1, starting at nucleotide 1345 of *rbcL*, 5′-CGGGAAGCCTGTCGTTGGTC; rbcL3, starting at nucleotide 1373 of *rbcL*, 5′-AGTTGGCCGCCGCCGCCTGCGAA; rbcL6, nucleotide −130; and 5′-CGCAGATTTTTCTCGCAACC (see Fig. 3). The eight nucleotides of the 3′ end of rbcL6 were found also at location 2478 of the 23 S rRNA, and therefore this oligonucleotide was used as well in the RT-PCR of this gene (see Fig. 4), tRNAu, starting at nucleotide 342 of the tRNA^{fmet} 5′-TGAGCTGTTTTACGTCTTCC (see Fig. 4).

The Construction of Knock-out Mutants of Synechocystis—Knock-out of the corresponding gene was performed by inserting the kanamycin resistance expression cassette, which was PCR-amplified from the SK-Kan plasmid, into the gene using oligonucleotide primers with the corresponding restriction sites (20, 21). The PAP (putative), PNPase, RNase II, and RNase E genes (see Table II for the identification of the genes) were PCR-amplified from genomic DNA, isolated as described previously (20), and cloned into the PUC57T plasmid (Fermentus Inc.). The nucleotide sequences of the PCR primers are available upon request. The kanamycin cassette was cloned into the *Avr*II sites of the putative PAP, the *Acc65I* sites of PNPase, *Nhe*I/*Stu*I of RNase II, and *Bsp*MI/*Sty*I of RNase E. Each plasmid contained about 200–500 bp of the corresponding 5′ and 3′ ends interrupted by the 1,200 bp of the kanamycin resistance expression cassette. The plasmids were transformed into *Synechocystis* PCC6803 cells, as described previously (22). The transformed cells were spread on a nitrocellulose membrane, grown on increasing concentrations of kanamycin (5–500 μg/ml), and analyzed by PCR for the homoplasmic presence of the disrupted gene. No homoplasmic cells could be obtained in all of the genes disrupted in this work, even after transferring the colonies eight times on plates containing 500 μg/ml kanamycin. This indicated that the expression of these genes is required for the viability of the cells.

Expression of Putative PAP in E. coli—The putative PAP of *Synechocystis* was PCR-amplified from genomic DNA and cloned into the *E. coli* pQE31 expression vector (New England Biolabs, Inc.) using the *Sma*I and *Pst*I restriction endonuclease sites. Expression and purification on a NTA-agarose column were performed, as described previously (9).

In Vitro Polyadenylation and Nucleotidyltransferase Activity Assays—An *in vitro* polyadenylation assay, using either the recombinant putative PAP protein or the yeast PAP (Amersham Biosciences), was performed as described previously (10). Briefly, [^{32}P]-labeled RNA corresponding to the 3′ end of the spinach chloroplast gene *petD* was incubated with 1 mM ATP in Buffer E (20 mM HEPES, pH 7.9, 70 mM KCl, 16.25 mM $MgCl_2$, 0.1 mM EDTA, 4 mM dithiothreitol, and 5% glycerol). Following incubation, the RNA was purified and analyzed using denaturing polyacrylamide gel and autoradiography. For the nucleotidyltransferase activity assay, the recombinant protein (10 ng) was incubated with *E. coli* tRNA (Sigma) in a buffer containing CTP, ATP, and [^{32}P]ATP (23). Following incubation, the RNA was purified and analyzed by denaturing polyacrylamide gel electrophoresis and autoradiography.

Size-exclusion Chromatography—Size-exclusion chromatography was performed by applying *Synechocystis* soluble proteins to a Superdex 200 column in Buffer E at a flow rate of 0.5 ml/min (9). Each fraction was then loaded on a heparin column. The bound proteins were eluted, precipitated by cold acetone, and analyzed by SDS-PAGE and immunoblot using specific antibodies against the *Arabidopsis* RNase E. The Superdex 200 column was calibrated with the following protein standards: thyroglobolin, 669 kDa; catalase, 232 kDa; aldolase, 158 kDa; bovine serum albumin, 67 kDa; and casein, 30 kDa.

Production of Antibodies—For the production of antibodies, the *Arabidopsis* RNase E was expressed in *E. coli*, tagged with His_6 using the pQE expression system, purified by nickel-nitrilotriacetic acid affinity chromatography, and injected into a rabbit. Specific antibodies were prepared as described previously (24) and were used to detect the protein. The protein amount was determined using the Bio-Rad assay.

RESULTS

RNA Polyadenylation in Synechocystis—The addition of poly(A) and poly(A)-rich tails to the RNA of *E. coli*, the chloroplast, and the mitochondria of higher plants has been studied in recent years. Although the analysis of poly(A) tails in *E. coli* disclosed homogenous, 30–60-nt-long poly(A) tails located at the 3′ end, as well as at internal sites, poly(A) tails containing other nucleotides were described in stationary grown bacteria and when the gene of PAP was inactivated (7). This strain, containing an inactivated PAP, is viable, because PNPase polyadenylates the RNA. Most of the polyadenylating sites in chloroplasts were located internally and not at the 3′ end; they were several hundred nucleotides long and heterogeneous, consisting of about 70% adenosines, 20% guanosines, and the rest of nucleotides, uridines, and cytidines (17). This heterogeneity of the polyadenylated tails was later explained by the lack of equivalent *E. coli* PAP enzyme. Indeed, it was found that PNPase is the only polyadenylation enzyme in the chloroplast (10). Therefore, the nature of the polyadenylated tail could enlighten us regarding the polyadenylating enzyme, which is homogenous for PAP and heterogeneous for PNPase. Because cyanobacteria is believed to be closely related to the evolutionary ancestor of the chloroplast, we intended to analyze which is the major polyadenylating enzyme in this organism, PAP as in *E. coli* or PNPase as in the chloroplast.

To detect RNA polyadenylation in *Synechocystis*, and because we had predicted that only a minute fraction of the RNA is polyadenylated at the steady-state level, we first enriched the polyadenylated RNA by applying the total RNA to the oligo(dT) affinity column. The oligo(dT) purification step was necessary, because several trials to carry out this experiment on total RNA had previously failed. The RNA bound to the column was radioactively labeled at the 3′ end with [^{32}P]pCp and then digested for completion with RNase A (cutting after C and U residues) and RNase T1 (cleaving after G residues). Therefore, only poly(A) sequences present at the 3′ end of the RNA molecules remained intact and radioactively labeled. As a control, we used total RNA isolated from spinach leaves, in which we expected to get a ladder of poly(A) tails of all lengths (17). In addition, because phosphate starvation was reported recently (25) to enhance RNA stability significantly in the chloroplast of *Chlamydomonas reinhardtii*, we performed the same experiment on culture that was starved for phosphate. An analysis of the products on polyacrylamide gel and autoradiography revealed that the total spinach leaf RNA was degraded into a ladder of A-containing homopolymers, as reported previously (17) for polyadenylated RNA (Fig. 1). The ladder shape of these results reflected the formation of poly(A) tails of every length because of the metabolism of the polyadenylated tails (26, 27). The products of *Synechocystis* RNA also resulted in a

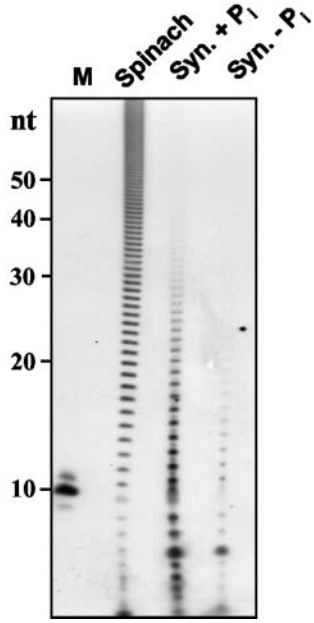

FIG. 1. **Detection of poly(A)-tailed RNA in *Synechocystis*.** Total RNA was isolated from spinach leaves and *Synechocystis* cells that were transferred to a growth medium for 2 days either with (*Syn.* + P_i) or without (*Syn.* − P_i) phosphate. Total RNA from spinach and purified poly(A) RNA from *Synechocystis* were labeled with [^{32}P]pCp at the 3′ end and digested with RNase T1 and RNase A. The remaining poly(A) tails were then fractionated on denaturing PAGE and detected by autoradiography. The equivalent amounts of 200 and 8 μg of total *Synechocystis* and spinach RNAs, respectively, were analyzed. An end-labeled, 10-nt oligonucleotide was run in the same gel as a size marker (*M*).

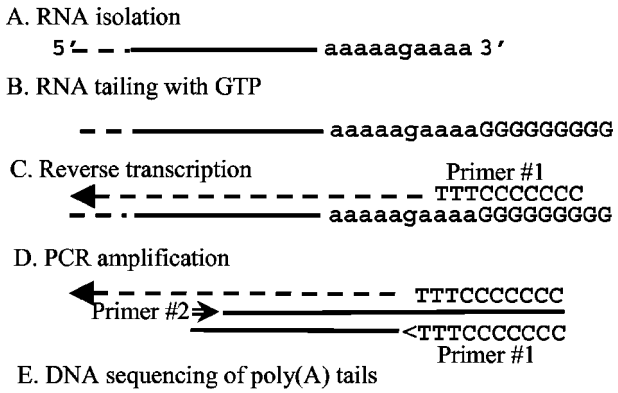

FIG. 2. **Method used for determining poly(A) tail sequences and lengths.** Total RNA was purified from *Synechocystis* cells (*A*) and polyguanylated using *E. coli* PNPase and GTP (*B*). This RNA was then reverse-transcribed using oligo(dC_9T_6) (*Primer #1*) as a primer (*C*). The reverse-transcribed cDNA product was then PCR-amplified with oligo(dC_9T_6) and a gene-specific primer #2 (*D*). The PCR product was sequenced to determine the poly(A) tail length and sequence (*E*).

ladder of shorter tails of up to about 30 nucleotides long (Fig. 1). Because *Synechocystis*-polyadenylated RNA was first isolated on an oligo(dT) column, we could not determine whether the stronger signal observed in the non-phosphate depleted cells indicated a greater amount of polyadenylated tails under these conditions. However, this difference was observed in several repetitions of this experiment. Roughly 25 times the amount of *Synechocystis* RNA was used as a starting material to obtain a similar signal (Fig. 1). This may indicate that the amount of polyadenylated tails in *Synechocystis* is about 25 times less the amount of mRNA in the plant cell. Together, these results implied that *Synechocystis* RNA is polyadenylated, suggesting that this polyadenylation produces homogenous poly(A) tails of up to about 30 nucleotides long. If only these homogenous tails are present, then we can expect a poly(A) polymerase and not PNPase to be the major polyadenylating enzyme, similar to the situation in *E. coli* and unlike the case in the chloroplast. Indeed, a gene termed PAP was identified in the fully sequenced *Synechocystis* genome (see below). However, because only poly(A) RNA was analyzed in this experiment because of the oligo(dT) column purification step, the other possibility is that there are heterogeneous tails in *Synechocystis* containing only short adenosine stretches that were not purified by the oligo(dT) purification step and not detected in these experiments.

To examine this possibility, we analyzed the location, length, and sequences of polyadenylated tails of this prokaryote using reverse-transcription PCR. In previous experiments, we used oligo(dT)-primed cDNA for the analysis of the chloroplast poly(A) tails (17). Although tails of several hundred nucleotides long were observed, we could not assure that the actual tails were not even longer. Therefore, we modified this procedure to analyze the full-length of the poly(A) tail. First, the RNA molecules were polyguanilated by adding GTP and the *E. coli* PNPase (Fig. 2). The transcript was then reverse-transcribed using the d(C_9T_6) primer, and the cDNA was amplified using the same primer, in addition to the gene-specific one (Fig. 2). The previous oligo(dT)-primed RT-PCR method was also used, and no significant differences were obtained between the two (17). The *rbcL* gene encoding the large subunit of the ribulose bisphosphate carboxylase was chosen as a representative protein-encoding transcript. An inverted repeat sequence located between nucleotides 1,470 and 1,545 (counting started from the first AUG) suggested the formation of a stem-loop structure typically of prokaryotic and organelle genes at the 3′ end. Many polyadenylated tails were observed in the 3′ region of this transcript ranging in length from 15 to 172 nt (Fig. 3). Similar to the situation observed previously in the chloroplast, the polyadenylation sites were distributed throughout the analyzed region, including the amino acid coding region, the 3′ untranslated region, and the putative stem-loop structure. In addition, again similar to the situation in the chloroplast, the sequences were heterogeneous, containing 60% or more adenosines, about 15–25% guanosines, and the rest, cytidines and uridines, making them purine-rich sequences (Fig. 3). The search for polyadenylated sequences at the 5′ untranslated region of the *rbcL* transcript revealed polyadenylation sites located in this region as well (Fig. 3). In addition, in several cases the *rbcL* gene-specific primers annealed to other transcripts, such as heme oxygenase, CDP glucose dehydratase, and several open reading frames related to unknown genes, revealing polyadenylation sites in these RNAs (Table I). The tails presented in Figs. 3 and 4 and Table I are part of the large collection obtained throughout the course of this work. No significant differences were observed between the oligo(dT)- and the d(C_9T_6)-primed systems except that, as expected, the tails observed using the second system were longer.

The *Synechocystis* genome contains one intron in the $tRNA^{fmet}$ gene. This single intron belongs to self-splicing group I harboring an open reading frame encoding specific DNA endonuclease (28). Because the degradation of spliced introns is very fast, and no polyadenylation of intron sequences has been analyzed yet, with the exception of one chloroplast intron that was characterized recently (29) to harbor a short poly(A) tail, we looked for polyadenylation of this transcript. Indeed, 11 polyadenylated transcripts were detected in 10 different sites of this intron (Fig. 4*A*). The tails were heterogonous, similar to those obtained in the *rbcL* transcript, and were between 16 and 128 nt long. Interestingly, no poly(A) tails were observed at the 3′ end of the intron (nucleotide number 655), which is the 3′

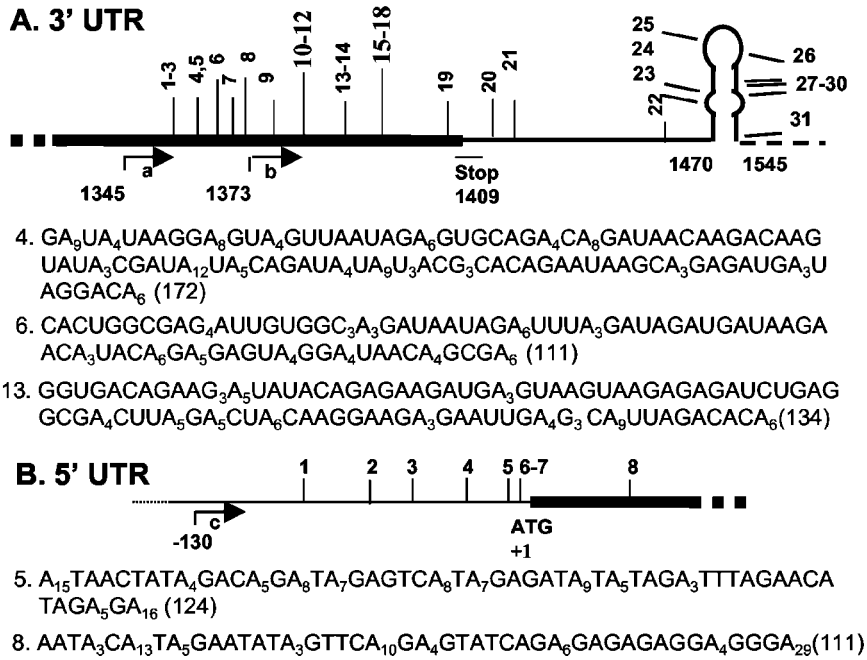

FIG. 3. **poly(A)-rich addition to *rbcL* transcripts in *Synechocystis*.** The 3′ and 5′ parts of the *rbcL* transcript (*A* and *B*, respectively) are shown schematically. The nucleotides are numbered from the translation initiation codon. The *thick line* represents the amino acid coding, and the *thin line* represents the 3′ and 5′ untranslated regions. The primers *a*, *b*, and *c* were used for RT-PCR. A predicted stem-loop structure at the 3′ end is shown, as well as 31 poly(A)-rich addition sites at the 3′ part and eight sites at the 5′ part. The sequences of tails 4, 6, and 13 at the 3′ part, as well as 5 and 8 at the 5′ parts, are presented *below*, and the length is indicated in *parentheses*.

TABLE I

poly(A)-rich tailed RNAs detected in several genes of Synechocystis

Polyadenylated transcripts identified when analyzing the reverse-transcribed PCR clones using specific primers for the *rbcL* gene. Each clone presented here contained a nucleotide sequence of the corresponding gene elongated by the poly(A)-rich tail at the indicated nucleotide (counting started from the ATG of the first methionine). The length of the tails in nucleotides is indicated.

Name of gene	Gene	Location	Length of tail
Unknown	slr1270	939	29
Heme oxygenase	sll1875	772, 785, 812, 815, 816, 817(2), 832, 863	17, 41, 18, 28, 36, 20, 21, 25, 19
Unknown	sll1414	529, 647	27, 17
CDP glucose dehydratase	slr0984	575, 644, 672	127, 69, 79
ORF376	sll0597	884	75
Unknown	sll0926	859	57
Unknown	sll0135	790	73

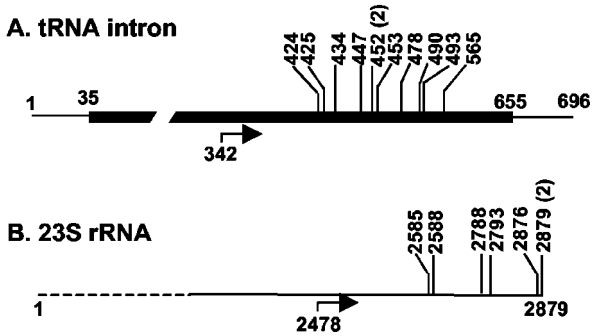

FIG. 4. **poly(A)-rich addition to tRNA^{fmet} intron and 23 S rRNA of *Synechocystis*.** The tRNA^{fmet} and the 23 S rRNA genes are shown schematically (*A* and *B*, respectively). The nucleotides are numbered from the transcription initiation site. The *thick line* represents the intron sequence, and *interrupted dashed lines* indicate that the scheme is not to scale. The location of the two gene-specific oligonucleotides primers used for the RT-PCR, as well as the location of the poly(A)-rich tails, are indicated with *arrows*. The tails were similar to those presented in Fig. 3, ranging in length from 16 to 128 nt. *(2)* indicates that two different tails were identified at the same position.

end resulting from the splicing event (Fig. 4*A*). We also examined the 23 S rRNA and found polyadenylation sites located internally and at the 3′ end, suggesting that the relatively stable rRNA is also degraded by the polyadenylation-dependent degradation mechanism (Fig. 4*B*).

Together, the nucleotide sequence heterogeneity of the tails of all RNA examined suggested that RNA polyadenylation in *Synechocystis* is carried out by PNPase and not PAP, similar to the chloroplast and unlike *E. coli*. However, because analyzing the genome sequence of *Synechocystis* disclosed a gene encoding a putative PAP homologue, we next asked whether the product of this gene is really a poly(A) polymerase enzyme.

The Product of the Putative PAP Gene of Synechocystis Displays Nucleotidyltransferase and Not PAP Activity—The *E. coli* PAP is closely related to the nucleotidyltransferase family (the tRNA CCA-adding enzymes) and displays a high homology to the amino acid sequence (30, 31). Actually, the homology is so high that the function of a gene product as either PAP or nucleotidyltransferase could not be predicted from the amino acid sequence and should be tested experimentally. A gene in the *Synechocystis* genome is identified as putative PAP (Sll1253), suggesting that PAP is polyadenylating the RNA. However, the heterogeneous nucleotide sequences of the poly(A)-rich tails suggest that PNPase is the polyadenylation enzyme. Therefore, we decided to analyze the activity of the corresponding gene product. The gene was PCR-amplified from a genomic DNA, cloned into a bacterial expression vector, expressed and purified to near-homogeneity, and assayed for either PAP or nucleotidyltransferase activities (Fig. 5). The recombinant protein was active as a nucleotidyltransferase using *E. coli* tRNA as the substrate (Fig. 5*B*). In contrast, no polyadenylating activity was observed in the assay in which the activity of the yeast enzyme was easily detected (Fig. 5*C*). While this manuscript was being reviewed, an analysis of the nucleotidyltransferase activity of this enzyme was published (32). In agreement with our results it was found that this polypeptide adds the A residue to the tRNA in *Synechocystis*

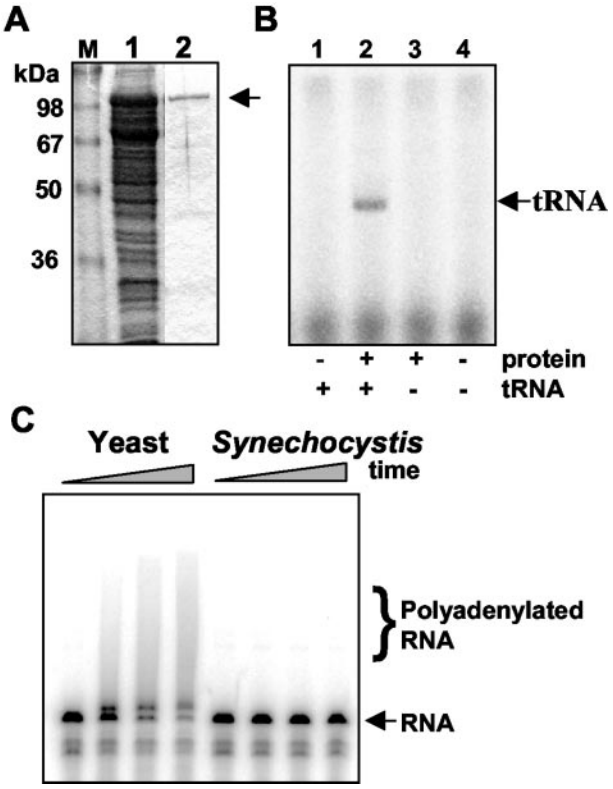

FIG. 5. **Analysis of the putative poly(A) polymerase protein for polyadenylation and nucleotidyltransferase activity.** *A*, the putative PAP open reading frame of *Synechocystis* was expressed in *E. coli*. The bacteria proteins were analyzed by SDS-PAGE electrophoresis (*lane 1*), and the putative PAP protein was purified on an affinity column (*lane 2*). In the *lane marked M*, molecular markers were fractionated as shown on the *left*. *B*, the purified protein was assayed for nucleotidyltransferase activity using tRNA as a substrate and [^{32}P]ATP, as described under "Experimental Procedures." In *lane 1*, no protein was added to the assay as a control. In *lane 2*, the protein and *E. coli* tRNA as a substrate were added to the assay. In *lane 3*, only the protein without tRNA was present, and in *lane 4*, neither tRNA nor the protein was present. Following incubation for 20 min, the tRNA was purified and analyzed by denaturing PAGE and autoradiography. The *arrow* indicates the migration of tRNA in this gel. *C*, the *Synechocystis* putative PAP is not active in RNA polyadenylation. A [^{32}P] RNA of 120 nt corresponding to the spinach chloroplast *petD* gene was incubated with ATP and either the yeast PAP (*left four lanes*) or the putative *Synechocystis* PAP (*right four lanes*). Following incubation for 0, 20, 40, and 60 min, the RNA was purified and analyzed by denaturing PAGE and autoradiography.

whereas a second polypeptide (NP_442458) adds the CC residues (32). Taken together, these results showed that the putative PAP (Sll1253, NP_441479) identified in the genome sequence of *Synechocystis* is actually a nucleotidyltransferase enzyme and not PAP.

Deletion of Genes for Putative PAP, PNPase, RNase II, and RNase E Resulted in Lethality of the Synechocystis Cells—In *E. coli*, the inactivation of genes encoding PAP or PNPase does not result in lethality, because PNPase polyadenylates the RNA in the absence of PAP and RNase II, or RNase R degrades the polyadenylated transcripts in the absence of PNPase. However, if cyanobacteria is similar to the chloroplast in which no PAP equivalent exists, and PNPase performs both the polyadenylation and degradation processes, then we could expect that knocking out PNPase would result in lethality. To determine whether this is indeed the case, we constructed a plasmid harboring a kanamycin resistance expression cassette surrounded by the N and C terminus of the PNPase gene as targets for replacement by a homologous recombination of the endogenous gene. The plasmid was transformed into *Synechocystis*, and the cells were grown on agar plates containing kanamycin as a selectable marker. Once colonies were observed, they were transferred to new plates containing elevated concentrations of the antibiotics. PCR analysis was performed to observe that the cells became homogenic and that only genome copies in which the PNPase was disrupted were present. However, even after more than eight transfers were made, and cells were grown in the presence of a high concentration of kanamycin (500 μg/ml), we were not able to obtain homoplasmic cells in which no uninterrupted PNPase gene could be detected (Table II). Therefore, we concluded that the disruption of PNPase is lethal for *Synechocystis*. Lethality also occurred when the putative PAP (shown here and in Ref. 32 to be a nucleotidyltransferase), RNase II and RNase E genes were analyzed in the same way (Table II). In *E. coli*, only the inactivation of RNase E of these enzymes is lethal, probably because of the inability to process tRNAs (33, 34). Although lethality because of the inactivation of PNPase could be explained in *Synechocystis*, because this is the only polyadenylation enzyme, unlike the situation in *E. coli*, the reason for the lethality caused by the inactivation of RNase II is still unknown. Further investigation into the differences among *E. coli*, cyanobacteria, and the chloroplast is therefore necessary.

TABLE II
The Synechocystis genes that were inactivated in this work

The genes indicated were interrupted with a kanamycin cassette inserted into the middle of each one. No viability was concluded when no homoplasmidity was observed following eight transfers of the cells on medium containing the highest concentration of the antibiotic.

Name	Gene	Amino acids	Viability
PAP (putative)	Sll1253	942	No
PNPase	Sll1043	718	No
RNase II/R	Sll1290	667	No
RNase E	Slr1129	674	No

Unlike E. coli, RNase E in Synechocystis Is Not Associated in a Multicomponent Complex—In *E. coli*, RNase E is associated together with PNPase, RNA-helicase, the enzyme enolase, RNA components, and possibly other proteins in a multicomponent complex termed a degradosome. The molecular mass of this complex was estimated to be about 500 kDa and even more (35). In *E. coli*, there is another enzyme that is homologous to the N terminus catalytic part of RNase E, the *cafA* gene product, RNase G. This enzyme is not associated in the multicomponent degradosome complex, and therefore the *Synechocystis* RNase E, which displays sequence homology only to the N terminus catalytic half of the *E. coli* RNase E, is related more to RNase G. The C terminus half of the *E. coli* protein serving as the scaffold for binding the other components of the degradosome shares no homology with the *Synechocystis* protein (9, 14, 36). Indeed when the *Synechocystis* RNase E was expressed in *E. coli*, it was purified without being associated with other members of the *E. coli* degradosome (14). In addition, we have shown recently (9) that no multicomponent degradosome complex such as the *E. coli* type exists in the chloroplast. Here, we wanted to determine whether the *Synechocystis* RNase E is associated in a high molecular weight complex similar to *E. coli* but different in the chloroplast. To this end, *Synechocystis* protein extract was fractionated on a size-exclusion column, and the RNase E protein was detected by specific antibodies. The *Synechocystis* RNase E is composed of 674 amino acids and migrates on SDS-PAGE as a 100-kDa band (14).[2] Because this protein is low in abundance, it is very difficult to be detected in the total protein extract. Therefore, we fractionated each frac-

[2] V. Liveanu, T. Nakamura, and G. Schuster, submitted for publication.

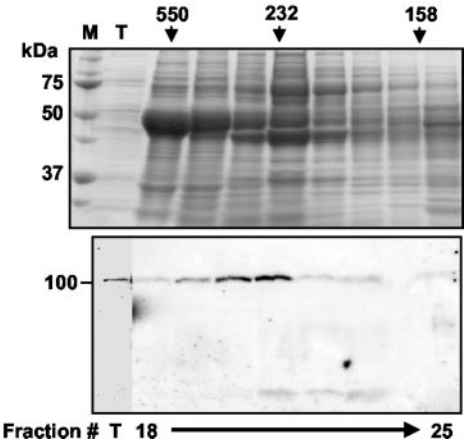

FIG. 6. ***Synechocystis* RNase E is not associated in a high molecular weight complex.** A soluble protein extract of *Synechocystis* cells was fractionated on a size-exclusion Superdex 200 column. The proteins of each fraction were further purified on a heparin column and then fractionated on SDS-PAGE and stained (*top panel*), and the RNase E was detected by immunoblotting decorated with specific antibodies (*bottom panel*). The elution profile of several molecular weight markers, as indicated under "Experimental Procedures," is indicated on the *top*.

tion of the size-exclusion column on a heparin column and analyzed the bound proteins by immunoblot using specific antibodies to the *Arabidopsis* RNase E. We used this method, because the recombinant *Synechocystis* RNase E bound to a heparin column and cross-reacted to the *Arabidopsis* RNase E antibodies.[3] Analyzing the protein pattern of the size-exclusion column fractions revealed a good separation of the protein complexes according to their molecular mass, the most remarkable of which was the ribulouse-bisphosphate carboxylase that peaked at 550 kDa (the large subunit being the most abundant protein) (Fig. 6*A*). The RNase E signal clearly peaked at about 250–300 kDa, indicating that this protein is not associated with a multicomponent complex such as the *E. coli* degradosome (Fig. 6*B*). It is not yet possible to conclude whether it is associated with other proteins, forms a homocomplex, or exists as one polypeptide. However, this molecular mass cannot account for the *E. coli*-type degradosome. Therefore, as for the polyadenylation enzyme and with the absence of the degradosome complex, the situation in *Synechocystis* is more similar to that of the chloroplast than that of *E. coli*.

DISCUSSION

In this work, we analyzed the RNA polyadenylation and degradation mechanisms of the cyanobacteria *Synechocystis* PCC6803. The RNA degradation mechanism has been described in recent years and found to be very similar in *E. coli* and the chloroplast of higher plants. In both cases, the initial event is an endoribonucleolytic cleavage of the transcript, followed by the addition of poly(A)-rich sequences in the chloroplast and poly(A) tails in *E. coli*. The polyadenylated RNA molecules then become preferred substrates for the exoribonucleases that continue the degradation process (1, 2, 6, 8). However, major differences were described between the *E. coli* and the higher plant chloroplast systems. The high molecular weight complex, the degradosome, exists in *E. coli* but not in the chloroplast (9). RNA polyadenylation is performed in *E. coli* primarily by the PAP enzyme, resulting in homogenous poly(A) tails, whereas the PNPase is responsible for this step in the chloroplast of higher plants, resulting in heterogeneous poly(A)-rich tails (10). However, only homogenous poly(A) tails were observed in the chloroplast of the green algae *C. reinhardtii*, suggesting that a PAP enzyme possibly exists there, similar to the situation in *E. coli* (19). These observations raised several interesting evolution-related questions. What was the primarily prokaryotic RNA degradation mechanism? Did the formation of the degradosome complex, RNase G, and the PAP enzyme occur during the evolution of the RNA degradation mechanism in *E. coli*? If so, the answer to these questions will probably lead to a better understanding of the ancient prokaryotic RNA degradation system and how the present systems were evolutionarily developed.

To answer these questions, we analyzed the RNA polyadenylation and degradation mechanisms in the photosynthetic prokaryote, which is believed to be closely related to the evolutionary ancestor of the chloroplast, the cyanobacteria. We found that the RNA polyadenylation and degradation mechanisms in cyanobacteria resembled those of the chloroplast of higher plants but differed in *E. coli*. Therefore, the RNA degradation mechanism described in the chloroplast and cyanobacteria probably represents the more ancient evolutionary system, and *E. coli* modified it by evolutionarily forming the degradosome and PAP functions. This suggestion is also supported by the observations that the degradosome complex is not required for viability in *E. coli* and that the PAP is very homologous to the nucleotidyltransferases, the enzymes editing the CCA 3′ tail of the tRNA molecules (6). The formation of the degradosome complex harboring the *E. coli* RNase E enzyme could also be the reason why another RNase E type enzyme, RNase G, exists in *E. coli*, which is very homologous to the N terminus half of RNase E and not included in the degradosome complex. Therefore, revealing the biological function of the degradosome will probably disclose why *E. coli* and some related bacteria have acquired this phenomenon during their evolution. It should be also noted that a degradosome differing from that of *E. coli* was identified in the *Rodobacter capsulatus* bacteria (37). Moreover, analyzing RNA polyadenylation in the Gram-positive *Streptomyces coelicolor* bacteria revealed heterogeneous poly(A)-rich tails, suggesting a lack of the PAP enzyme and further indicating that this enzyme is restricted to *E. coli* and possibly some other related bacteria (38). Interestingly, as described above, only homogenous poly(A) tails were observed in the chloroplast of the green algae *C. reinhardtii*, suggesting that perhaps PAP is the polyadenylating enzyme (19).

When the gene for PAP was inactivated in *E. coli*, the cells were found to be viable, because the PNPase functioned in RNA polyadenylation during the degradation process. The tails in these cells, where PNPase was the polyadenylating enzyme, were heterogeneous, reflecting the situation in cyanobacteria and the chloroplast of higher plants (7). The amino acid sequence of the PAP of *E. coli* is highly homologous to the enzyme nucleotidyltransferase, as shown for *Bacillus subtilis* (31) and *Synechocystis* (32) (this work); the function could not be predicted from the amino acid sequence and should be determined experimentally. This observation suggests that *E. coli* acquired the PAP function very late in its evolution by converting the nucleotidyltransferase enzyme, which adds the CCA triplet to the 3′ end of the tRNA, to the PAP, which adds poly(A). The activity of both enzymes includes the addition of the adenosine nucleotide to the 3′ end of an RNA molecule without using a template (30). A detailed study of the structures and functions of these proteins is required to understand the molecular basis of how the nucleotidyltransferase was converted into PAP in *E. coli*. A recent analysis of the crystal structure of the *Bacillus stearothermophilus* enzyme represents a big step forward toward achieving this goal (39).

As described above, RNA polyadenylation is performed by

[3] V. Liveanu, T. Nakamura, and G. Schuster, submitted for publication.

PNPase in the chloroplast, the cyanobacteria, and the *E. coli* strain lacking PAP. Exonucleolytic RNA degradation is then performed by PNPase and RNase II/RNase R. The crystal PNPase structure of the *Streptomyces antibioticus* bacteria revealed a trimetric structure that includes six RNase PH domains and three of the RNA-binding S1 and KH domains (40, 41). This is also the number of RNase PH, S1, and KH domains found in the exosome high molecular weight complex involved in the RNA degradation of yeast and mammalian cells (42). The results of this work, suggesting PNPase as being the evolutionary ancient protein complex for RNA polyadenylation and degradation, together with its similarity to the eukaryotic exosome in the number of RNase PH and RNA-binding domains and structural organization, suggest an evolutionary relationship between the prokaryotic PNPase and the eukaryotic exosome RNA degradation complexes. Therefore, the PNPase could be termed the prokaryotic exosome.

The addition of non-coding nucleotides (mostly As) to the 3′ end of the chloroplast *trnV* intron has been reported recently (29). The tails were one to 20 nt long and almost all As. Surprisingly, no poly(A)-rich tails were identified at the 3′ end when the single intron of $tRNA^{fmet}$ was analyzed in this work. However, many polyadenylation sites were detected between the RT-PCR primer location and the 3′ end of the intron, and the tails were heterogeneous and relatively long. This result suggests that the degradation of this intron is performed by a similar mechanism to that of mRNAs, initial endonucleolytic cleavages, and subsequently polyadenylation and exonucleolytic degradation by PNPase and possibly RNase II/R. Further investigation into the polyadenylation of chloroplast introns is required to determine whether the same mechanism is responsible for their degradation. Because the degradation of introns is relatively fast, our results suggest that RNA degradation using the PNPase as the polyadenylating enzyme could take place when rapid degradation of mRNAs and introns takes place and when the degradation of slow turnover molecules, such as rRNA, occurs.

Why have the RNA degradation and polyadenylation mechanisms in *E. coli* undergone the evolutionary modifications described above? Clearly additional data on the evolution of these processes in other prokaryotes and organelles are required to answer this question. It may be related to the relatively rapid RNA turnover and short half-lives of *E. coli* transcripts in comparison to those of the chloroplast, minutes as opposed to hours. However, the half-lives of several minutes and even less were measured in cyanobacteria, shown here to lack PAP and the degradosome (11–13).

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