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Polyadenylation of three classes of chloroplast RNA in *Chlamydomonas reinhardtii*

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ABSTRACT

Three classes of RNA, represented by *atpB* and *petD* mRNAs, Arg and Glu tRNAs, and 5S rRNA, were found to exist in polyadenylated form in *Chlamydomonas reinhardtii* chloroplasts. Sequence analysis of cDNA clones derived from reverse transcriptase-polymerase chain reaction protocols used to select polyadenylated RNAs revealed that, at least for the mRNAs and tRNAs, there are three apparent types of polyadenylation. In the first case, the poly(A) tail is added at or near the mature 3′ end, even when this follows a strong secondary structure. In the second case, the tail is added to pre-mRNA or pre-tRNA, suggesting a possible competition between polyadenylation and RNA-processing pathways. Finally, in all cases, the poly(A) tail can be added internally, possibly as a part of an RNA-decay pathway. The tails found in *Chlamydomonas* chloroplasts differ from those of spinach chloroplasts in adenine content, being nearly homopolymeric (>98% adenine) versus 70% in spinach, and are similar in length to those of *Escherichia coli*, being mostly between 20 and 50 nt. In vitro assays using a *Chlamydomonas* chloroplast protein extract showed that a 3′ end A_{25} tail was sufficient to stimulate rapid degradation of *atpB* RNA in vitro, with a lesser effect for *petD*, and only minor effects on *trnE*. We therefore propose that polyadenylation contributes to mRNA degradation in *Chlamydomonas* chloroplasts, but that its effect may vary.

Keywords: photosynthesis; posttranscriptional control; RNA stability; RT-PCR

INTRODUCTION

Degradation of RNA is an important aspect of gene regulation in all organisms. Yet, the biological pathways mediating RNA degradation are relatively unexplored (Hajnsdorf et al., 1995; O'Hara et al., 1995). One particularly interesting posttranscriptional modification that affects RNA stability is polyadenylation. In bacterial cells, polyadenylation is associated with RNA instability, whereas the function of polyadenylation of nucleus-encoded mRNAs is to enhance their stability and promote translational initiation (Baker, 1993). In vascular plant chloroplasts, which can be thought of as a prokaryotic compartment in a eukaryotic cell, poly(A)-rich sequences at the 3′ ends of endonucleolytic cleavage products of mRNAs accelerate RNA degradation in vitro (reviewed in Hayes et al., 1999; Schuster et al., 1999). In addition, blocking RNA polyadenylation in chloroplasts inhibits its degradation (Lisitsky et al., 1997a). In contrast, polyadenylation in plant mitochondria has only a modest effect on RNA stability in vitro (Gagliardi & Leaver, 1999; Lupold et al., 1999). Thus, in different organisms and subcellular compartments, polyadenylation appears to play variable roles in modulating RNA stability and gene expression.

In *Escherichia coli*, the first indication of a biological role for polyadenylation came from the finding that a mutation of the *pcnB* gene, which encodes a poly(A) polymerase (Cao & Sarkar, 1992), caused a dramatic decrease in the rate of degradation of RNA I, an antisense RNA that blocks initiation of replication of ColE1-type plasmids (He et al., 1993; Xu et al., 1993). Concomitantly, RNA I was found to be adenylated at its 3′ end and at internal sites only in *pcnB*$^+$ cells, but not in *pcnB* mutants (Xu et al., 1993). Subsequently it was demonstrated that the exonucleolytic decay of *rpsO* mRNA follows endonucleolytic cleavage and polyadenylation at the resultant 3′ termini (Haugel-Nielsen et al., 1996; Hajnsdorf & Régnier, 1999). The process of poly(A)-mediated RNA degradation is carried

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out at least in part by a multienzyme complex called the degradosome, which contains RNase E, the exonuclease polynucleotide phosphorylase (PNPase), as well as other proteins (Blum et al., 1999), possibly including poly(A) polymerase (Raynal & Carpousis, 1999).

In chloroplasts, the PNPase homolog 100RNP/PNPase has been implicated in poly(A)-meditated RNA degradation, based on in vitro experiments (Lisitsky et al., 1997b). A model has been suggested in which mRNA degradation is initiated by an endonuclease cleavage(s), followed by polyadenylation and exonucleolytic degradation (Lisitsky et al., 1996). The observation that blocking polyadenylation of RNA in chloroplasts inhibits its degradation suggests that polyadenylation is an obligate step for mRNA degradation, at least in green plastids (Lisitsky et al., 1997a), and it has also been suggested that polyadenylation accelerates mRNA decay in dark-adapted spinach plants (Kudla et al., 1996).

Both the polyadenylation literature and other studies make it clear that mRNA degradation is a major player in chloroplast gene regulation. For example, chloroplast–RNA stability changes dramatically during plant development (Deng & Gruissem, 1987) and stabilities of chloroplast mRNAs fluctuate in synchronized cells of the green alga *Chlamydomonas reinhardtii* (Salvador et al., 1993). Furthermore, several nuclear genes have been identified in *Chlamydomonas* whose products specifically regulate chloroplast–mRNA stability (Nickelsen, 1998). Yet, the existence of polyadenylation has not been addressed in *Chlamydomonas* chloroplasts and, indeed, has only been reported for one plant system.

Chlamydomonas is a key biological system for investigations of chloroplast–RNA metabolism, and this report represents an initial effort to characterize RNA polyadenylation in this organism. Upon sequencing cDNAs corresponding to poly(A)$^+$ chloroplast RNAs, we found that not only mRNAs, but also tRNAs and 5S rRNA can be polyadenylated, and that both incompletely processed, mature, and partially degraded transcripts are substrates for this activity. Because in vitro assays show that poly(A) tails can destabilize at least some RNAs, we suggest that the poly(A)-mediated pathway contributes to RNA turnover in *Chlamydomonas* chloroplasts.

RESULTS

Identification and analysis of polyadenylated mRNAs

Total RNA was isolated from continuously light-grown *Chlamydomonas* cells and used as a template for cDNA synthesis. The reverse-transcription primer contained an 18-nt adapter sequence followed by 17 Ts, that could anneal to poly(A) tails. Following reverse transcription, PCR was carried out using the adapter primer and a gene-specific primer, as shown for *atpB* in Figure 1C (primer a). This gene was chosen because we have previously characterized its RNA maturation pathway (Stern & Kindle, 1993), as well as *cis* elements and *trans* factors affecting its RNA stability (Stern et al., 1991; Levy et al., 1997).

When RT-PCR products generated with the adapter primer and primer a were cloned and sequenced, they were found to be mostly nonspecific (data not shown). To increase specificity, products of the first RT-PCR reaction were reamplified with the nested primer b and the adapter primer. Because the nested PCR products still appeared diffuse when analyzed by agarose gel electrophoresis, *E. coli* clones were screened by PCR using the adapter primer and a third gene-specific oligonucleotide, primer c. Using this strategy, approximately 40% of positive colonies contained the expected products for *atpB*, that is the correct sequence of *atpB* including all gene-specific primers, a poly(A) tail of at least 17 nt, and the adapter-primer sequence. A similar strategy was used to clone poly(A)$^+$ cDNAs for *petD*, another chloroplast gene we have studied extensively (Sakamoto et al., 1994a, 1994b; Sturm et al., 1994). The difficulty in obtaining the desired products most likely stemmed from two factors: the instability of poly(A)$^+$ RNA in chloroplasts (see below), and the use of total RNA, which contains a high proportion of nucleus-encoded poly(A)$^+$ transcripts. Total RNA was used because it is difficult to isolate large amounts of purified chloroplasts from *Chlamydomonas*.

Results for *atpB* mRNA are shown in Figure 1. Figure 1A gives the relevant part of the *atpB* RNA sequence, and shows that all 12 poly(A) addition sites were in the 3′ untranslated region (UTR), although the gene-specific primers used were well inside the coding region (see Fig. 1C). Of the addition sites, numbers 2–4 were clustered around the mature *atpB* 3′ end, whereas sites 8–9 coincided with an endonuclease cleavage site (ECS), the target of an activity that carries out the first step in *atpB* mRNA maturation (Stern et al., 1991). The remaining sites were between the mature 3′ end and the ECS, or slightly downstream of the ECS. These data suggest that polyadenylation can occur before, during, or following 3′ end maturation, or based on site 1, during RNA degradation.

The frequencies of *atpB* cDNAs isolated are shown in Figure 1B. Of the 45 cDNAs isolated, approximately half were polyadenylated at the mature 3′ end or ECS. The lengths of the poly(A) tails ranged from 17 nt, equal to the number of Ts in the RT primer, to 102 nt (Table 1). Because the RT primer could anneal to tails shorter than 17 nt, or in the middle of tails longer than 102 nt, this range may not fully represent their actual in vivo status (see below). Most

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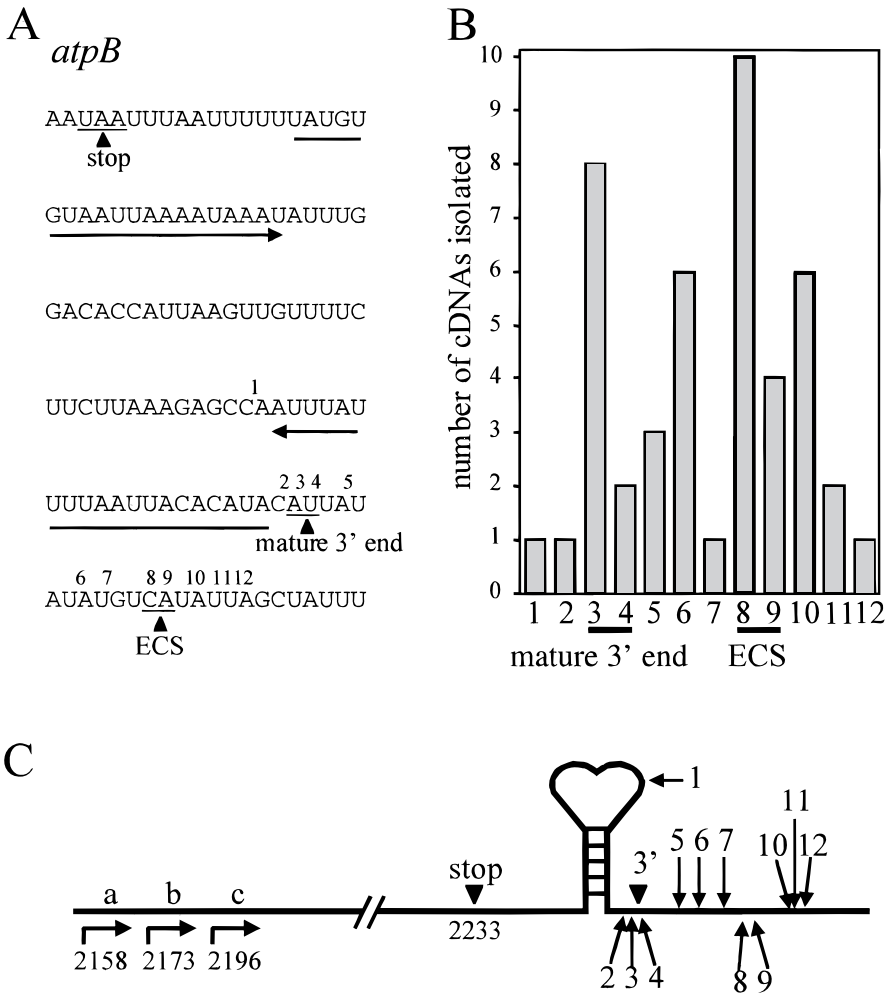

FIGURE 1. Polyadenylation sites in *atpB* mRNA based on analysis of cDNA clones. **A**: Sequence of *atpB* mRNA, beginning shortly upstream of the translation termination codon. Numbered bases indicate the sites of polyadenylation; some sites are ambiguous; for example site 1 could be at the C or at either of the following As. The horizontal arrows indicate two inverted repeats that can form part of the stem-loop structure shown in **C**. Two underlined bases are at the mature 3′ end, and the downstream underlined bases labeled ECS indicate the endonuclease cleavage site that functions in 3′ end maturation. **B**: Comparison of number of cDNAs isolated corresponding to each poly(A) addition site. The poly(A) addition sites shown on the abscissa correspond to those indicated in **A** and **C**. **C**: PCR cloning strategy and schematic representation of poly(A) addition sites. Arrows a, b, and c represent tandem primers used for RT-PCR and PCR screening as described in Materials and Methods, with numbering according to the GenBank sequence. Primer a anneals approximately 170 nt upstream of the mature 3′ end.

poly(A) tails obtained for *atpB* and the other genes discussed below were adenosine ribohomopolymers, with the frequency of guanosines, cytosines, and uridines being less than 2%. This contrasts with the tails of spinach chloroplast mRNAs, which were essentially poly(A)-rich (Lisitsky et al., 1996).

TABLE 1. Summary of polyadenylation of chloroplast RNAs.

RNA	Poly(A) cDNA clones obtained[a]			Range of poly(A) tail length (nt)
	Internal	Mature 3′ end	Precursor	
atpB	2	10	33	17–102[b]
petD	9	2	1	17–22[b]
5S	4	0	0	17–59[b]
$tRNA^{Arg}$	6	0	0	17–66[b]
$tRNA^{Glu}$	12	1[c]	3	5–85

[a]The values indicate the number of cDNA clones obtained by sequencing RT-PCR products. "Internal" indicates that the site was upstream of the major mature RNA 3′ end; "precursor" indicates that the addition site was downstream of the major mature 3′ end.

[b]The shortest tails (17 nt) are equivalent to the number of Ts present in the RT primer; the actual tail may have been less than 17 nt.

[c]This RT-PCR clone was obtained from G-tailed total RNA using the adapter-C_5T_3 primer as described in Materials and Methods.

To see whether the results obtained for *atpB* might be typical for *Chlamydomonas* chloroplast polyadenylation, a similar experiment was carried out for *petD*. Like *atpB* mRNA, *petD* mRNA has a terminal 3′ stem-loop structure; however, its maturation pathway has not been carefully characterized. Most likely, *petD* 3′ maturation begins with endonuclease cleavage at the $tRNA^{Arg}$ RNase P site, followed by exonucleolytic trimming; $tRNA^{Arg}$ is immediately downstream of *petD*. Figure 2A shows that *petD* polyadenylation appears to differ qualitatively from that of *atpB*. In the case of *petD*, 9 of 12 cDNAs corresponded to poly(A) addition within the coding region, with the remaining 3 addition sites at or near the mature 3′ end. The way in which *petD* and *atpB* polyadenylation differ may be because of the structures of the RNAs, as discussed below, although we cannot be certain that with the number of cDNAs isolated for *petD*, our results are statistically significant. The lengths of *petD* poly(A) tails were found to range from 17 to 22 nt (Table 1), somewhat shorter than those for *atpB*. Together, the sequences of the *petD* and *atpB*

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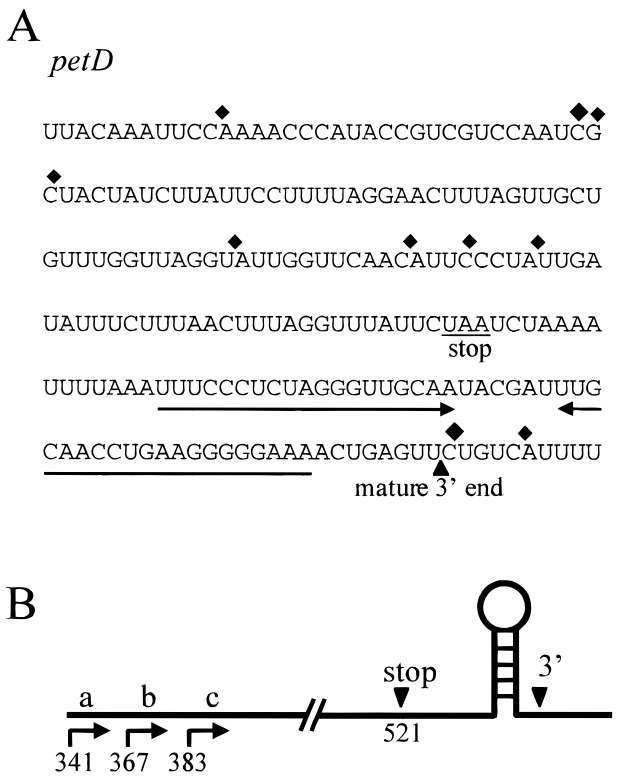

FIGURE 2. Polyadenylation sites in *petD* mRNA based on analysis of cDNA clones. **A**: Sequence of *petD* mRNA, beginning approximately 135 nt upstream of the translation termination codon. Sites of polyadenylation are indicated by filled diamonds. Single representatives for each site were found, except where a larger diamond is drawn; in these cases, two clones were found. The horizontal arrows indicate two imperfect inverted repeats that can form part of the stem-loop structure shown in **B**. **B**: PCR cloning strategy and schematic representation of poly(A) addition sites, labeled as for Figure 1C.

cDNA clones strongly suggest that at least two mRNAs can be polyadenylated in *Chlamydomonas* chloroplasts.

Polyadenylation of tRNAs and 5S rRNA

Although polyadenylation of mRNA has been reported in chloroplasts, mitochondria, and bacteria, only one study (Li et al., 1998b) has revealed polyadenylation of so-called "stable RNA species," for example tRNA, rRNA, and M1 RNA, the catalytic subunit of RNase P. These RNAs had very short (1–7 nt) tails, and accumulated only in exoribonuclease-deficient genetic backgrounds.

To see whether this class of RNA could be polyadenylated in chloroplasts, we used a modified version of the strategy described above for mRNAs. For $tRNA^{Arg}$ and $tRNA^{Glu}$, the short length of the target RNA precluded using a series of nested primers, so we used instead a single, short primer that annealed to the 5′ end of the mature tRNA and extended 13 nt. For 5S rRNA, two nested primers were used. Following RT-PCR, the sequences of cloned products were determined, and only those with the correct sequence downstream of the primer were considered to be valid.

Figure 3A shows results for the two tRNAs. All poly(A)$^+$ clones for $tRNA^{Arg}$ had the tail added within the D-loop, the exact position being ambiguous within 2 nt because of an adenosine in the tRNA sequence. Table 1 shows that unlike in *E. coli*, the tails were of lengths similar to those of mRNAs. For $tRNA^{Glu}$, a more complex pattern was found. Although several addition sites occurred in the D-loop, five clones were also found corresponding to polyadenylation in the anticodon loop, one at the mature 3′ end following the terminal CCA, and two in a putative precursor, that lacked the CCA but included sequences corresponding to genomic DNA downstream of the mature 3′ end. These findings thus differ from bacterial tRNA polyadenylation in terms of addition site and tail length.

The sites of 5S rRNA polyadenylation are shown in Figure 3B. Four cDNAs corresponding to poly(A)+ RNAs were isolated and all addition sites were internal, unlike the situation in *E. coli*. The analysis of 5S rRNA was problematic, as many cloned PCR products were nonspecific. Again, the tail lengths were similar to those found for mRNAs (Table 1). Taken together, the data for stable RNAs in chloroplasts suggest that most, if not all RNAs in this organelle may be subject to transient polyadenylation in vivo. Because the vast majority of the addition sites were internal to the RNAs, we suggest that poly(A) tails are often added either to incompletely synthesized and/or partially degraded transcripts.

Determination of poly(A) tail length

Most work on organelle or bacterial polyadenylation has used a strategy relying on reverse transcription following annealing of an oligo(dT) primer, which can in principle anneal anywhere within a poly(A) tail, or even to a tail shorter than the number of Ts in the primer. In one *E. coli* study (Sarkar, 1997), an "adapter RNA" was ligated to an RNA pool, allowing cDNAs with very short tails to be amplified. To determine the actual lengths of poly(A) tails in *Chlamydomonas* chloroplasts, we added poly(G) sequences of about 20 nt to total RNAs using yeast poly(A) polymerase (see Materials and Methods). This will yield an A-G junction at the 3′ end of the poly(A) tail. We then used one of two primers, either adapter-C_5T_8 or adapter-C_3T_{10}, for reverse transcription and then amplified cDNAs as before. This was done for *atpB* and $tRNA^{Glu}$. Analysis of sequences obtained from 14 cDNA clones revealed tails from 5 nt ($tRNA^{Glu}$) to 85 nt, with the majority in the range of 20 to 50 nt. This corresponds well to the lengths of tails found using the conventional method (Table 1), and also allowed us to find shorter tails like those of bacteria. Furthermore, the distribution of poly(A) addition sites did not differ from those found using the conventional method.

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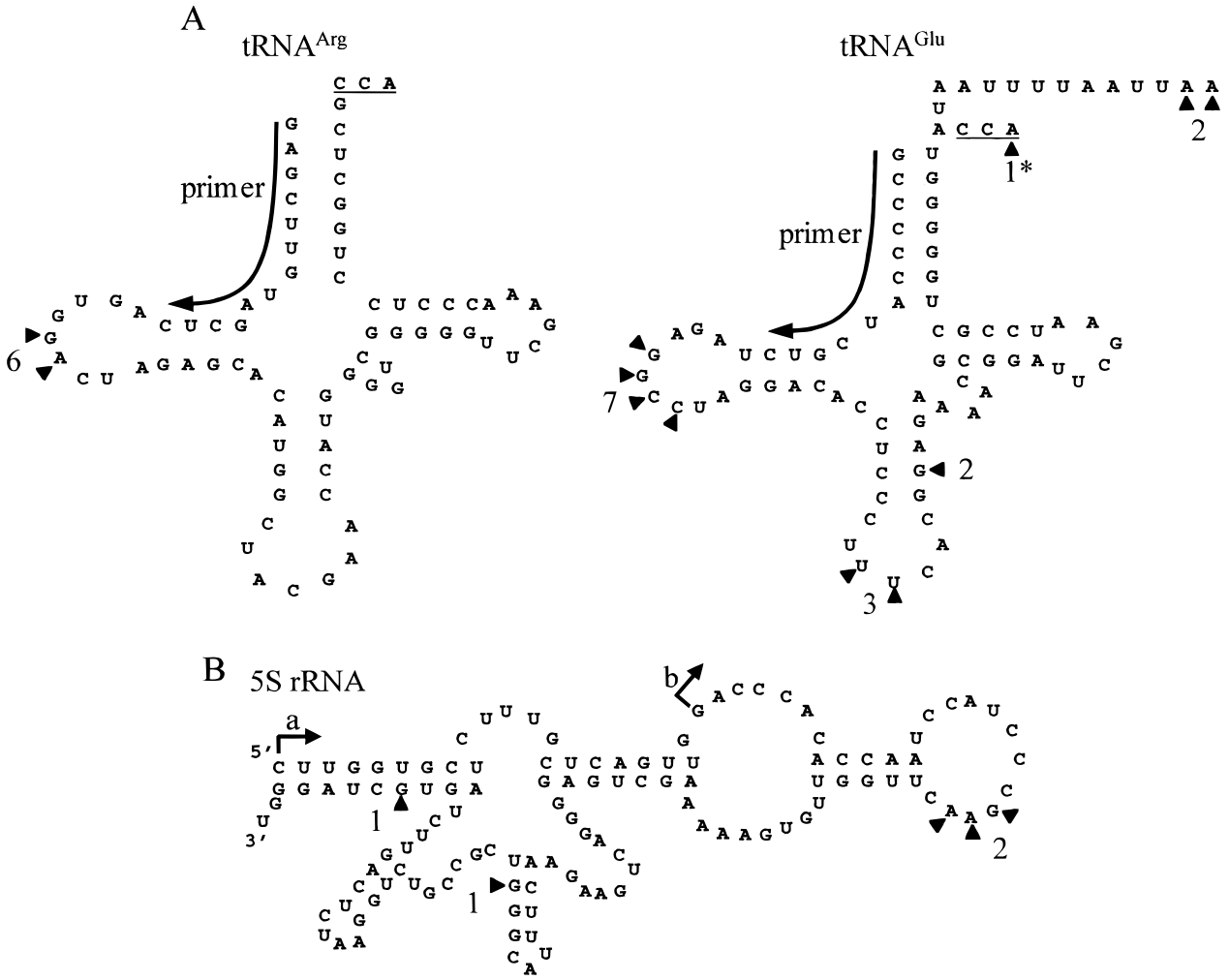

FIGURE 3. Polyadenylation sites in stable RNAs. The poly(A) addition sites in $tRNA^{Arg}$ and $tRNA^{Glu}$ (**A**) or 5S rRNA (**B**) are indicated by arrowheads, along with the number of cDNAs obtained corresponding to each site. Some sites, for example in the D-loop of $tRNA^{Arg}$, are ambiguous because one of the bases is an A. The site in $tRNA^{Glu}$ marked by an asterisk corresponds to a cDNA containing five adenines, obtained using the primer adp-C_5T_3 and G-tailed total RNA (see Materials and Methods). The primers used for RT-PCR are shown as curved lines for the tRNAs (**A**), and as primers a and b for 5S rRNA (**B**).

Stabilities of poly(A)-tailed and nonadenylated mRNAs in vitro

To determine whether the poly(A) tail increases or decreases RNA stability, synthetic RNAs with or without 25 added adenines (A_{25} tail) were incubated in a protein extract generated from purified *Chlamydomonas* chloroplasts. The A_{25} tail was chosen because it appeared to correspond to the average size of tail found from the cDNA analysis.

Figure 4 shows results when transcripts originating from *atpB* were used. For this assay, the A_{25} tail was added to site 1, which is in the 3′ UTR loop (Fig. 1), and the A+ and A− molecules were mixed together so that their relative stabilities could easily be compared, but they were also assayed separately. It is immediately obvious that transcripts with the A_{25} tail were rapidly degraded (Fig. 4, top band), with only 10% of the starting material remaining after 15 min. Furthermore, no discrete degradation intermediates were observed when the A_{25} transcript was assayed alone (Fig. 4, lower panel, A+). These results suggest that the poly(A) tail confers instability by promoting rapid and processive degradation.

In contrast, otherwise identical transcripts lacking the A_{25} tail were shortened within 5 min, generating a stable product that remained during the 60-min reaction time (Fig. 4, lower bands). This product, which is approximately 10 nt shorter than the starting transcript, most likely marks an unpredicted secondary structure that is blocking exonucleolytic digestion, and also accumulated when this substrate was assayed alone (Fig. 4, lower panel, A−). Because this product does not accumulate when the substrate contains A_{25}, we suggest that the A+ and A− RNAs are recognized by different ribonuclease activities, or that the A_{25} tail stim-

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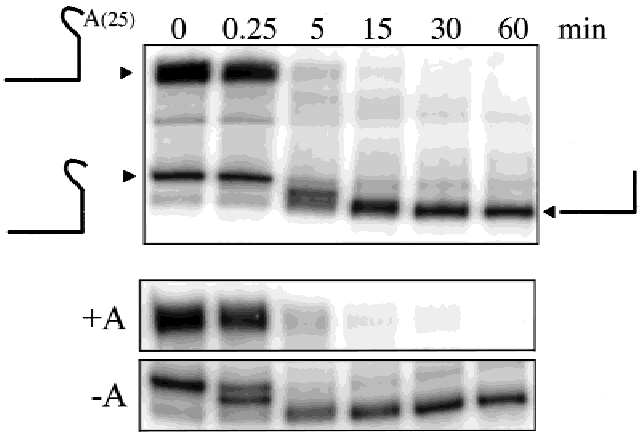

FIGURE 4. In vitro RNA-degradation assay performed with a mixture of transcripts with (top) or without (bottom) an A_{25} tail generated from a partial *atpB* gene, with the 3′ end corresponding to site 1 in Figure 1C. The schematic structures are shown as partial versions of the full-length *atpB* 3′ UTR, to aid in interpretation of the data. Reaction times in min are shown across the top of the figure. The bottom two panels (+A and −A) show time courses in which the two transcripts were assayed independently; the time points are identical to those in which they were mixed.

ulates the exonuclease to pass through secondary structures.

To determine whether the conclusion that A_{25} can destabilize RNAs in vitro could be generalized for mRNAs, we conducted a similar assay using *petD*, with the tail added between the stop codon and the downstream stem-loop. Figure 5 shows that whereas 50% of the polyadenylated transcripts were degraded within 30 min, there was no appreciable change in the amount of the nonadenylated ones. Again, when the transcripts were assayed independently, the results did not change (Fig. 5, lower panels). Although these results differed quantitatively from those obtained for *atpB*, as the apparent half-lives were longer, the A+ transcript was still less stable. The quantitative difference may reflect a sensitivity of the ribonuclease activity(ies) to RNA sequence and/or structure.

In vitro stability of polyadenylated tRNA

Although polyadenylation has previously been shown to destabilize mRNA, its effect on tRNA stability has not been examined. If the internally polyadenylated tRNA molecules we found are indeed degradation intermediates, we might expect that poly(A) addition would promote their decay. To test this we generated three short (35–60 nt) transcripts from the $tRNA^{Glu}$ gene, to which A_{25}, A_5, or no tail was added. The poly(A) addition site in the anticodon loop rather than the D-loop was chosen (Fig. 3A), as this would generate substrates of a reasonable length.

Results of a typical assay are shown in Figure 6. In the top panel, the A_{25}, A_5, and A− transcripts were

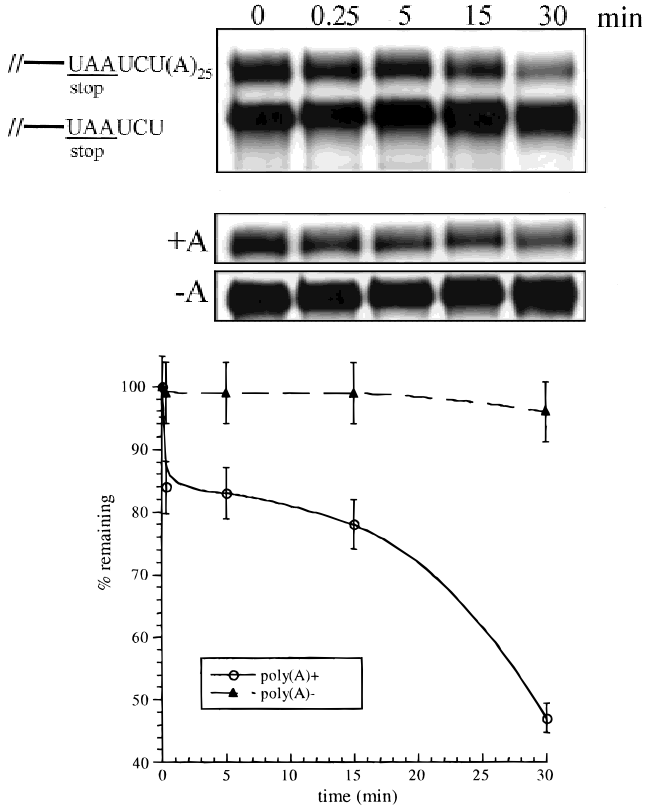

FIGURE 5. In vitro RNA degradation assay performed with a mixture of transcripts with or without an A_{25} tail generated from a partial *petD* gene; the site of poly(A) addition is shown relative to the translation termination codon. Reaction times in min are shown across the top of the figure. The bottom two panels (+A and −A) show time courses in which the two transcripts were assayed independently; the time points are identical to those in which they were mixed. Below the gel pictures is a graph showing average values for disappearance of the A+ and A− transcripts, with initial values set to 100% and the vertical bars showing standard errors.

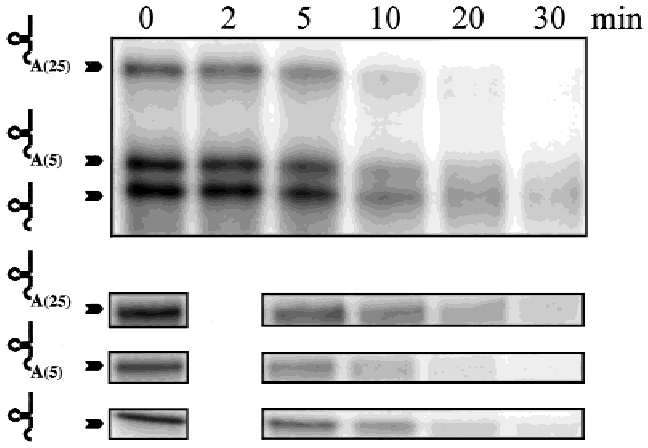

FIGURE 6. In vitro RNA-degradation assay performed with partial $tRNA^{Glu}$ transcripts without added adenosines, or with 5 or 25 adenosines as indicated. The site of added adenosines can be seen in Figure 3A. The three transcripts were mixed and assayed together (top panel), or assayed individually (bottom three panels). Reaction times in min are shown across the top of the figure; the 2-min time point was not assayed for the individual transcripts.

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mixed together, whereas they were assayed separately in the bottom panels. It is clear that each of these short RNA molecules is relatively unstable in the chloroplast protein extract, with degradation rates of the A+ transcripts being near that of the *petD* A+ substrate, but slower than that of the *atpB* A+ RNAs (Figs. 4 and 5). Unlike the mRNA substrates, however, the nonadenylated molecule was also unstable, with most disappearing within 30 min. This may be ascribed to its short length and thus to an inability to form any structures that impede exonucleolytic digestion. When mixed together, we found that the A_{25} transcript was slightly less stable that the nonadenylated one, but the difference was minor. We speculate that the short lengths of the molecules make them subject to a background of nonspecific degradation that mostly masks any destabilizing effect of the poly(A) tails.

DISCUSSION

Based on cDNA sequence analysis, we have shown polyadenylation of the three major classes of RNA in *Chlamydomonas* chloroplasts. Previously, only mRNA adenylation had been reported in chloroplasts and in bacteria. In the only case in which adenylation of tRNA and rRNA had been reported, both the sites and sizes of tails differed from the results reported here. We have not tested directly what proportion of chloroplast RNAs is polyadenylated in vivo, but we suggest that they are an extreme minority, as a multistep PCR protocol was required to isolate the corresponding cDNAs. Similar conclusions can be drawn from bacteria, where such RNAs accumulate only in exonuclease-deficient strains (Xu & Cohen, 1995), and in plant mitochondria and chloroplasts, where similar PCR protocols were required (Lisitsky et al., 1996; Lupold et al., 1999). This contrasts with the abundance and stability of poly(A)+ transcripts, both in the cytosol and in animal mitochondria (Ojala et al. 1981), in which polyadenylation is an essential activating step in gene expression.

The polyadenylation sites found in this study contrast with those identified previously in both bacteria and chloroplasts, and differed between the two mRNAs analyzed. For *atpB* mRNA, 45 cDNAs were isolated and 12 different polyadenylation sites were identified in the 3′ UTR and downstream region (Fig. 1). No sites within the coding region were found; however, that the coding region can be polyadenylated is exemplified by the results for *petD* (Fig. 2). The *atpB* coding region may have a secondary structure or protecting ribosomes that prevent endoribonucleases from cleaving the transcripts to initiate polyadenylation. Protection of the mRNA coding region by ribosomes against RNase E cleavage has recently been shown in bacteria (Braun et al., 1998).

Stern and Kindle (1993) showed that 3′ end maturation of *atpB* mRNA is carried out by 3′-to-5′ exonuclease activity, following cleavage by an endonuclease activity 10 nt downstream of the mature 3′ end. Fourteen polyadenylation sites were found to be located at or near the endonuclease cleavage site, indicating that polyadenylation followed endonuclease cleavage, presumably supplanting RNA maturation. This result suggests that there is a regulatory or stochastic competition among these activities. One polyadenylation site was found in the *atpB* loop region, consistent with RNA degradation being promoted by destruction of a protective 3′ secondary structure, a pathway for which there is considerable experimental support in chloroplasts (Stern et al., 1991; Drager et al., 1996; Lee et al., 1996; Yang & Stern, 1997).

A novel result obtained in this study was finding polyadenylation of two tRNAs and 5S rRNA. Although several A's could be found at the mature termini of such RNAs in exonuclease-deficient *E. coli* strains (Li et al., 1998b), in *Chlamydomonas* chloroplasts virtually all of the addition sites were internal, with the D-loop and anticodon loop being preferential sites in tRNA (Fig. 3A). Several ribonucleases are known that cleave anticodon loops, one being nuclease S1 (Harada & Dahlberg, 1975), another being the product of the *E. coli prr* locus, which cleaves $tRNA^{Lys}$ (Amitsur et al., 1989; Meidler et al., 1999), and a third being the Rn nuclease that was isolated from rye, and shown also to cleave within the D-loop but not the T_{ψ}-loop (Przykorska et al., 1992). The finding of 5S rRNA polyadenylation is consistent with a report that this RNA is associated with degradosomes in *E. coli* cells (Bessarab et al., 1998), although specific decay intermediates were not observed in that study. Finally, two $tRNA^{Glu}$ cDNAs had poly(A) added to an apparent precursor species, consistent with what was found for *atpB*, and suggesting that in effect, any RNA, whether a precursor, mature, or partially degraded (or synthesized) species, is a substrate for poly(A) addition in chloroplasts.

The lengths of poly(A) tails for all the RNAs analyzed ranged from 5 nt to >100 nt, with the shorter tails being detected following G-tailing and reverse transcription with a hybrid primer. The lower part of this range is consistent with bacterial tails, whereas the higher end exceeds the lengths of bacterial and mitochondrial tails found to date, but is consistent with results from spinach chloroplasts. Based on *E. coli* in vitro experiments, there is no positive correlation between the length of tail and RNA degradation rate (Blum et al., 1999), but this topic has not been carefully investigated in chloroplasts.

In this article, we have shown that an A_{25} tail can strongly destabilize an *atpB* mRNA segment, whereas transcripts lacking the tail were shortened but otherwise stable (Fig. 4). The shortening suggests the presence of a secondary structure that may be blocking

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an exoribonuclease, but the addition of poly(A) overcame this apparent structure. This suggests that the two molecules are degraded by different activities, perhaps an RNase II-like one for the A− substrate, and a PNPase-like activity for the A+ substrate. PNPase is known to degrade, in particular, polyadenylated RNAs in *E. coli*, and can proceed through secondary structures (Hajnsdorf et al., 1996). A PNPase homolog has been identified in chloroplasts (Li et al., 1996; Lisitsky et al., 1997b), and has been proposed to be a component of a chloroplast degradosome (Kudla et al. 1996), complexed with PAP (Li et al. 1998a), consistent with this hypothesis.

In vitro results for *petD* and *trnE* differed from those for *atpB*, in that poly(A)+ *petD* RNA was not as strongly destabilized, and for *trnE* no significant difference was seen between A+ and A− transcripts. Although in vitro experiments may not be definitive, these results raise the possibility that poly(A) tails do not destabilize all transcripts equally in chloroplasts. If poly(A) did not have a meaningful function in chloroplast RNA decay, however, it is very unlikely that poly(A)+ transcripts would be so rare, which is suggested by the multiple PCR steps required to amplify the cDNAs, or that poly(A)+ *atpB* transcripts would be exceedingly unstable in chloroplast protein extracts in which nonpolyadenylated *atpB* pre-mRNAs are processed normally and to stable products (Stern & Kindle, 1993). Eventually, genetic tests should clearly define a role for chloroplast polyadenylation.

The stabilities of specific plastid mRNAs change during chloroplast and leaf development in higher plants (Klaff & Gruissem, 1991; Kim et al., 1993) and in synchronized cells of *Chlamydomonas* (Salvador et al., 1993), and it is possible that polyadenylation plays a role when rapid adjustments of RNA levels are required. However, measured half-lives of chloroplast mRNAs are generally long relative to those of their bacterial counterparts, and it is therefore possible that endonucleolytic cleavage, rather than polyadenylation itself, is the rate-limiting factor in RNA decay. Furthermore, recent studies showing an active 5′-to-3′ RNA degradation pathway in *Chlamydomonas* chloroplasts (Drager et al., 1998, 1999; Nickelsen et al., 1999) suggest that this activity, too, will need to be taken into account to fully understand the regulation of chloroplast RNA stability.

MATERIALS AND METHODS

Culture conditions, RNA isolation, and chloroplast protein extract preparation

The strain used in this study was the cell wall-deficient mutant CC406 (*Chlamydomonas* Genetic Center, Duke University). Cells were grown in TAP medium (Harris et al., 1994) under constant fluorescent light or under a 12-h-light and 12-h-dark cycle. Total RNA was isolated from early log phase cultures (1–2× 10^6 cells/mL). Chloroplast soluble protein extracts were prepared as described previously (Stern & Kindle, 1993).

Identification of polyadenylation sites

Reverse transcriptase (RT)-PCR was carried out as described by Lisitsky et al. (1996), with modifications. Total RNA (2 μg) was primed with the adapter-dT_{17} oligonucleotide (Lisitsky et al., 1996) for the RT reaction, which was carried out using Superscript II (Gibco-BRL) under conditions prescribed by the manufacturer at 45 °C for 1 h. The cDNAs were amplified by PCR using one or two successive gene-specific primer(s) and the adapter primer, and the PCR products were ligated into plasmid pUC57 (MBI Fermentas). Positive plasmids were identified by PCR screening of *E. coli* colonies with a gene-specific primer and the adapter primer, and their inserts were sequenced. For the *atpB* gene (EMBL accession number M13704), three tandem primers at positions 2158 (a; 20 mer), 2173 (b; 20 mer), and 2196 (c; 18mer) were used to select gene-specific PCR products, as shown in Figure 1C. For the *petD* gene (EMBL accession number X56700), the three tandem primers were at positions 341 (a; 17 mer), 367 (b; 17 mer), and 383 (c; 19 mer), as shown in Figure 2B.

PCR was carried out as follows for mRNAs. Primer a was used for the first PCR reaction, with preheating for 1 min at 94 °C, followed by 50 cycles of 1 min each at 94 °C, 55 °C (*petD*), or 51 °C (*atpB*), and 72 °C, with additional *Taq* DNA polymerase being added after 25 cycles, and a final extension at 72 °C for 10 min. When primer b was used for the second (nested) PCR, 40 cycles were used with the same temperatures, except the annealing temperature 52 °C was used for *petD* and 51 °C for *atpB*, and the final extension was for 25 min. For PCR colony screening (primer c), preheating was at 94 °C for 2 min, followed by 35 cycles of 1 min at 94 °C, 45 °C, and 72 °C, with a final extension for 10 min. For 5S rRNA, the first primer (19 mer) used for PCR abutted the mature 5′ end, and cycles were as for *petD* (primer a). For the second primer (18 mer), which begins immediately downstream of the first one and was used for nested PCR and PCR colony screening, cycles were as for *petD*, except the annealing temperature was 55 °C. For tRNAs, the sole primer abutted the mature 5′ end, and was used for PCR and colony screening. For RT-PCR, cycles were as for *petD* except the annealing temperatures were 44 °C for $tRNA^{Arg}$ and 54 °C for $tRNA^{Glu}$, and the final extension was for 25 min. For colony screening, cycles were as for *petD* except the annealing temperatures were 44 °C for $tRNA^{Arg}$ and 54 °C for $tRNA^{Glu}$. The sequences of all primers are available upon request.

To determine the lengths of poly(A) tails, total RNA (2 μg) was tailed using GTP and 600 U yeast poly(A) polymerase (United States Biochemicals). This reaction was carried out at 37 °C for 20 min using buffer supplied by the manufacturer and a final GTP concentration of 0.5 μm. Reverse transcription was primed at the border between the poly(A)-tails and the oligo(G) stretch using the primer adapter-C_5T_8 or adapter-C_3T_{10}. PCR reactions, cloning, and screening were carried out as explained in the preceding paragraph.

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In vitro transcription and RNA degradation assays

Transcripts corresponding to segments of *atpB* and *petD* mRNAs were generated from PCR products using T7 RNA polymerase. The T7 promoter was incorporated into the upstream primer, whereas the downstream primer was gene specific and in some cases, ended with the desired number of Ts. To create $tRNA^{Glu}$ substrates, two primers were annealed to give a partially double-stranded template, with the double-stranded portion containing the T7 promoter. Primer sequences are available upon request. In vitro transcription was carried out as described previously (Stern & Gruissem, 1987) with modifications. The *atpB* and *petD* templates were incubated with 2.5 μm (α-^{32}P)UTP and 125 μm cold UTP, as well as 500 μm ATP, CTP, and GTP. The $tRNA^{Glu}$ templates were synthesized using 5 μm (α-^{32}P)UTP, no cold UTP, as well as the other rNTPs at 500 μm. Transcripts were then gel purified and assayed in the chloroplast-soluble protein extract (protein concentration = 0.3 μg/μL) at 30 °C, as described previously (Stern & Kindle, 1993). All quantification was carried out using the Storm system (Molecular Dynamics Inc., Sunnyvale, California), after resolving the reaction products using denaturing polyacrylamide gel electrophoresis.

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REFERENCES

Amitsur M, Morad I, Kaufmann G. 1989. In vitro reconstitution of anticodon nuclease from components encoded by phage T4 and *Escherichia coli* CTr5X. *EMBO J 8*:2411–2415.

Baker EH. 1993. Control of poly(A) length. In: Belasco JG, Brawerman G, eds. *Control of mRNA stability.* New York: Academic Press Inc. pp 367–415.

Bessarab DA, Kaberdin VR, Wei CL, Liou GG, Lin-Chao S. 1998. RNA components of *Escherichia coli* degradosome: Evidence for rRNA decay. *Proc Natl Acad Sci USA 95*:3157–3161.

Blum E, Carpousis AJ, Higgins CF. 1999. Polyadenylation promotes degradation of 3′-structured RNA by the *Escherichia coli* mRNA degradosome in vitro. *J Biol Chem 274*:4009–4016.

Braun F, Le Derout J, Regnier P. 1998. Ribosomes inhibit an RNase E cleavage which induces the decay of the *rpsO* mRNA of *Escherichia coli*. *EMBO J 17*:4790–4797.

Cao GJ, Sarkar N. 1992. Identification of the gene for an *Escherichia coli* poly(A) polymerase. *Proc Natl Acad Sci USA 89*:10380–10384.

Deng XW, Gruissem W. 1987. Control of plastid gene expression during development: The limited role of transcriptional regulation. *Cell 49*:379–387.

Drager RG, Girard-Bascou J, Choquet Y, Kindle KL, Stern DB. 1998. In vivo evidence for 5′ → 3′ exoribonuclease degradation of an unstable chloroplast mRNA. *Plant J 13*:85–96.

Drager RG, Higgs DC, Kindle KL, Stern DB. 1999. 5′ to 3′ exoribonucleolytic activity is a normal component of chloroplast mRNA decay pathways. *Plant J 19*:521–531.

Drager RG, Zeidler M, Simpson CL, Stern DB. 1996. A chloroplast transcript lacking the 3′ inverted repeat is degraded by 3′ → 5′ exoribonuclease activity. *RNA 2*:652–663.

Gagliardi D, Leaver CJ. 1999. Polyadenylation accelerates the degradation of the mitochondrial mRNA associated with cytoplasmic male sterility in sunflower. *EMBO J 18*:3757–3766.

Hajnsdorf E, Braun F, Haugel-Nielsen J, Le Derout J, Régnier P. 1996. Multiple degradation pathways of the *rpsO* mRNA of *Escherichia coli*. RNase E interacts with the 5′ and 3′ extremities of the primary transcript. *Biochimie 78*:416–424.

Hajnsdorf E, Braun F, Haugel-Nielsen J, Regnier P. 1995. Polyadenylation destabilizes the *rpsO* mRNA of *Escherichia coli*. *Proc Natl Acad Sci USA 92*:3973–3977.

Hajnsdorf E, Régnier P. 1999. *E. coli* rpsO mRNA decay: RNase E processing at the beginning of the coding sequence stimulates poly(A)-dependent degradation of the mRNA. *J Mol Biol 286*:1033–1043.

Harada F, Dahlberg JE. 1975. Specific cleavage of tRNA by nuclease S1. *Nucleic Acids Res 2*:865–871.

Harris EH, Boynton JE, Gillham NW. 1994. Chloroplast ribosomes and protein synthesis. *Microbiol Rev 58*:700–754.

Haugel-Nielsen J, Hajnsdorf E, Regnier P. 1996. The *rpsO* mRNA of *Escherichia coli* is polyadenylated at multiple sites resulting from endonucleolytic processing and exonucleolytic degradation. *EMBO J 15*:3144–3152.

Hayes R, Kudla J, Gruissem W. 1999. Degrading chloroplast mRNA: The role of polyadenylation. *Trends Biochem Sci 24*:199–202.

He L, Soderbom F, Wagner EG, Binnie U, Binns N, Masters M. 1993. *PcnB* is required for the rapid degradation of RNA I, the antisense RNA that controls the copy number of ColE1-related plasmids. *Mol Microbiol 9*:1131–1142.

Kim M, Christopher DA, Mullet JE. 1993. Direct evidence for selective modulation of *psbA*, *rpoA*, *rbcL*, and 16S RNA stability during barley chloroplast development. *Plant Mol Biol 22*:447–463.

Klaff P, Gruissem W. 1991. Changes in chloroplast mRNA stability during leaf development. *Plant Cell 3*:517–530.

Kudla J, Hayes R, Gruissem W. 1996. Polyadenylation accelerates degradation of chloroplast mRNA. *EMBO J 15*:7137–7146.

Lee H, Bingham SE, Webber AN. 1996. Function of 3′ non-coding sequences and stop codon usage in expression of the chloroplast *psaB* gene in *Chlamydomonas reinhardtii*. *Plant Mol Biol 31*:337–354.

Levy H, Kindle KL, Stern DB. 1997. A nuclear mutation that affects the 3′ processing of several mRNAs in *Chlamydomonas* chloroplasts. *Plant Cell 9*:825–836.

Li QS, Gupta JD, Hunt AG. 1996. A plant poly(A) polymerase requires a novel RNA-binding protein for activity. *J Biol Chem 271*:19831–19835.

Li QS, Gupta JD, Hunt AG. 1998a. Polynucleotide phosphorylase is a component of a novel plant poly(A) polymerase. *J Biol Chem 273*:17539–17543.

Li Z, Pandit S, Deutscher MP. 1998b. Polyadenylation of stable RNA precursors in vivo. *Proc Natl Acad Sci USA 95*:12158–12162.

Lisitsky I, Klaff P, Schuster G. 1996. Addition of poly(A)-rich sequences to endonucleolytic cleavage sites in the degradation of spinach chloroplast mRNA. *Proc Natl Acad Sci USA 93*:13398–13403.

Lisitsky I, Klaff P, Schuster G. 1997a. Blocking polyadenylation of mRNA in the chloroplast inhibits its degradation. *Plant J 12*:1173–1178.

Lisitsky I, Kotler A, Schuster G. 1997b. The mechanism of preferential degradation of polyadenylated RNA in the chloroplast: The exoribonuclease 100RNP-polynucleotide phosphorylase displays high binding affinity for poly(A) sequence. *J Biol Chem 272*:17648–17653.

Lupold DS, Caoile AGFS, Stern DB. 1999. Polyadenylation occurs at multiple sites in maize mitochondrial *cox2* mRNA and is independent of editing status. *Plant Cell 11*:1565–1578.

Meidler R, Morad I, Amitsur M, Inokuchi H, Kaufmann G. 1999. Detection of anticodon nuclease residues involved in $tRNA^{Lys}$ cleavage specificity. *Mol Biol 287*:499–510.

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Nickelsen J. 1998. Chloroplast RNA stability. In: Rochaix J-D, Goldschmidt-Clermont M, Merchant S, eds. *The molecular biology of chloroplasts and mitochondria in Chlamydomonas*. Dordrecht: Kluwer Academic Publishers. pp 151–163.

Nickelsen J, Fleischmann M, Boudreau E, Rahire M, Rochaix J-D. 1999. Identification of *cis*-acting RNA leader elements required for chloroplast *psbD* gene expression in *Chlamydomonas*. *Plant Cell 11*:957–970.

O'Hara EB, Chekanova JA, Ingle CA, Kushner ZR, Peters E, Kushner SR. 1995. Polyadenylation helps regulate mRNA decay in *Escherichia coli*. *Proc Natl Acad Sci USA 92*:1807–1811.

Ojala D, Montoya J, Attardi G. 1981. tRNA punctuation model of RNA processing in human mitochondria. *Nature 290*:470–474.

Przykorska A, el Adlouni C, Keith G, Szarkowski JW, Dirheimer G. 1992. Structural specificity of Rn nuclease I as probed on yeast tRNA(Phe) and tRNA(Asp). *Nucleic Acids Res 20*:659–663.

Raynal LC, Carpousis AJ. 1999. Poly(A) polymerase I of *Escherichia coli*: Characterization of the catalytic domain, an RNA binding site and regions for the interaction with proteins involved in mRNA degradation. *Mol Microbiol 32*:765–775.

Sakamoto W, Chen X, Kindle KL, Stern DB. 1994a. Function of the *Chlamydomonas reinhardtii petD* 5′ untranslated region in regulating the accumulation of subunit IV of the cytochrome b_6/f complex. *Plant J 6*:503–512.

Sakamoto W, Sturm NR, Kindle KL, Stern DB. 1994b. *petD* mRNA maturation in *Chlamydomonas reinhardtii* chloroplasts: The role of 5′ endonucleolytic processing. *Mol Cell Biol 14*:6180–6186.

Salvador ML, Klein U, Bogorad L. 1993. Light-regulated and endogenous fluctuations of chloroplast transcript levels in *Chlamydomonas*. Regulation by transcription and RNA degradation. *Plant J 3*:213–219.

Sarkar N. 1997. Polyadenylation of mRNA in prokaryotes. *Annu Rev Biochem 66*:173–197.

Schuster G, Lisitsky I, Klaff P. 1999. Update on chloroplast molecular biology: Polyadenylation and degradation of mRNA in the chloroplast. *Plant Physiol 120*:937–944.

Stern DB, Gruissem W. 1987. Control of plastid gene expression: 3′ inverted repeats act as mRNA processing and stabilizing elements, but do not terminate transcription. *Cell 51*:1145–1157.

Stern DB, Kindle KL. 1993. 3′ end maturation of the *Chlamydomonas reinhardtii* chloroplast *atpB* mRNA is a two-step process. *Mol Cell Biol 13*:2277–2285.

Stern DB, Radwanski ER, Kindle KL. 1991. A 3′ stem/loop structure of the *Chlamydomonas* chloroplast *atpB* gene regulates mRNA accumulation in vivo. *Plant Cell 3*:285–297.

Sturm N, Kuras R, Buschlen S, Sakamoto W, Kindle KL, Stern DB, Wollman FA. 1994. The *petD* gene is transcribed by functionally redundant promoters in *Chlamydomonas reinhardtii* chloroplasts. *Mol Cell Biol 14*:6171–6179.

Xu F, Cohen SN. 1995. RNA degradation in *Escherichia coli* regulated by 3′ adenylation and 5′ phosphorylation. *Nature 374*:180–183.

Xu F, Lin Chao S, Cohen SN. 1993. The *Escherichia coli pcnB* gene promotes adenylylation of antisense RNAI of ColE1-type plasmids in vivo and degradation of RNAI decay intermediates. *Proc Natl Acad Sci USA 90*:6756–6760.

Yang J, Stern DB. 1997. The spinach chloroplast endoribonuclease CSP41 cleaves the 3′ untranslated region of *petD* mRNA primarily within its terminal stem-loop structure. *J Biol Chem 272*:12784–12880.