Addition of destabilizing poly(A)-rich sequences to endonuclease cleavage sites during the degradation of chloroplast mRNA

 polyadenylation/mRNA decay/posttranscriptional modification/psbA mRNA/spinach

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ABSTRACT In this work, we report the posttranscriptional addition of poly(A)-rich sequences to mRNA in chloroplasts of higher plants. Several sites in the coding region and the mature end of spinach chloroplast psbA mRNA, which encodes the D1 protein of photosystem II, are detected as polyadenylated sites. In eukaryotic cells, the addition of multiple adenosine residues to the 3′ end of nuclear RNA plays a key role in generating functional mRNAs and in regulating mRNA degradation. In bacteria, the adenylation of several RNAs greatly accelerates their decay. The poly(A) moiety in the chloroplast, in contrast to that in eukaryotic nuclear encoded and bacterial RNAs, is not a ribohomopolymer of adenosine residues, but clusters of adenosines bounded mostly by guanosines and rarely by cytidines and uridines; it may be as long as several hundred nucleotides. Further analysis of the initial steps of chloroplast psbA mRNA decay revealed specific endonuclease cleavage sites that perfectly matched the sites where poly(A)-rich sequences were added. Our results suggest a mechanism for the degradation of psbA mRNA in which endonucleolytic cleavages are followed by the addition of poly(A)-rich sequences to the upstream cleavage products, which target these RNAs for rapid decay.

The addition of multiple adenosine residues to the 3′ end of eukaryotic cell transcripts plays a key role in generating functional mRNA and in regulating mRNA decay (1–3). The poly(A) tail is formed by the addition of about 250 adenylate residues to a 3′ end generated by endonucleolytic cleavage of the precursor RNA (4). Polyadenylation is performed by the enzyme poly(A)-polymerase and is accompanied by the complex assembly of proteins (5). More recently, poly(A) sequences were also described for bacterial RNAs (6–12). Polyadenylation greatly accelerated the decay of several Escherichia coli RNAs, and it was therefore suggested to play a role in regulating mRNA decay (6–12).

During leaf development and plastid differentiation, the levels of many plastid mRNAs vary dramatically. RNA processing and differential stability are important factors that contribute to the developmental mRNA accumulation. In higher plant chloroplast, mRNAs are transcribed as precursor RNAs that undergo a variety of maturation events, including cis- and trans-splicing, cleavage of polycistronic messages, processing of 5′ and 3′ ends, and RNA editing (13–17). A general characteristic of the plastid protein coding region is the presence of inverted repeats sequences in the 3′ untranslated region (UTR), which form a stem–loop structure when transcribed to RNA. The 3′ ends of the chloroplast mRNAs are located several nucleotides 3′ to these stem–loop structures, which were nevertheless shown to not function as efficient transcriptional terminators (18). Instead, these structures serve as efficient RNA processing elements in vitro and are capable of stabilizing upstream RNA fragments in vivo and in vitro (18–20).

To study the degradation pathways of mRNA in the chloroplast of higher plants, an in vitro degradation system based on lysed spinach chloroplasts has been recently developed (21). It was shown that the degradation of the psbA mRNA is initiated by endonucleolytic cleavages within the amino acid coding region of the message, followed by subsequent decay that is facilitated by exonucleolytic activities (21). Nevertheless, the precise mechanism in which the stability of a specific RNA is posttranscriptionally modulated in the chloroplast during plant development and in response to physiological changes (such as light intensity and quality) is still not understood (22). Furthermore, the prokaryotic nature of the chloroplast decoding machinery, in which transcription and translation can theoretically be coupled, requires a mechanism to rapidly degrade immature RNAs, preventing translation of aberrant proteins.

The discovery that polyadenylation of bacterial mRNA significantly affects transcript stability and may trigger rapid degradation prompted us to look for posttranscriptional polyadenylation of mRNA in higher plant chloroplasts. The results presented in this paper show that poly(A)-rich sequences are posttranscriptionally attached to the plastid psbA mRNA at specific positions. Unlike eukaryotic and bacterial mRNAs, the sequence moiety does not consist exclusively of poly(A) but is rather composed mostly of purines, 70% adenosines and 25% guanosines. Cytidines and uridines make up the remaining 5%. Specific endonucleolytic cleavage sites that perfectly matched the sites where the poly(A)-rich sequences are added were identified. In vitro analysis of the chloroplast polyadenylation activity revealed specificity to ATP and GTP, reflecting the composition of the poly(A)-rich tails. Furthermore, the activity is specific for the substrate structure, as unstructured RNAs are polyadenylated with high efficiency compared with those molecules forming the stem–loop structure characteristic of the mature plastid mRNA 3′ end. Polyadenylated RNA was rapidly degraded when incubated in chloroplast extract. The implications of these results are discussed below.

MATERIALS AND METHODS

Plant Growth, Chloroplast Isolation, and RNA Extraction. Chloroplasts were isolated on Percoll gradients from leaves of hydroponically grown spinach plants (Spinacia oleracea cv. Viroflay) under a 10.5 h light/13.5 h dark cycle as described (23). RNA was extracted from leaves or chloroplasts and depleted of DNA by DNase digestion as described (23).

Determination of Poly(A) Tail Length. Chloroplast and total leaf RNAs (10 μg) were 3′ end-labeled with [32P]pCp and T4

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RNA ligase before digestion with 25 μg RNase A and 200 units of RNase T1 for 1 h at 37°C (12). Poly(A) tails were resolved in 12% polyacrylamide sequencing gels containing 7 M urea.

**PCR Amplification and Identification of Polyadenylation Sites.** Chloroplast RNA (10 μg) was used to synthesize oligo(dT)-primed cDNA with the dT-adaptor primer [5′-GACTCGAGCTCGACATCGA(T)5] using avian myeloblastosis reverse transcriptase at 37°C for 10 min followed by incubation at 42°C for 2 h. This cDNA was amplified by PCR with one of the following primers: 5′-2′-GATCAGG-GAAACCCACAG; 5′-5′-ACTTGGGCTGATATC; or 5′-SL, CAAAAAAGAAATCGGTTATTGC, oligonucleotides extending from positions 669 to 685, 941 to 954, and 1127 to 1149 of the psbA gene, respectively (24) (see Fig. 1A), and the adapter oligonucleotide 5′-GACTCGAGCTCGACATCG [identical to the sequence of nucleotides 3′ to the (T)5 of the dT-adaptor oligonucleotide]. Amplification was carried out for 50 cycles of 1 min each at 94°C, 55°C, and 72°C, with the addition of extra enzyme after 25 cycles. PCR products were cloned, and colonies that hybridized to the dT-adaptor oligonucleotide and to the psbA gene-specific probe were sequenced.

**Determination of the Initial Cleavage Sites in the Degradation of psbA mRNA.** Intact chloroplasts were isolated, lysed, and incubated at 25°C for 15 min at a protein concentration of 10 mg/ml in 20 mM Hepes, pH 7.9/60 mM KCl/10 mM MgCl2/0.1 mM EDTA/2 mM DTT/0.5 mg/ml yeast tRNA/20% glycerol (21). Reactions were terminated as described (21), and RNA was extracted. For high resolution RNA blot analysis, the RNA was resolved in a denaturing 5% polyacrylamide gel, electroblotted to a nylon membrane, and hybridized with a 32P-labeled oligonucleotide complementary to positions 1084–1105 (5′-GCCCCCTTTACCTTTACTAACTC) (24). Primer extension analysis was performed as published (21). Primers used were 5′-GAAAGAATTGAAAATGAGGAG and 5′-GCCCCCTTTACCTTTACTAACTC (complementary to positions 801–823 and 893–1110, respectively).

**Soluble Protein Extract, in Vitro Processing, and Polyadenylation.** A soluble protein extract capable of 3′ end processing of chloroplast RNAs was prepared from isolated intact chloroplasts as described (25). The plasmids used for *in vitro* transcription of 3′ UTR mRNAs of spinach chloroplast genes *psbA* and *petD* have been described (23, 25). RNAs resembling the mature 3′ end processing products were obtained by *in vitro* processing of precursors RNA in scaled-up reactions and resolation of the product. The plasmid for transcription of *petD* 3′ end RNA was linearized by DpnI to generate the RNA that 3′ ended in the stop codon of the amino acid coding region (23). RNAs were transcribed using T7 RNA polymerase and were radioactively labeled with [α-32P]UTP to a specific activity of 8 × 106 cpm/fmol (25). The full-length transcription products were then purified on 5% denaturing PAGE gels. *In vitro* RNA processing experiments were carried out as described (25). Briefly, *in vitro* synthesized RNA (2 fmol) was incubated in the chloroplast soluble protein extract (1 mg/ml) for 1 h or for the times indicated in the figure legends. After incubation, the RNA was isolated and analyzed by gel electrophoresis and autoradiography. *In vitro* polyadenylation experiments were performed as the *in vitro* processing assays but with the addition of 0.5 mM ATP (or the respective nucleotides) to the reaction mixture. Polyadenylation reaction buffer (supplied with the pol(A) polymerase obtained from United States Biochemical) were performed according to the manufacturer’s instructions.

**RESULTS**

**Detection of Polyadenylated RNA in Chloroplast and Identification of Polyadenylated Sites.** To detect polyadenylated RNA in the chloroplast, intact chloroplasts from spinach leaves were purified on Percoll gradients and washed several times to reduce the contamination of cytoplasmic polyadenylated RNA to a minimum. RNA was extracted from these chloroplast preparations and analyzed by Northern blotting using an oligo(dT) probe. A low hybridization signal was reproducibly detected in chloroplast RNA preparations obtained from mature leaves that were illuminated before the purification of chloroplasts. In another experiment, *psbA* RNA was purified by hybridization of chloroplast RNA to a DNA fragment corresponding to the *psbA* gene. This hybrid-selected *psbA* RNA showed a detectable hybridization signal with oligo(dT) probe, whereas the control *in vitro* synthesized *psbA* RNA did not (not shown). Therefore, illuminated mature spinach leaves were used for further studies that focused on polyadenylation of *psbA* RNA. Detection and analysis of polyadenylated *psbA* mRNA was performed by cloning and sequencing of the respective cDNAs. Chloroplast RNA isolated from light-adapted mature leaves was used as a template to synthesize cDNA primed with oligo(dT) and a flag oligonucleotide (see Materials and Methods). To precisely determine the site of the poly(A) addition, as well as the posttranscriptionally added sequences, the *psbA*–poly(A) junctions were amplified by PCR, cloned, and sequenced. Our experimental procedure included gel purification and cloning in bulk of the major PCR fragments. Positive colonies were selected by hybridization to both *psbA* gene-specific and oligo(dT) probes. With use of this strategy, about 50 colonies out of several hundred were obtained when primers 5′-2′ or 5′ were used (Fig. 1A). From these, 15 were sequenced, and about equal numbers of sequences 1–5 presented in Fig. 1A were obtained. For the primer 5′-SL, which is located 12 nucleotides in front of the 3′ end of the RNA, only 2 colonies out of several hundred were found to hybridize to the *psbA* and oligo(dT) probes (numbers 6 and 7 in Fig. 1). Sequences cloned are part of the *psbA* mRNA that continues at different points in the 3′ direction as adenine–rich sequences that were not found in the DNA sequence of the gene (Fig. 1A). The *psbA*–poly(A) junctions are located at several positions within the amino acid coding sequence (numbers 1–5 in Fig. 1A) and at the 3′ end of mature *psbA* mRNA (numbers 6 and 7 in Fig. 1A). The 3′ UTRs of most chloroplast transcription units contain inverted repeats that can fold into stem–loop structures (18). The mature 3′ end of the mRNA, which is formed by processing of a longer precursor RNA, is located immediately at the 3′ end of the stem–loop structure (Fig. 1A) and, like bacteria mRNAs, is mostly not polyadenylated in its steady-state form. Therefore, the detection of only two *in vitro*-amplified poly(A)-rich sequences added to the mature 3′ end of the *psbA* mRNA, compared with about 50 clones added to nucleotides in the *psbA* coding region, indicated that it is polyadenylated at a low frequency.

**Analysis of the Posttranscriptionally Added Sequences.** The posttranscriptionally added RNA tails detected in chloroplasts, unlike those of mRNAs encoded by nuclear and bacterial genes, are not adenosine ribohomopolymers. The tail is a mixture of mostly adenosines (70%), guanosines (25%), and cytosines and uridines (5%) (Fig. 1A). In general, sequences added posttranscriptionally to the *psbA* mRNA or respective fragments are mostly purines and rarely pyrimidines. However, only poly(A)-rich sequences could be amplified by using oligo(dT) to prime the cDNA synthesis. Other posttranscriptionally added sequences of nucleotides, if they exist in the chloroplasts, would not have been detected since the RNA tails vary, with the longest one recovered (number 3 in Fig. 1A) being 270 nucleotides. However, as an oligo(dT) primer was used for reverse transcription, it is likely that the poly(A)-rich sequence that served as a template for this clone was even longer because of the annealing position of the primer. We speculate, therefore, that the poly(A)-rich sequences added posttranscriptionally to *psbA* mRNA may be as much as several hundred nucleotides long.
endonucleolytic cleavage within the polyadenylation might occur at sites that are generated by prior developed (21). We employed this assay to analyze whether with primers corresponding to the 3' (Fig. 2A). The open box denotes the coding region. The single line represents the 5' UTR in which the inverted repeats are symbolized by a stem–loop structure. The pbsA gene is numbered according to Zurawski et al. (24), and the nucleotides where poly(A)-rich sequences had been added are numbered 1–7, underlined, and printed in boldface type. The poly(A)-rich addition sites coincident with endonucleolytic cleavage sites are circled (numbers 2, 3, and 5). The 3' end of the mature pbsA mRNA is located at nucleotides 1159A and 1160G, immediately following the inverted repeats that form the stem–loop structure (24); two of the poly(A)-rich addition sites, 6 and 7, were located at this position. The gene-specific primers are indicated by arrows. The poly(A)-rich stretches that are shown below, numbered 1–7, are those that were posttranscriptionally added to sites 1–7, respectively. The nucleotides that are not adenines are shaded. (B) Size of poly(A) tracts in chloroplast RNA. Total RNA from mature leaf cells (T) and purified chloroplast RNA (Cp) were labeled with [32P]pCp, followed by complete digestion of the RNAs with RNase T1 and RNase A. An end-labeled, 35-nt-long oligonucleotide was run in the same gel as a size marker (M).

**Fig. 1.** Posttranscriptional addition of poly(A)-rich sequences to spinach chloroplast pbsA mRNA. (A) Nucleotide sequences and locations of the poly(A)-rich stretches that were PCR amplified from oligo(dT)-primed chloroplast cDNA. A schematic representation of the pbsA RNA 3' region is shown. The open box denotes the coding region. The single line represents the 5' UTR in which the inverted repeats are symbolized by a stem–loop structure. The pbsA gene is numbered according to Zurawski et al. (24), and the nucleotides where poly(A)-rich sequences had been added are numbered 1–7, underlined, and printed in boldface type. The poly(A)-rich addition sites coincident with endonucleolytic cleavage sites are circled (numbers 2, 3, and 5). The 3' end of the mature pbsA mRNA is located at nucleotides 1159A and 1160G, immediately following the inverted repeats that form the stem–loop structure (24); two of the poly(A)-rich addition sites, 6 and 7, were located at this position. The gene-specific primers are indicated by arrows. The poly(A)-rich stretches that are shown below, numbered 1–7, are those that were posttranscriptionally added to sites 1–7, respectively. The nucleotides that are not adenines are shaded. (B) Size of poly(A) tracts in chloroplast RNA. Total RNA from mature leaf cells (T) and purified chloroplast RNA (Cp) were labeled with [32P]pCp, followed by complete digestion of the RNAs with RNase T1 and RNase A. An end-labeled, 35-nt-long oligonucleotide was run in the same gel as a size marker (M).

Sensitivity of Poly(A)-Rich Tails to Ribonuclease Digestion. The discovery of A- and G-rich instead of exclusively poly(A) sequences prompted us to examine whether this observation can be generalized for posttranscriptionally added RNA tails in spinach chloroplasts. Total leaf RNA and chloroplast RNA were labeled at the 3' end with [32P]pCp and T4 RNA ligase and then completely digested with RNase A (cutting after C and U residues) and RNase T1 (cleaving after G residues). Analysis of the products on a 12% polyacrylamide gel revealed that the total leaf RNA was degraded into a ladder of A-containing homopolymers, as previously reported for poly(A)-mRNA, an in vivo transcript. (Fig. 3A). Isolated intact chloroplasts were lysed and incubated in the presence of 0.5 mg/ml tRNA, which was shown to inhibit the activity of fast proceeding exonucleases (21). Several major degradation intermediates that probably result from endonucleolytic cleavages can be observed by high-resolution RNA blot analysis (Fig. 2A). The majority of these molecules can only be detected with primers corresponding to the 3' end of pbsA mRNA, indicating a rapid degradation of the 5' fragment. The exact positions of the cleavage sites were determined by primer extension (Fig. 2B) and are indicated in Fig. 1A by circled numbers. Three of the positions identified perfectly matched one nucleotide 3' to the sites of poly(A)-rich addition, implying that addition of the poly(A)-rich sequence can occur following cleavage of the mRNA by an endonuclease. The 5' moiety of the cleaved pbsA mRNA, which undergoes addition of the poly(A)-rich sequence, is rapidly degraded.

**In Vitro Polyadenylation of Synthetic RNAs in the Chloroplast Protein Extract.** To characterize the biochemical properties of the enzyme activity responsible for adding the poly(A)-rich sequences, we tested whether synthetic RNA can be polyadenylated by components of the chloroplast soluble protein extract. This chloroplast protein extract allows accurate transcription and 3' end processing of chloroplast RNA (18, 23, 25). As depicted in Fig. 3A, synthetic transcripts corresponding to the precursor of the pbsA 3' end were adenylated by an activity in this extract to the same extent as with yeast poly(A) polymerase. Accumulation of polyadenylated RNA in the chloroplast protein extract is only transient: under our experimental conditions, polyadenylated pbsA RNA was fully degraded after 90 min of incubation (Fig. 3A). The transient accumulation of the in vitro polyadenylated RNA is reminiscent of the low steady state concentration of polyadenylated RNAs in vivo, which are only detected by PCR. Specificity of the in vitro polyadenylation activity to different nucleotides was tested by replacing the ATP with each of the other nucleotides. Addition of about 200 residues of ribonucleoprotein was observed with GTP or ATP, but not with UTP or CTP (Fig. 3B). This specificity of activity toward ATP and GTP is in good agreement with the poly(A)-rich sequences detected in vivo, which were 95% adenosines and guanosines (Fig. 1). However, we cannot exclude the possibility that in addition to the poly-purine sequences, poly-pyrimidine

**Fig. 1.** Posttranscriptional addition of poly(A)-rich sequences to spinach chloroplast pbsA mRNA. (A) Nucleotide sequences and locations of the poly(A)-rich stretches that were PCR amplified from oligo(dT)-primed chloroplast cDNA. A schematic representation of the pbsA RNA 3' region is shown. The open box denotes the coding region. The single line represents the 5' UTR in which the inverted repeats are symbolized by a stem–loop structure. The pbsA gene is numbered according to Zurawski et al. (24), and the nucleotides where poly(A)-rich sequences had been added are numbered 1–7, underlined, and printed in boldface type. The poly(A)-rich addition sites coincident with endonucleolytic cleavage sites are circled (numbers 2, 3, and 5). The 3' end of the mature pbsA mRNA is located at nucleotides 1159A and 1160G, immediately following the inverted repeats that form the stem–loop structure (24); two of the poly(A)-rich addition sites, 6 and 7, were located at this position. The gene-specific primers are indicated by arrows. The poly(A)-rich stretches that are shown below, numbered 1–7, are those that were posttranscriptionally added to sites 1–7, respectively. The nucleotides that are not adenines are shaded. (B) Size of poly(A) tracts in chloroplast RNA. Total RNA from mature leaf cells (T) and purified chloroplast RNA (Cp) were labeled with [32P]pCp, followed by complete digestion of the RNAs with RNase T1 and RNase A. An end-labeled, 35-nt-long oligonucleotide was run in the same gel as a size marker (M).
Fig. 2. Determination of the initial cleavage sites in the degradation of psbA mRNA. (A) Characterization of psbA degradation intermediates by high-resolution RNA blot analysis. Lysed chloroplasts from spinach leaves were incubated at 25°C for 180 min, and the RNA was recovered and separated in denaturing polyacrylamide gels, which were electroblotted to nylon membranes and hybridized with a 32P-radiolabeled oligonucleotide complementary to the 5′ or 3′ end of psbA mRNA. (B) Determination of the initial psbA decay intermediate cleavage sites by primer extension analysis. The 5′ ends of the degradation intermediates were determined by primer extension analysis. Oligonucleotide primers complementary to positions 801–823 (Upper) or complementary to positions 1084–1105 (Lower) of the coding region of psbA mRNA were used. Lanes G, A, T, and C show the sequencing reactions of the corresponding cloned fragments. Nucleotide numbers of reverse transcriptase stops in the psbA gene are indicated. Positions of the three corresponding poly(A)-rich addition sites are given in parentheses.

Fig. 3. Polyadenylylation of synthetic psbA transcripts in vitro using an extract of soluble chloroplast proteins. (A) Radioactive RNA corresponding to the unprocessed 3′ UTR of the chloroplast psbA mRNA was incubated in the presence of 0.5 mM ATP with chloroplast extract (Cp Extract) or yeast poly(A) polymerase. Lane −, RNA that was incubated for 60 min without addition of protein. The lengths of the RNAs in nucleotides are shown at the left. (B) The synthetic RNA described in A was incubated for 1 h with the chloroplast protein extract in the presence of 0.5 mM ATP (lane A), CTP (lane C), GTP (lane G), or UTP (lane U). (C) RNA terminating with a stem–loop structure is poorly polyadenylylated in chloroplast protein extract. Synthetic RNAs corresponding to the unprocessed precursor, mature 3′ end and part of the coding region of the petD 3′ end RNA, were incubated for the times indicated in the figure with chloroplast extract in the presence of 0.5 mM ATP. A schematic representation of the RNA substrates is shown on the right. The open box denotes the amino acid coding region of the mRNA.

...stretchs are also present in the chloroplast and were not detected in this work as the result of using oligo(dT) primer for the reverse transcription–PCR. In addition, 0.5 mM ATP and GTP inhibited the in vitro 3′ end processing reaction usually observed; the stable, right size, processing product of 219 nucleotides does not accumulate (Fig. 3). A similar polyadenylation activity was obtained using a soluble protein extract isolated from chloroplasts of the green alga Chlamydomonas reinhardtii (V. Liveanu, unpublished results). In addition to the psbA 3′ end RNA, in vitro polyadenylation activity was also observed for synthetic RNAs corresponding to rbcL (encoding the large subunit of the ribulose-1, 5-bisphosphate carboxylase), petID (encoding subunit IV of cytochrome bf) (Fig. 3B), and rps14 (encoding 30S ribosomal subunit protein 14) precursor 3′ ends that were tested (not shown).

A 100-kDa RNA-binding chloroplast protein (100RNP-PNPase) has been recently described as displaying an exonuclease activity and sequence similarity to the bacterial enzyme polynucleotide phosphorylase (PNPase) (26). To determine if the activity described here reflected artificial polymerase activity of the 100RNP-PNPase, the chloroplast protein extract was depleted of this enzyme by fractionation on a single-stranded DNA-cellulose or on heparin agarose columns binding the 100RNP-PNPase (26). Polyadenylation activity was observed exclusively in the 100RNP-PNPase-depleted fractions (the unbound fraction of the columns) (data not shown). Because immunoblots using specific antibodies to the 100RNP-PNPase (26) demonstrated that the unbound frac-
an inefficient substrate for polyadenylation activity in vivo and in vitro.

Polyadenylated RNA Is Specifically Degraded in the Chloroplast Protein Extract. To determine whether polyadenylation affects the stability of RNA when incubated in the chloroplast protein extract, a psbA precursor 3′ end RNA was polyadenylated in vitro, re-isolated, and analyzed in the standard in vitro 3′ end processing assay. Fig. 4A shows that nonpolyadenylated psbA 3′ end RNA is processed and a stable product of correct size accumulates, as previously described (18, 23, 25). When the polyadenylated precursor 3′ end RNA was incubated with the chloroplast protein extract, it was not degraded without the accumulation of a stable product, and after 2 h of incubation, most of it could no longer be detected (Fig. 4A). To verify that destabilization of the psbA 3′ RNA did not occur as a result of the addition of 200 nucleotides to the 3′ end of the RNA, an RNA of 550 nucleotides extended into the vector sequences was transcribed using the plasmid of the psbA 3′ end DNA sequence digested with PvuII instead of HindIII (25). When this RNA was incubated with chloroplast protein extract, it was not degraded, as compared with the polyadenylated RNA (data not shown). Previous studies have shown that RNA that does not carry a stem–loop structure at their 3′ end could be isolated from oligo(dT)-primed cDNA libraries, suggesting that polyadenylation of mRNA occurs in that organelle (28). Surprisingly, the detection of poly(A)-containing RNA from plastids of maize was described 20 years ago (29). The rediscovery of posttranscriptional addition of poly(A)-rich sequences in the chloroplast demonstrates that mRNA polyadenylation is a universal posttranscriptional modification, occurring in all major genetic systems.

In higher plant chloroplasts, psbA mRNA stability is modulated during leaf development and in response to physiological changes such as dark–light transitions (13–16, 30–32). The results of this work suggest that targeting of mRNA for rapid degradation could be achieved by the addition of poly(A)-rich sequences to an endonucleolytic cleavage product, full-length transcribed molecule, or possibly an unprocessed transcribed precursor RNA. Our in vitro experiments imply that precursor RNA to which poly(A)-rich sequence is added will be degraded and does not accumulate as a 3′ end processed stable product of correct size (Fig. 4). In other words, following transcription, the precursor psbA4 RNA in the chloroplast can either undergo 3′ end processing to generate a stable product or the addition of poly(A)-rich sequences and subsequent rapid degradation. In vitro, the respective pathway depends on the concentration of ATP present in the reaction. Processing occurs at an ATP concentration of less than 0.5 mM, and polyadenylation followed by rapid decay occurs at high concentration (Fig. 3). In the chloroplast, however, other factors such as redox potential, photosynthetic electron flow, posttranslational modifications of the enzymes involved, or specific regulatory proteins may determine which way the precursor RNA will go.

Our experiments showed that mRNAs terminated with a poly(A)-rich tail will be detected at the 3′ end of mRNA fragments cleaved endonucleolytically, as well as at the 3′ end of mature mRNAs. Poly(A)-rich tailed RNAs are highly unstable; therefore, they could only be detected by the sensitive technique of reverse transcription–PCR. We suggest that degradation of chloroplast psbA mRNA is initiated by endonucleolytic cleavages, followed by the addition of a poly(A)-rich sequence that may target this RNA for rapid degradation, possibly to prevent translation of aberrant RNAs.

Despite clear indications that polyadenylation of mRNA exists in bacteria, for about 20 years it was considered to be exclusively one of the unique properties of nuclear-encoded mRNA in eukaryotic cells (1–7). It is believed to function in both mRNA turnover and in facilitating translation (1–4). However, polyadenylation of several RNAs has been recently reported in bacteria (6–12). In these cases, it was suggested that this posttranscriptional modification is part of a mechanism that targets the corresponding RNA for rapid degradation (6, 9–12). Polyadenylation of RNA in the mitochondria was described a long time ago (34). In addition, cDNAs of mitochondria-encoded genes harboring poly(A) stretches in the 3′ end could be isolated from oligo(dT)-primed cDNA libraries, suggesting that polyadenylation of mRNA occurs in that organelle (28).

Fig. 4. Effect of polyadenylation on the 3′ end processing and degradation of RNAs in vitro. In vitro-synthesized [32P]RNA corresponding to the 3′ end of the psbA precursor RNA (A) or the petD amino acid coding region and part of the 3′ UTR (B), and the same RNAs that were first polyadenylated in vitro, were incubated in the chloroplast protein extract without the addition of ATP, either each alone (A) or as a mixture (B). Samples were taken at the times indicated in the figure and analyzed by denaturing gel electrophoresis and autoradiography.
3’ end by the addition of poly(A)-rich sequence. In this case, what will be the initial process to target this mRNA for degradation at an indicated time? A possible scenario is endonucleolytic cleavage removing the stem–loop 3’ end, allowing the addition of poly(A)-rich sequences and targeting for degradation. An endonucleolytic cleavage has been shown to be the initial step in the degradation of many bacterial and nuclear-encoded RNAs (1–3).

Recent work in bacteria suggests that polyadenylated RNAs are degraded by the coordinated activity of the endonuclease RNase E and the exonuclease polynucleotide phosphorylase (PNPase), which have been found in the same multiprotein complex in E. coli cells (33). An analogous high-molecular-weight enzymatic complex that contains a PNPase-like exoribonuclease and a site-specific endoribonuclease has been recently described in spinach chloroplasts (26). This suggests a similarity between bacteria and chloroplast in the regulation of RNA degradation by the addition of poly(A)-rich sequences to endonucleolytic cleavage sites. For example, when the endonuclease of the high-molecular-weight complex, a 67-kDa protein that crossreacts to antibodies raised against bacterial endonuclease RNase E, was analyzed in vitro, it cleaved synthetic RNA corresponding to the 3’ end of the petD mRNA in the amino acid coding region (26). Ongoing experiments are exploring the possibility that the chloroplast 67-kDa endonuclease can mediate degradation of mature RNA by generating truncated RNA, which is elongated by poly(A)-rich sequences and subsequently degraded.

We have demonstrated that poly(A)-rich sequences are posttranscriptionally added to chloroplast mRNA at 3’ termini generated by endonuclease(s) cleavages and exonucleases (the mature 3’ end). In this respect, chloroplast poly(A) polymerase(s) are similar to eukaryotic enzymes that polyadenylate mRNA at processing sites (4, 5). The chloroplast poly(A) polymerase(s) may have affinity either for the RNA processing complexes as in mammalian cells (4, 5) or for other features of mRNA sequence or structure. The results presented here suggest that poly(A)-rich tails play a major role in the rapid degradation of intermediary products of mRNA decay as well as precursor and mature mRNA.

**Note Added in Proof.** Polyadenylation of chloroplast mRNA has also been described in a recent paper by Kudla et al. (35).

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