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Update on Chloroplast Molecular Biology

Polyadenylation and Degradation of mRNA in the Chloroplast[1]

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Chloroplast development is characterized by the synthesis and assembly of the photosynthetic complexes of the thylakoid membranes. This maturation process requires the coordinated expression of many nuclear- and chloroplast-encoded genes. As chloroplasts are semiautonomous organelles, they possess their own genome with its inherent transcriptional and translational machinery. However, nuclear-encoded gene products are also necessary for all of the processes occurring in the chloroplast. In this *Update* we will focus on the control of chloroplast gene expression at the posttranscription stage. Posttranscriptional processes are widely used in controlling the steady-state levels of plastid mRNAs, and are mediated mainly by nuclear-encoded proteins, suggesting a way in which the nucleus can modulate gene expression in the chloroplast. For example, mutants of nuclear-encoded genes affecting the accumulation of specific chloroplast transcripts have been described in maize, Arabidopsis, and the green algae *Chlamydomonas reinhardtii* (Goldschmidt-Clermont, 1998).

mRNAs of higher plant and green algae chloroplasts 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 (Fig. 1). A general feature of plastid protein-encoding genes is the presence of inverted repeat sequences in the 3′-UTR, which form a stem-loop secondary structure when transcribed to RNA. The 3′ end of mature chloroplast mRNAs is located several nucleotides 3′ to this stem-loop structure (Fig. 1). Contrary to similar structures found in bacterial mRNA, these elements do not function as transcriptional terminators in chloroplasts; instead, they serve as RNA-processing elements capable of stabilizing upstream RNA fragments in vivo and in vitro (Barkan and Stern, 1998).

Different aspects of chloroplast mRNA processing and stability have been the subject of recently published reviews (Barkan and Stern, 1998; Drager and Stern, 1998; Goldschmidt-Clermont, 1998; Nickelsen, 1998) and will not be discussed here. We will focus on recent discoveries concerning the molecular mechanism of mRNA polyadenylation and degradation in the chloroplast, and the proteins involved.

HOW CAN mRNA DEGRADATION CONTROL GENE EXPRESSION DURING CHLOROPLAST DEVELOPMENT?

During leaf development and plastid differentiation, the levels of many plastid mRNAs vary dramatically. The concentration of a specific mRNA is determined by its transcription rate in comparison with its degradation rate. Run-on experiments revealing relative rates of RNA synthesis showed that the different mRNA steady-state levels cannot be related to gene-specific transcriptional activity (Gruissem, 1989). Therefore, changes in the degradation rate of specific mRNAs during chloroplast development occur. To study the degradation of mRNAs in the chloroplast of higher plants in vivo, spinach and barley plants were treated with transcription inhibitors. The rate of decay of chloroplast-encoded mRNAs was determined by quantitative northern analysis using gene-specific probes. The half-life of several chloroplast mRNAs, such as *psbA*, changed during development from young to mature leaves, whereas that of others, such as *rbcL*, did not.

These results showed that differential mRNA stability contributes to chloroplast mRNA concentrations during leaf development (Klaff and Gruissem, 1991; Kim et al., 1993). Analysis of the transcription, translation, and mRNA levels of 15 plastid genes during barley chloroplast development revealed a dynamic modulation of gene expression and mRNA stability (Rapp et al., 1992). Furthermore, enhanced levels of *psbA* mRNA in mature barley chloroplasts were due primarily to its selective stabilization. Although data about the RNA elements and the proteins involved in this process are slowly emerging (see below), the precise mechanism by which the stability of a specific chloroplast mRNA changes during plant development and in response

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Abbreviations: PNPase, polynucleotide phosphorylase; RT, reverse transcription.

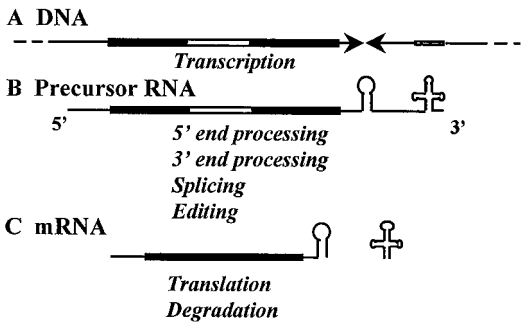

Figure 1. A, RNA metabolism in the chloroplast. Schematic representation of a monocistronic transcription unit in the chloroplast. UTRs are marked with a thin black line; sequences that code for amino acids with a thick black line; and introns with a thick white line. The inverted repeats characterizing the 3′-UTR are symbolized by two arrowheads. A tRNA gene usually presenting 3′ to the inverted repeats is shown as a dashed line. B, The precursor RNA transcribed from this transcription unit undergoes 5′- and 3′-end processing to generate the 5′ and 3′ ends of the mRNA, respectively. C, The 3′ end is located several nucleotides 3′ of the stem-loop structure formed by the inverted-repeats sequence. The introns are removed by splicing, and the tRNA gene is processed by RNaseP. The mRNA is then translated and later degraded.

to physiological changes (such as light intensity or quality) is still not understood.

cis-REGULATORY ELEMENTS CONFERRING STABILITY AND/OR INSTABILITY OF CHLOROPLAST mRNAs

A central issue for understanding the regulation of mRNA stability is the identification of *cis*-regulatory elements. These elements are defined by their ability to transfer properties of a certain mRNA to a reporter gene. In general, this is achieved by constructing chimeric genes consisting of the respective RNA element and a reporter gene, such as the *Escherichia coli* GUS gene, and transforming these constructs into a recipient organism. Chloroplasts of the green algae *C. reinhardtii* have been routinely transformed, and, recently, techniques for higher plant chloroplast transformation have become available. Nevertheless, most information on *cis*-regulatory elements in chloroplasts is still derived from *C. reinhardtii*. In addition, a wide variety of nuclear mutants affecting mRNA stability have been described for *C. reinhardtii* and higher plants, providing an additional genetic tool for understanding mRNA-degradation pathways (for review, see Goldschmidt-Clermont, 1998).

A well-characterized example of an impaired mRNA metabolism in *C. reinhardtii* is the nuclear mutation *nac2-26*, which results in the decreased stability of *psbD* mRNA. Fusing the 5′-UTR of the *psbD* mRNA to *aadA* (aminoglycoside adenyltransferase) as a reporter gene showed a destabilized chimeric transcript in the mutant background and normal accumulation in the wild type, reflecting the properties of the *psbD* mRNA. These data indicate that the 5′-UTR of the mRNA includes a determinant for *psbD* mRNA degradation. Instability of the *psbD* mRNA in the mutant correlates with a 47-kD protein binding to the *psbD* leader that is present in wild-type *C. reinhardtii* chloroplasts but not in *nac2-26* cells, making this protein a candidate for a gene-specific, nuclear-encoded, *trans*-acting factor that stabilizes the mRNA (Nickelsen et al., 1994).

A more complex mechanism for the regulation of mRNA stability has been proposed for the *C. reinhardtii petD* mRNA. Extensive mutational analysis and experiments using reporter constructs revealed that sequences within the 5′-UTR are essential for translation and affect RNA stability. In all mutants in which translation was compromised, *petD* mRNA accumulated to a lower level than in wild-type strains, indicating that mRNA stability is not only regulated by RNA-binding proteins but may also be linked to translatability (Sakamoto et al., 1994). The role of nuclear-encoded factors for *petD* mRNA stability was confirmed by F16, a nuclear mutant harboring the mutation mcd1-1, which failed to accumulate *petD* mRNA. The analysis of this mutant suggested that the mcd1-1 gene product is involved in protein binding the 5′-UTR to prevent digestion of the mRNA by a 5′ to 3′ exonuclease (Drager et al., 1998).

In chloroplasts of higher plants information on *cis*-regulatory elements for mRNA stability has been obtained from mutational studies and from the recent construction of transplastomic tobacco plants. Similar to the situation in *C. reinhardtii*, no general scheme emerged, but several parameters contributing to the stability of a certain mRNA were observed. Nuclear mutants in which many chloroplast mRNAs were associated with abnormally few ribosomes showed that the level of *rbcL* mRNA was reduced 4-fold, indicating that the *rbcL* mRNA is destabilized as a consequence of its decreased polysome association (Barkan, 1993). For the same mRNA, the analysis of constructs consisting of the 5′-UTR fused to a reporter gene showed that mRNA accumulation in the dark is mediated by this region, whereas a leaderless molecule cannot be detected. That study also provided evidence that *rbcL* mRNA stability is regulated via the 5′-UTR (Shiina et al., 1998).

In addition to the 5′-UTR and its role in mRNA accumulation in chloroplasts, the 3′-UTR is also remarkably important. As described above, most chloroplast mRNAs are flanked by a stem-loop structure 3′ of their coding region that takes part in the mature 3′-end processing (for review, see Barkan and Stern, 1998). In addition, these elements are important for impeding the progress of processive exoribonucleases, which can be shown in vitro using a soluble extract from higher plant chloroplasts, and of synthetic RNA fragments as the substrates (Barkan and Stern, 1998; Drager and Stern, 1998). Partial or complete in vivo deletion of the *atpB* gene stem-loop in transformed *C. reinhardtii* chloroplasts led to a dramatic decrease in the accumulation of 3′-end-processed mRNA, whereas the transcription rate of this gene remained unaffected. This result indicated that the stem-loop structure is required for the correct 3′-end processing and mRNA accumulation (for review, see Barkan and Stern, 1998; Drager and Stern, 1998).

The stem-loop structure can be replaced in vivo by a stretch of 18 guanosines, which also serves as a barrier for a 3′ to 5′ exonuclease in vitro. The correct 3′-end process-

ing of the mRNA mediated by the structural element is nevertheless essential, since it is required for, or strongly stimulates, its translation in *C. reinhardtii* chloroplasts (Rott et al., 1998).

The results summarized in this section suggest that *cis* elements responsible for the modulation of mRNA stability of specific genes are mainly localized in the 5′-UTR. On the other hand, RNA structural elements located in the 3′-UTR are required for correct processing and are therefore necessary for mRNA stability. We describe below the search for the molecular mechanism of mRNA degradation and the proteins involved.

WHICH ARE THE RNA DEGRADATION ENZYMES IN THE CHLOROPLAST?

The in vitro RNA processing and degradation system, in which a synthetic RNA is processed or degraded when incubated with soluble chloroplast extract, was utilized to isolate the enzymes involved. The proteins were fractionated using conventional biochemical separation methods, and the purified fractions were analyzed for activity until one or more polypeptides were present. The search for the RNase involved in the 3′-end processing of chloroplast mRNAs yielded 100RNP (100-kD RNA-binding protein; Hayes et al., 1996). Purified 100RNP has biochemical properties similar to PNPase, one of the two exonucleases discovered to date in bacterial cells. Furthermore, the deduced amino acid sequence of the chloroplast 100RNP cDNA was highly homologous to the bacterial PNPase. Does this result imply that the chloroplast RNA processing and degradation system is similar to recently discovered mechanisms in *E. coli*? (Nierlich and Murakawa, 1996; Carpousis et al., 1999). Together with the discoveries about the mechanisms of mRNA polyadenylation and degradation, which we will describe later, the answer to this question appears to be yes. Nevertheless, unlike bacteria, plastid mRNA metabolism and its associated enzymes are controlled by the nucleus and may be regulated by light or by the redox state of the chloroplast (Hayes et al., 1996).

IS THERE A CHLOROPLAST DEGRADOSOME SIMILAR TO BACTERIA?

The *E. coli* RNA degradosome is a multienzyme complex consisting of the exoribonuclease PNPase, the endonuclease RNase E, a DEAD-box ATP-dependent RNA-helicase, and the enzyme enolase (Carpousis et al., 1999). This high-molecular-mass protein complex is important in RNA processing and mRNA degradation in the bacterial cell, since two of its components, PNPase and RNase E, have been shown to be key elements in these processes. The chloroplast 100RNP/PNPase was isolated in a high-molecular-mass complex of about 600 kD. A 67-kD protein cross-reacting with antibodies prepared against RNase E of *E. coli* and displaying endoribonuclease activity was copurified with that complex (Hayes et al., 1996). Therefore, it is tempting to suggest that a complex similar to the bacterial degradosome exists in the chloroplast, preserving its ancestral prokaryotic origin (Carpousis et al., 1999).

In both bacteria and chloroplasts, it appears that not all of the PNPase population is associated with the degradosome (Carpousis et al., 1994; Lisitsky et al., 1997b). The question of whether different forms of the 100RNP/PNPase are involved in each RNA metabolic activity, such as 3′-end processing and degradation, therefore, remains open. In vitro experiments using synthetic RNAs and purified 100RNP/PNPase have shown much higher enzyme activity on polyadenylated RNA (see below). This selectivity to polyadenylated RNA resulted from the high-affinity binding of the 100RNP/PNPase to poly(A) sequences (Lisitsky et al., 1997b). It is interesting that a similar function and mode of action have recently been reported for the *E. coli* PNPase and for another exoribonuclease, RNase II (Coburn and Mackie, 1996; Lisitsky and Schuster, 1999). Therefore, identification, isolation, and characterization of the other chloroplast exoribonucleases will determine whether the preference for poly(A)-rich RNAs is only intrinsic to the 100RNP/PNPase, or if it is shared by several or all of the chloroplast exonucleases. In addition, a protein complex composed of several RNA-degradation enzymes was recently identified and isolated in yeast, and was named the exosome (Mitchel et al., 1997; Jacobs et al., 1998). Are the bacterial degradosome, the chloroplast degradosome, and the yeast cytoplasm exosome related to each other functionally and/or evolutionarily? This interesting question is now under intensive study.

POLYADENYLATION OF mRNA IN EUKARYOTIC CELLS

Posttranscriptional addition of a poly(A) tail to the 3′ end of mRNA was first identified and characterized in eukaryotic cells for viral and nuclear-encoded mRNAs. In these cells the poly(A) tails are formed by the addition of about 250 adenylate residues to a 3′ end generated by endonucleolytic cleavage of the precursor RNA. A complex assembly of proteins is required, along with the activity of poly(A) polymerase. The result of this process is that most of the mRNA molecules are polyadenylated (Wahle and Keller, 1996).

What is the function of the poly(A) tail? Many studies have revealed that the long poly(A) tail of eukaryotic nuclear-encoded mRNAs is an important determinant of their maturation and initiation of translation. When referring to maturation, we include the transfer of the precursor RNA from the nucleus to the cytoplasm and the determination of stability. In yeast, deadenylation is a major step in the degradation pathway of nuclear-encoded mRNAs. The poly(A) tail and the protein that bound to it were found to be very important for the initiation of the translation process. How can the poly(A) located in the 3′ end of the RNA molecule control the translation starting at the 5′ end? A model invoking mRNA circularization has been proposed whereby the mRNA 5′ and 3′ ends can interact with each other. In this way, the poly(A)-binding protein that is associated with the poly(A) tail stimulates the binding of the 40S ribosomal subunit to mRNA by associating with the translation initiation factor eIF4G, which also binds to eIF4E and the 5′ cap of mRNA. The circularization of the

mRNA in this way is required for the initiation of translation (Sachs et al., 1997).

IS mRNA POLYADENYLATED IN PROKARYOTE CELLS?

For a long time, polyadenylation was believed to be exclusively associated with eukaryotic mRNAs. Other RNAs, such as rRNAs, tRNAs, and RNAs in prokaryotes, were believed not to be polyadenylated. Most of these RNA molecules do not have a poly(A) tail in their 3′ end. Nevertheless, poly(A) tails have recently been detected in bacteria (Sarkar, 1997). The polyadenylated RNA accounts for only a tiny fraction of the population of the same RNA in the cell. This fraction increases severalfold in mutant bacteria cells that lack exoribonuclease(s) activity. On the other hand, in mutants in which RNA polyadenylation was inhibited due to the lack of poly(A) polymerase enzymes, the half-life of the RNA molecules dramatically increased.

What do these results suggest? They imply that, unlike the nucleus and cytoplasm of eukaryotic cells, where the poly(A) tail is important for the stability, maturation, and translation of mRNA and the deadenylation of the long poly(A) tail in part of the mRNA degradation pathway, the addition of poly(A) tails in bacterial mRNAs promotes their degradation. Taken together, polyadenylation of RNA molecules in bacteria cells is a part of the molecular mechanism of RNA degradation in bacteria. Is polyadenylation required for RNA degradation, or is there an additional nonpolyadenylated-dependent degradation pathway in the cell? Is the polyadenylation-dependent degradation pathway specific to certain types of RNA molecules such as mRNA? What is the sequence of events in this RNA-degradation pathway and what is the rate-limiting step? These questions are now under intensive investigation (Blum et al., 1999).

IS RNA POLYADENYLATED IN THE CHLOROPLAST?

Chloroplasts evolved from free-living prokaryotes that were introduced into eukaryotic cells in an endosymbiotic event(s). Many characteristics of the gene expression machinery of the chloroplast resemble those of the bacteria. However, some characteristics of the gene expression apparatus in the chloroplast are similar to the eukaryotic system. For example, chloroplast genes are usually interrupted with introns that have not been found in bacteria. The question then arose, does RNA polyadenylation occur in the chloroplast? Is it similar to the eukaryotic nuclear-encoded genes, such as bacteria, or is it a unique feature?

It is interesting to note that poly(A) RNA was detected in the chloroplast more than 20 years ago (Haff and Bogorad, 1976). Using hybridization experiments with ctDNA and ^{125}I-labeled RNA from maize seedlings, it was determined that about 6% of the poly(A)-containing RNA hybridized to ctDNA, and that the chloroplast poly(A) tracts averaged about 45 nucleotides in length. Nevertheless, like the situation in the polyadenylation of bacterial RNA, polyadenylation has long been regarded as a feature of eukaryotic nuclear and viral mRNAs. However, polyadenylation of prokaryotic and organellar mRNAs has recently returned as the focus of research as part of the mRNA degradation mechanism.

To detect polyadenylated RNA in the chloroplast, the powerful method of RT-PCR was used. RNA was isolated from purified chloroplasts and oligo(dT)-primed cDNA was synthesized from the polyadenylated RNA molecules. The cDNA corresponding to a specific gene was PCR amplified using a gene-specific primer on one side and a $(dT)_n$ tail on the other (Kudla et al., 1996; Lisitsky et al., 1996). Analyzing the nucleotide sequences of the poly(A) tails of chloroplast RNAs revealed several interesting features. First, compared with poly(A) tails in bacteria and yeast, the chloroplast poly(A) tails are very long. Several tails of 270 nucleotides were detected, compared with only 40 to 60 nucleotides in bacteria and yeast. Second, unlike eukaryotic nuclear-encoded and bacterial RNAs, the poly(A) moiety in the chloroplast was not found to be a ribohomopolymer of adenosine residues, but rather was composed of clusters of adenosines mostly bound by guanosines, and, on rare occasions, by cytidines and uridines. A chloroplast poly(A)-rich tail usually contains 70% adenosines, 25% guanosines, and 5% cytidines and uridines, making them purine-rich sequences (Lisitsky et al., 1996). Why are the poly(A) tails heterologous in the chloroplast but not in nuclear-encoded or bacteria RNA? Is there a biological function for this heterogeneity, or does it just reflect less specificity of the chloroplast poly(A) polymerase enzyme? Currently, we do not know the answers to these questions. Other nucleotides in addition to adenosine were recently found in RNA isolated from *E. coli* cells under stationary growth conditions (Cao and Sarkar, 1997). The third phenomenon is related to the location of the polyadenylation sites in the RNA molecule and will be discussed below. Two possibilities for the formation of polyadenylated sites are truncated transcription termination and cleavage of mature, full-length RNA.

Most of the polyadenylation sites that were found by RT-PCR of oligo(dT)-primed chloroplast RNA were localized within the amino acid-coding region of the mRNA. RT-PCR clones of mRNA polyadenylated at the 3′ end were also obtained, but were at least 50 times lower in frequency (Lisitsky et al., 1996). This result indicated that most truncated mRNAs are polyadenylated. How did the truncated RNA molecules undergoing the addition of poly(A)-rich tails originate? The truncation may originate from either early transcription termination or cleavage of a full-length transcript.

Two observations suggest that in vivo polyadenylation occurs subsequent to the cleavage of an mRNA as part of the specific degradation pathway. First, five of the polyadenylation sites mapped by RT-PCR in spinach *psbA* mRNA perfectly matched endonucleolytic cleavage sites that were mapped by a primer extension in the lysed chloroplast mRNA-degradation system (Fig. 2) (Lisitsky et al., 1996). Since mapping the endonucleolytic cleavage site by primer extension marks the first nucleotide at the 5′ end of the distal cleavage product, and the poly(A) tail is added to the 3′ end of the proximal product, the nucleotide labeled by primer extension was the adjusted nucleotide 3′ to the polyadenylated one (Fig. 2). Second, polyadenylation sites

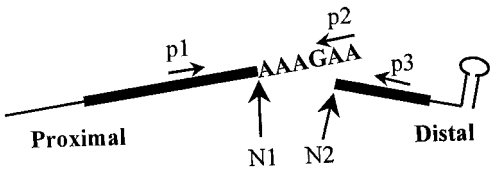

Figure 2. Experimental design used to analyze polyadenylation of endonucleolytically cleaved mRNA. The mRNA 3′- and 5′-UTRs are symbolized by thin lines and the coding region by a thick black line. RT-PCR of purified chloroplast RNA using the primers p1 and p2 revealed clones whereby the poly(A)-rich sequences were added to the last 3′ nucleotide of the proximal cleavage product (N1). The 5′ nucleotide of the distal cleavage product (N2) was determined by primer extension analysis (using the primer p3) of endonucleolytically cleaved RNA in the lysed chloroplast mRNA degradation system (see text). N2 was found to be one nucleotide 3′ to N1. Therefore, polyadenylation occurs at the 3′ end of the proximal endonucleolytic cleavage product.

located in the 3′-UTR of the *petD* mRNA determined by RT-PCR were mapped to the positions observed as cleavage sites of the purified endoribonuclease p67 on synthetic transcribed RNA corresponding to the *petD* 3′-UTR (Kudla et al., 1996). These results strongly suggest that most of the polyadenylated mRNAs result from endonucleolytic cleavage of full-length transcripts. Nevertheless, the possibility cannot be ruled out that some of the polyadenylated RNA molecules in the chloroplast are the result of polyadenylation of truncated transcribed molecules. These molecules must immediately enter the degradation pathway, because otherwise they might serve as the templates for the translation of truncated, defective proteins. Full-length polyadenylated transcripts were also detected, albeit in very small amounts relative to the nonpolyadenylated or truncated polyadenylated transcripts.

IS POLYADENYLATION REQUIRED FOR CHLOROPLAST mRNA DEGRADATION?

The question of whether polyadenylation is required for mRNA degradation, or whether other decay pathways also exist for chloroplast mRNAs, was approached via studies using the lysed-chloroplast system and the polyadenylation inhibitor 3′-dATP (cordycepin triphosphate). Blocking the polyadenylation of RNA inhibits RNA degradation and has similar, if not identical, effects as the direct blocking of the exonucleases by the addition of excess yeast tRNA (Fig. 3) (Lisitsky et al., 1997a). In both treatments the full-length mRNA was endonucleolytically cleaved to distinct degradation products that accumulated instead of being exonucleolytically degraded. Therefore, the addition of poly(A)-rich sequences to the endonucleolytic cleavage products of mRNA is required to target these molecules for rapid exonucleolytic degradation in the chloroplast. A system that degrades mRNA without the addition of poly(A)-rich sequences to the endonucleolytic cleavage product either does not exist or was inactive under this experimental system.

THE BIOCHEMISTRY OF POLYADENYLATION IS ELUCIDATED USING AN IN VITRO SYSTEM

An in vitro polyadenylation system was used to elucidate the biochemistry of mRNA polyadenylation and degradation activities, to isolate the proteins involved, and to reconstitute their activities. In vitro-transcribed RNAs corresponding to the chloroplast RNAs could be polyadenylated at their 3′ end using a soluble chloroplast protein extract complemented by the addition of ATP (Lisitsky et al., 1996). In vitro analysis of chloroplast polyadenylation activity revealed specificity to ATP and GTP, reflecting the composition of the poly(A)-rich tails observed by RT-PCR described above. In this respect, it is interesting to note that poly(A)- and poly(G)-polymerase activities were purified from wheat chloroplasts 25 years ago (Burkard and Keller, 1974). Furthermore, in vitro polyadenylation activity is dependent on the substrate structure. Unstructured RNAs were polyadenylated in a highly efficient manner compared with those molecules forming the stem-loop structure characteristic of the mature plastid mRNA 3′ end (Lisitsky et al., 1996). Again, this observation is in agreement with the RT-PCR clones obtained, where polyadenylated RNA molecules at the mature 3′ end (characterized by a stem-loop structure) were found 50 less times than those at the middle of the RNA molecule.

CAN THE POLYADENYLATED-DEPENDENT DEGRADATION OF CHLOROPLAST RNA BE MIMICKED IN VITRO?

RNA can be synthesized in a test tube using a DNA template of the corresponding nucleotide sequence of the interested gene and an RNA polymerase from a bacteriophage. RNA degradation by chloroplast proteins can be followed by incubating a chloroplast soluble protein ex-

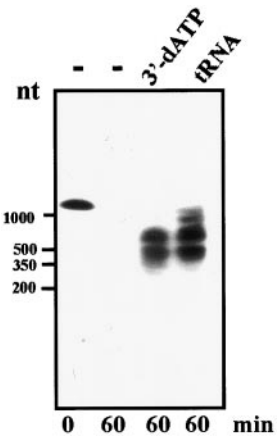

Figure 3. Similar inhibition of mRNA degradation by blocking polyadenylation and inhibiting the exoribonucleases. Lysed chloroplasts were incubated for 0 or 60 min in the presence or absence (−) of 2 mM 3′-dATP (cordycepin triphosphate) or 0.5 mg/mL yeast tRNA (tRNA), as specified in the figure. RNA was extracted from equal amounts of lysed chloroplasts and analyzed by an RNA gel blot that was hybridized with a *psbA*-specific probe. The migration of DNA size markers is indicated on the left as the number of nucleotides (nt).

tract harboring the ribonucleases, as well as the other RNA-binding proteins and components of this process, together with a radioactive-labeled synthetic RNA. In vitro-transcribed, synthetic polyadenylated RNA was rapidly degraded compared with the same nonpolyadenylated RNA incubated in a soluble chloroplast protein extract. Competition experiments revealed that polyadenylated RNA molecules are more efficient competitors for the degradation machinery than nonpolyadenylated molecules. These results suggest that poly(A)-rich tails play a major role in the rapid degradation of intermediate products of mRNA decay in the chloroplast by targeting the cleavage products for rapid degradation, due to their high affinity to chloroplast exonuclease(s) (Kudla et al., 1996; Lisitsky et al., 1996, 1997b). A possible scenario for the situation in the chloroplast is that the relative concentration of the exonucleases is such that they are all occupied in degrading polyadenylated endonucleolytic cleavage products. In this scenario, only polyadenylated RNA molecules will be degraded, as was described above for the experiments using polyadenylation inhibition in the lysed chloroplast system.

WHAT IS THE BIOCHEMICAL MECHANISM OF PREFERENTIAL DEGRADATION OF POLYADENYLATED RNA?

The 100RNP/PNPase discussed above, similar to the bacterial PNPase, is a processive exoribonuclease binding to the 3′ end, digesting the RNA nucleotide by nucleotide without dissociating from the molecule. As described above, this protein could be obtained as a purified single polypeptide. Therefore, it was possible to determine whether the purified enzyme would retain the preferential degradation activity to polyadenylated RNA observed with the chloroplast protein extract. The other possibility was that other auxiliary proteins are required. In competition experiments using isolated, purified 100RNP/PNPase, the polyadenylated RNA competed with the nonpolyadenylated RNA for the exonuclease, as shown for the soluble protein extract (Lisitsky et al., 1997b).

The results implied that competition for polyadenylated RNA is an intrinsic phenomenon of the enzyme as one polypeptide. Therefore, competition for polyadenylated RNA does not depend on the association with the multiprotein complex, the degradosome described above. Is the enzyme's preferred activity to polyadenylated RNA due to the higher binding activity of this protein to a poly(A)-rich sequence or to the faster degradation activity of polyadenylated RNA? Affinity-binding assays of the 100RNP/PNPase to poly(A), as well as to other RNA molecules, displayed higher binding affinity of this protein to poly(A) than to other RNA molecules (Lisitsky et al., 1997b). On the other hand, the degradation rate was similar for all RNA molecules examined. These results suggest that the preferential degradation of polyadenylated RNA in the chloroplast is based on the exoribonuclease 100RNP/PNPase's high binding affinity to the poly(A) sequence. This polypeptide possibly harbors a poly(A) high-affinity binding site in addition to the RNA degradation active site. In addition, the possibility should be emphasized that another, as-yet-unidentified chloroplast exoribonuclease(s) also binds polyadenylated RNA with higher affinity than nonpolyadenylated RNA. Higher in vitro degradation activity of bacterial RNase II to polyadenylated RNA was recently detected (Coburn and Mackie, 1996). Similarly, the *E. coli* PNPase was recently found to bind polyadenylated RNA with higher affinity than other RNA molecules (Lisitsky and Schuster, 1999).

THE MOLECULAR MECHANISM OF mRNA DEGRADATION IN THE CHLOROPLAST

Our recent model of the mRNA degradation pathway in the chloroplast is presented in Figure 4. The initial event is endonucleolytic cleavage(s) producing RNA molecules with no stem-loop structure at the 3′ end (Fig. 4B). RNAs ending in a stem-loop structure were poorly polyadenylated in vitro, and RT-PCR clones of poly(A)-rich sequences at the end of the mRNA molecule (characterized by a stem-loop structure) were obtained with much lower frequency than those having the additional site inside the coding region (Lisitsky et al., 1996). Therefore, we suggest that the stem-loop structure characterizing most of the chloroplast mRNAs, and shown to be an effective 3′-end processing signal, also serves as a poor polyadenylation site for preventing exonucleolytic degradation of the functional molecule. Following the endonucleolytic cleavage(s), the proximal fragments were polyadenylated by the addition of poly(A)-rich sequences (Fig. 4C). This stage is inhibited by 3′-dATP (cordycepin) and is required for the continuation of mRNA degradation in the chloroplast. Due to the higher affinity of this enzyme(s) to the poly(A)-rich sequence, the RNAs were rapidly digested only following

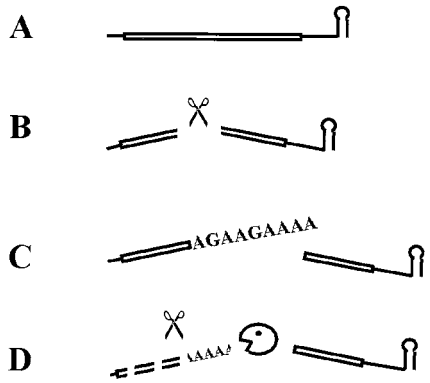

Figure 4. A model for the degradation pathway of mRNA in the chloroplast. A, Schematic representation of the *psbA* mRNA molecule. The white box represents the amino acid-coding region, and the stem-loop structures represent inverted repeats in the 5′- and 3′-UTRs that potentially form stem-loop structures. B, The initial step in the mRNA degradation process is suggested to be endonucleolytically cleaved by an as-yet-unidentified endonuclease(s). The endonuclease is schematically symbolized by scissors. C, A poly(A)-rich tail, which can be up to several hundred nucleotides in length, is then added to the 3′ end of the 5′ endonucleolytically cleaved product. D, The polyadenylated RNA molecule is rapidly degraded by an exonuclease(s), possibly the 100RNP/PNPase.

polyadenylation (Fig. 4D). This last stage can be slowed down by the addition of yeast tRNA (Lisitsky et al., 1997a).

The model suggests two mechanisms for modulating the half-life of a particular RNA molecule. The first implies that once the mRNA molecule is endonucleolytically cleaved, it is targeted for the degradation process. In this model the rate-limiting step is the initial endonucleolytic cleavage. Once this has occurred, cleaved mRNA will be rapidly polyadenylated and exonucleolytically degraded. In such a mechanism, the nature, specificity, and modulation of activity and/or expression of the endonuclease(s) determine the half-life of a particular mRNA molecule, and the activities of the poly(A) polymerase and exonuclease do not control the rate-limiting step. The second model suggests that other steps in the degradation pathway could limit the degradation rate. It is still unknown if the poly(A) tail length, which can amount to several hundred nucleotides, influences the degradation rate. Furthermore, the guanosine residues characteristic of the poly(A) tails of chloroplast *psbA* mRNA may be involved in modulating the activity of the respective enzyme. On the other hand, they may simply reflect the specificity of the poly(A) polymerase(s) (Burkard and Keller, 1974; Lisitsky et al., 1996). In vitro experiments in which synthetic transcribed RNA with poly(A) tails of different lengths and different proportions of guanosine residues were incubated with chloroplast protein extract revealed remarkable differences in degradation rates. The significance of these results in relation to the in vivo situation is still unclear and awaits further investigation.

So far, our model does not explain how the distal endonucleolytic cleavage product is degraded. One possibility is that many endonucleolytic cleavages along the mRNA occur until the small RNA fragment representing the 3′-UTR is degraded by the polyadenylation-dependent pathway, possibly by demolishing the stem-loop structure, thereby enabling polyadenylation. A low degree of polyadenylation of the mature 3′ end has already been obtained in vivo and in vitro (Lisitsky et al., 1996). Moreover, an additional exonucleolytic degradation pathway in the chloroplast that is independent of polyadenylation may exist. For example, evidence of 5′ to 3′ exonuclease activity was recently obtained in *C. reinhardtii* chloroplasts (Drager et al., 1998). Whether or not an additional degradation pathway exists, the results of the experiments using the polyadenylation inhibitor 3′-dATP (cordycepin) indicated that the *rbcL* and *psbA* mRNAs are exonucleolytically degraded only in the polyadenylation-dependent degradation pathway. To understand the role of this degradation pathway in the developmental regulation of plastid mRNA stability, the degradation pathway and the necessity for polyadenylation in etioplasts and root amyloplasts has to be determined. In these developmental stages the *psbA* and *rbcL* RNAs have short half-lives and are rapidly degraded.

Over the past few years, our understanding of the chloroplast mRNA degradation pathway has progressed significantly. For certain mRNAs, such as *psbA,* the different steps of its specific decay have been revealed. The succession of endonucleolytic degradation events is reminiscent of that of the prokaryotic ancestor. This is supported by the observation that one of the enzymes involved has been identified as sharing structural and functional homology with its prokaryotic counterpart. The chloroplast, however, is in part regulated by the nucleus and by external stimuli such as light. Moreover, the longevity of its mRNA better reflects the properties of plants than those of bacteria. Therefore, the chloroplast probably adopted an intermediate position by combining these different features. Following the biochemical pathway of plastid mRNA degradation, research will continue on the regulation of mRNA stability as part of the regulatory network determining leaf development and adaptation to environmental conditions.

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