Plant Mitochondrial Polyadenylated mRNAs Are Degraded by a 3′- to 5′-Exoribonuclease Activity, Which Proceeds Unimpeded by Stable Secondary Structures*

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Recently, we and others have reported that mRNAs may be polyadenylated in plant mitochondria, and that polyadenylation accelerates the degradation rate of mRNAs. To further characterize the molecular mechanisms involved in plant mitochondrial mRNA degradation, we have analyzed the polyadenylation and degradation processes of potato atp9 mRNAs. The overall majority of polyadenylation sites of potato atp9 mRNAs is located at or in the vicinity of their mature 3′-extremities. We show that a 3′- to 5′-exoribonuclease activity is responsible for the preferential degradation of polyadenylated mRNAs as compared with non-polyadenylated mRNAs, and that 20–30 adenosine residues constitute the optimal poly(A) tail size for inducing degradation of RNA substrates in vitro. The addition of as few as seven non-adenosine nucleotides 3′ to the poly(A) tail is sufficient to almost completely inhibit the in vitro degradation of the RNA substrate. Interestingly, the exoribonuclease activity proceeds unimpeded by stable secondary structures present in RNA substrates. From these results, we propose that in plant mitochondria, poly(A) tails added at the 3′ ends of mRNAs promote an efficient 3′- to 5′-degradation process.

The control of mRNA stability constitutes an important aspect of the regulation of gene expression in all organisms. Polyadenylation of mRNAs is involved in the control of mRNA stability, however, with two significantly different roles depending on the organism or subcellular compartment concerned. Polyadenylation is required for stabilizing virtually all nuclear-encoded mRNAs in all eukaryotes. In contrast, polyadenylation targets mRNAs for degradation in eubacteria (1, 2), chloroplasts (3–6), plant mitochondria (7–9), and trypanosome mitochondria (10). In eubacteria and chloroplasts, degradation of mRNAs is initiated by endonucleolytic cleavages. The resulting fragments are then degraded by 3′-to 5′-exonuclease activities (1, 5). In both systems, polyadenylation targets endonucleolytic cleavage products for rapid degradation. In chloroplasts, structured mature 3′ ends are poor substrates for polyadenylation as compared with the internal RNA fragments generated by endonuclease(s) (5, 6).

In plant mitochondria, molecular mechanisms involved in mRNA stability or degradation are still poorly understood (11). For instance, no proteins involved in these processes have been formally identified, although several candidate genes have now been identified since the completion of the nuclear genome sequence of Arabidopsis thaliana. Stable secondary structures can be predicted at the 3′-extremities of some but not all plant mitochondrial mRNAs (12). When present, these structures appear to be involved in stabilizing the transcripts (13–15) and in the correct processing of 3′-extremities rather than being signals for transcription termination (15). Mature 3′ termini of plant mitochondria mRNAs are generated by 3′-processing of longer pre-mRNA molecules (15), and stable mature mRNAs are not constitutively polyadenylated at their 3′-extremities. However, we have previously shown that sunflower atp1-orf322 mitochondrial mRNAs may be polyadenylated in vivo (7), the orf322 gene being the gene linked to the cytoplasmic male sterility phenotype in sunflower. We have reported that polyadenylation accelerates the degradation rate of the transcripts both in vivo and in vitro as compared with non-polyadenylated transcripts (7). Maize cox2 mRNAs may also be polyadenylated, although the influence of polyadenylation on the degradation rate in vitro is less pronounced (8). Recently, polyadenylation of pea atp9 and Oenothera atp1 transcripts has also been characterized (9). Both transcripts terminate with a stable double stem-loop structure, which compensates for some extent for the destabilization effect of polyadenylation in vitro (9).

In this study, we have determined the position of polyadenylation sites in potato mitochondria atp9 mRNAs and studied the influence of poly(A) tails on the degradation of atp9 mRNAs by mitochondrial RNase activities. The expression of the potato atp9 gene has been well documented (16, 17). The size of atp9 transcripts is relatively small, i.e. from 540 to 551 nucleotides. The 5′ termini are derived from transcription initiation (17), and the position of editing sites has been characterized within the open reading frame (16). Most importantly for our studies, a stable stem-loop is predicted within the 3′-UTR of atp9 mRNAs, and this putative secondary structure is located 67–71 nucleotides upstream of the mapped 3′-extremities of the mRNAs (17). Here, we show that most polyadenylation sites of atp9 transcripts are located in a small window of 10 nucleotides, which spans the 3′ mature ends. We show that an exoribonuclease activity is responsible for the degradation of polyadenylated mRNAs, and that the nucleotide composition and the size of the poly(A) tail added at the 3′-extremities of atp9 mRNAs can influence the degradation rate in vitro. Interest-
ingly, the progression of the exoribonuclease activity is not hindered by the presence of a stable secondary structure present in the 3'–UTR of atp9 mRNAs. The position of poly(A) tails and the ability of the exoribonuclease activity to progress through secondary structures of the RNA substrate indicate that poly(A) tails added at the 3′-extremities of mRNAs promote an efficient 3′- to 5′-degradation process in plant mitochondria.

EXPERIMENTAL PROCEDURES

Plant Material, cDNA Synthesis, and Polymerase Chain Reaction Amplification—Mitochondria were isolated from potato tubers (var Bintje) by differential centrifugations and purification on Percoll gradients as described previously (18). Mitochondrial RNAs (10 μg) from potato tubers were incubated with 10 units of DNase I for 30 min at 37 °C. After phenol-chloroform extraction and ethanol precipitation, RNAs were reverse-transcribed using an oligo(dT)18-adapter primer (Table I) and Moloney-murine leukemia virus reverse transcriptase (Stratagene). Aliquots of cDNA reactions were subjected to PCR amplification using the adapter primer in combination with an atp9 gene-specific primer (P1, P2, or P3, Table I) and Taq DNA polymerase (Life Technologies, Inc.). PCR amplification was performed using 30 cycles of 1 min at 94 °C, 50 °C, and 72 °C followed by one step of 5 min at 72 °C. PCR products from the whole reaction were purified using Qiagen PCR purification columns and cloned using a TOPO cloning kit (Invitrogen).

Separation of Two Distinct RNase Activities—Frozen mitochondrial pellets were reassembled at a protein concentration of 10 mg/ml in 50 mM MOPS, 250 mM KCl, 2 mM dithiothreitol, pH 7.5, and frozen/thawed five times. The mitochondrial lysate was centrifuged at 20,000 × g for 20 min at 4 °C. At this salt concentration, most RNase activities are recovered in the 20,000 × g supernatant (data not shown). Protein fractions corresponding to 0–20, 20–40, 40–60, and 60–80% saturation at 0 °C of ammonium sulfate were prepared, dialyzed against 10 mM MOPS, 50 mM KCl, 1 mM dithiothreitol, pH 7.5, at 4 °C, and concentrated by ultrafiltration. The ammonium sulfate fractions were assayed for RNase activities, and two distinct RNase activities (named 1 and 2) were identified. Most RNase activities 2 and 1 were recovered in the 20–40% and 60–80% fractions, respectively. The 40–60% fraction contained a mixture of both activities (data not shown). To further purify RNase activity 2, the 20–40% ammonium sulfate fraction was fractionated by anion-exchange chromatography using a 1-ml UNOSA column (Bio-Rad) previously equilibrated in 10 mM MOPS, pH 7.5. Elution steps were performed first using 100 mM and then 300 mM KCl in 10 mM MOPS, pH 7.5. The 300 mM KCl fraction contained most RNase activity 2 and was dialyzed against 10 mM MOPS, 50 mM KCl, 1 mM dithiothreitol, pH 7.5, at 4 °C and concentrated by ultrafiltration.

In Vitro RNA Degradation Assays—The non-polyadenylated RNA substrate in Figs. 3, 5, and 6 corresponds to the last 136 nucleotides of atp9 mRNA (sites a–c, respectively, in Fig. 1C). In only three out of five clones, poly(A) tails were detected 29 nucleotides 5′ to the putative stem-loop structure, within the stem-loop, or immediately 3′ to the stem-loop (sites a–c, respectively, in Fig. 1C). Polyadenylation sites 3′ to the stem-loop (site c) is the only site that is detected in a relatively A-rich region (4 A nucleotides in 5′). Therefore, site c is the only polyadenylation site in the 3′-UTR of atp9 mRNAs for which we cannot exclude an artificial priming of the oligo(dT)18-adapter primer during cDNA synthesis.

We then used primer P2 in RT-PCR experiments (see Fig. 1A for primer location) to detect possible additional polyadenylation sites within the open reading frame of atp9 mRNAs. Three discrete bands of 470, 270, and 200 bp, respectively, were observed (Fig. 1B). PCR products from whole PCR reactions were cloned. Of 49 clones, 47 clones were sequenced. Most of these polyadenylation sites (39 out of 45) were found at sites h–j that correspond to previously mapped 3′ termini (17). In only 3 out of 51 clones, poly(A) tails were detected 29 nucleotides 5′ to the putative stem-loop structure, within the stem-loop, or immediately 3′ to the stem-loop (sites a–c, respectively, in Fig. 1C).

Characterization of Polyadenylated atp9 mRNAs in Potato—Putative polyadenylation sites in atp9 mRNAs were identified by a similar RT-PCR strategy as was used in our study of the sunflower atp1-orf822 transcripts (7). First-strand cDNA was synthesized from potato mitochondrial RNAs using an oligo(dT)18-adapter primer. PCR amplification experiments were then conducted using an atp9 gene-specific primer (P1, P2, or P3) and an adapter primer. We first used the primer P3 designed to detect polyadenylation sites within the 3′-UTR of atp9 mRNAs (see Fig. 1A for primer location). A discrete band of 250 bp was amplified using the primer P3 and the adapter primer (Fig. 1B). Products from the whole PCR were cloned, and 51 atp9 clones were sequenced. 94% of the atp9 clones (48 out of 51 clones) contained a poly(A) tail located in a small region of 10 nucleotides (sites d–j in Fig. 1C). Most of these polyadenylated sites (39 out of 45) were found at sites h–j that correspond to previously mapped 3′ termini (17). In only 3 out of 51 clones, poly(A) tails were detected 29 nucleotides 5′ to the putative stem-loop structure, within the stem-loop, or immediately 3′ to the stem-loop (sites a–c, respectively, in Fig. 1C).

Analysis of Degradation Products by Thin Layer Chromatography—Aliquots of degradation assays were removed at the indicated time and extracted with phenol-chloroform. Aliquots were spotted onto polyethyleneimine-cellulose TLC plates (Sigma), and the chromatography was developed using 0.25 mM KH2PO4. Standards (UTP, UDP, UMP, or P i) were run alongside the samples.

RESULTS

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The underlined sequence in the T3-atp9 primer corresponds to the T3 RNA polymerase promoter sequence of either uniformly labeled with [α-32P]UTP or 5′-labeled using [γ-32P]ATP as indicated in the figure legends. A similar strategy was employed to generate non-polyadenylated and polyadenylated RNA substrates presented in Fig. 7 by using a cDNA clone corresponding to atp9 mRNAs polyadenylated at site i in Fig. 1C.

Degradation assays were performed in 10 mM MOPS, 50 mM KCl, 1 mM dithiothreitol, 1 mM MgCl2, 1 mM KH2PO4/K2HPO4, pH 7.5, 1 unit/μl RNase inhibitor, and 3.5 μg/μl protein extracts. The presence or absence of 1 mM ATP had little influence on the degradation rates and patterns produced by the ammonium sulfate extracts and was omitted when using the UNOSA fraction. At the indicated time, aliquots of the degradation reactions were extracted by phenol-chloroform and fractionated on an 6% (w/v) acrylamide 7M urea gel.

Analysis of Degradation Products by Thin Layer Chromatography—Aliquots of degradation assays were removed at the indicated time and extracted with phenol-chloroform. Aliquots were spotted onto polyethyleneimine-cellulose TLC plates (Sigma), and the chromatography was developed using 0.25 mM KH2PO4. Standards (UTP, UDP, UMP, or P i) were run alongside the samples.

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### Table I — List of PCR primers

| Oligo(dT)18-adapter primer | CGACCCCTTCGAGGGATCCCG(T)18 |
| Adapter primer | CGACCCCTTCGAGGGATCCCG |
| atp9 primer P1 | GCTTTCATGAAACATCTAAATG |
| atp9 primer P2 | CGAAGCTCAGAATGTTAGAAGGTC |
| atp9 primer P3 | TCATTCTAAGTCCTGATATCCG |
| atp9 primer P4 | GGGAGCCCCAGAGCACCCACC |
| T3-atp9 primer | TCTGAGAATTAAACCCCTCAACTAAGGTTAACTCGGAGAAGGCC |

7 The abbreviations used are: PCR, polymerase chain reaction; MOPS, 4-morpholino propane sulfonic acid; RT, reverse transcriptase; bp, base pair(s); UTR, untranslated region; ORF, open reading frame.
corresponds to mRNAs transcribed from an atp9 gene, which contains several nucleotide substitutions as compared with the genuine potato atp9 gene or an atp9 pseudogene previously identified (16) (GenBank™ accession numbers X63610 and X63611, respectively) (Fig. 2). This newly identified atp9 gene (GenBank™ accession number AJ344260) is probably a non-functional gene, as this sequence contains a deletion of four nucleotides as compared with the genuine atp9 ORF, which thus disrupts the open reading frame (Fig. 2). However, this gene is expressed at the RNA level, because we identified four editing sites within this sequence (Fig. 2). The poly(A) tails observed immediately 3' to a UAG codon situated at the position of the original stop codon in the genuine atp9 gene are encoded by the genomic sequence and thus do not correspond to post-transcriptional polyadenylation. The 470-bp band in Fig. 1B corresponds to atp9 mRNAs polyadenylated at their mature 3'-extremities at sites h and i. All previously characterized editing sites (16) were found edited in these clones. We could detect only 2 out of 49 clones that correspond to atp9 mRNAs, which are polyadenylated within the atp9 ORF. The poly(A) tails are located at nucleotides +60 and +63 respective to the first nucleotide of the start codon (Fig. 1D). Amplification products corresponding to these polyadenylated transcripts are not visible on ethidium bromide-stained gels (Fig. 1B), and their rare occurrence among cloned polyadenylated atp9 mRNAs indicates that they are of low abundance as compared with those transcripts polyadenylated at the 3'-extremities. The seven remaining clones corresponded to the expected atp9 sequences fused either to the 5'-extremities of atp9 mRNAs or to other mRNAs, such as atp1 or nad4 (data not shown), and were not further investigated.

Polyadenylation sites within the 5'-UTR were not detected, although the oligo(dT)18-adapter primer annealed to two relatively A-rich regions present 9 and 60 nucleotides 3' to primer P1 (data not shown). In conclusion, our results show that potato atp9 mRNAs may be polyadenylated in vivo, and that most of the characterized polyadenylation sites are present at their 3'-extremities.

Characteristics of atp9 mRNA Poly(A) Tails—The size of the cloned poly(A) tails of atp9 mRNAs usually corresponds to the size of the oligo(dT)18-adapter primer. However, poly(A) tails from 31 to 55 nucleotides were observed for seven clones. To analyze the nucleotide composition of these tails, 18 A nucleotides, which correspond to the length of the oligo(dT)18-adapter primer, were subtracted from each tail. Of the 136 remaining nucleotides, we detected only 5 guanosines and 131 adenosines (i.e. 96% of A nucleotides), indicating that the poly(A) tails are mainly homopolymeric. The RT-PCR strategy we employed to clone polyadenylated atp9 mRNAs cannot be used to estimate the real length of poly(A) tails in vivo, but our results indicate that some atp9 poly(A) tails may be as long as 55 nucleotides. Similar results were previously reported for maize cox2 mRNAs (8). Using primers P2 and P3, we also observed that four clones contained an additional C or a small nucleotide extension (ATAT) between the genome-encoded atp9 sequence and the poly(A) tail. We cannot exclude the generation of artifacts during cDNA synthesis or PCR amplification. However, this extension could correspond to the addition of non-genomically encoded nucleotides to the 3' termini of atp9 mRNAs. Such extensions have recently been characterized for several plant mitochondrial transcripts (9, 19).

Separation of Two Distinct RNase Activities—We detected two RNase activities (named 1 and 2) in potato mitochondrial extracts (Fig. 3A). These two distinct activities were separated from each other by ammonium sulfate precipitation (see under “Experimental Procedures”). RNase activities 1 and 2 were separately incubated in the presence of either non-polyadenylated or polyadenylated atp9 RNA substrates (Fig. 3A). Both RNA substrates correspond to the last 136 nucleotides of atp9 mRNAs, ending at site h in Fig. 1C, with the addition of 19 adenosine residues in the case of the polyadenylated substrate. As shown in Fig. 3A, RNase activity 1 degrades non-polyadenylated and polyadenylated atp9 RNA substrates at similar rates. Several faint degradation intermediates were detected. In contrast, the non-polyadenylated atp9 RNA substrate is stable in an RNase activity 2 protein extract, whereas the polyadenylated atp9 RNA substrate is degraded in this fraction (Fig. 3A). Using a PhosphorImager, we estimated in three separate experiments that from 55 to 74% of the input full-length polyadenylated substrate is degraded over a 60-min incubation with the RNase activity 2 fraction, whereas only 0–5% of the non-polyadenylated substrate was degraded. One
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Fig. 2. Nucleotide sequence comparison of the genuine atp9 gene with two atp9-related sequences present in potato mitochondria. The sequences a and b correspond, respectively, to an atp9 pseudogene (bases 1–184, GenBankTM accession number X63611) and to the genuine atp9 gene (bases 15–225, GenBankTM accession number X63609) as described previously (16). The sequence c corresponds to the novel atp9 sequence identified in this work (bases 465–673, GenBankTM accession number: AJ344260). Nucleotides conserved between at least two sequences are highlighted. Editing sites are indicated by lowercase letters. Two editing sites conserved between the genuine atp9 gene (b) and the novel atp9 sequence (c) are indicated by black arrowheads.

Fig. 3. Degradation of non-polyadenylated or polyadenylated atp9 RNA substrates by two distinct RNase activities. Non-polyadenylated or polyadenylated RNA substrates corresponding to the last 136 nucleotides of atp9 transcripts ending at site h in Fig. 1C were incubated with RNase activity 1, RNase activity 2, or phosphodiesterase as indicated at the top of each panel. Uniformly [α-32P]UTP-labeled non-polyadenylated or polyadenylated RNA substrates are indicated by No poly(A) and Poly(A), respectively. 5′-32P-labeled non-polyadenylated or polyadenylated RNA substrates are indicated by *No poly(A) and *Poly(A), respectively. Incubation times are shown in minutes (min) above each lane. Aliquots of degradation reactions were fractionated on 7 M urea/6% (w/v) acrylamide gels. Sizes of RNA standards are indicated in nucleotides. A, separation of RNAse activities 1 and 2 by ammonium sulfate precipitation is as described under “Experimental Procedures.” The protein fractions labeled RNase activity 1 and 2 correspond to ammonium sulfate fractions at 60–80% and 20–40% saturation at 0°C, respectively. A degradation intermediate is indicated by a white arrow. B, 5′-32P-labeled non-polyadenylated or polyadenylated RNA substrates were incubated with RNase activity 2. The degradation intermediate indicated by a white arrow as in A is produced by the removal of the poly(A) tail from the polyadenylated substrate. C, no degradation intermediates are observed after further purification of RNase activity 2 by anion-exchange chromatography. D, incubation of the 5′-32P-labeled polyadenylated RNA substrate with phosphodiesterase (3.5 × 10^-5 units/μl). An intermediate corresponding in size to the stalling of the phosphodiesterase at the predicted stem-loop structure is indicated by a black arrow.

Intermediate (marked by a white arrow in Fig. 3A) is generated during the degradation of the polyadenylated atp9 RNA substrate. This intermediate is similar in size to the non-polyadenylated RNA substrate and represents <30% of the input full-length polyadenylated substrate after an incubation of 60 min with the RNase activity 2 fraction. The intermediate is produced by the removal of the poly(A) tail from the polyadenylated substrate as shown by incubating 5′-32P-labeled RNA substrates with RNase activity 2 (Fig. 3B). However, this intermediate is no longer observed when RNase activity 2 is further purified by anion-exchange chromatography (Fig. 3C).

Thus, this additional purification step allows the elimination of either protein(s) or nucleic acids responsible for the formation of this intermediate (see under “Discussion”). This result shows that RNase activity 2 is not impeded at the junction between the poly(A) tail and the remaining RNA substrate, but that this property is dependent on the in vitro conditions, e.g. the degree of purity of the protein extract. Thus, the results presented in Fig. 3 demonstrate that polyadenylation promotes degradation of atp9 mRNAs in potato mitochondria by RNase activity 2.

The stability of the non-polyadenylated atp9 RNA substrate in the RNase activity 2 protein fraction indicates that this fraction is not contaminated by RNase activity 1. Conversely, an analysis of the degradation products of both RNase activities revealed that RNase activity 1 fraction is not contaminated by RNase activity 2 (see Fig. 5).

RNase Activity 2 Proceeds Unimpeded by Stable Secondary Structures—Remarkably, we did not observe a degradation intermediate that would correspond to the stalling of RNase activity 2 within or 3′ to the stable stem-loop structure predicted to exist in the 3′-UTR of atp9 mRNAs (Fig. 3C). However, the RNA substrate is structured as several degradation intermediates are observed when the polyadenylated atp9 RNA substrate is incubated in the presence of phosphodiesterase (Fig. 3D), an exonuclease known to be impeded by structured RNAs. Among the degradation intermediates, a fragment marked by a black arrow in Fig. 3D corresponds in size to the stalling of phosphodiesterase at the predicted stem-loop structure. This suggests that the predicted stem-loop structure exists, but results presented in Fig. 3C show that RNase activity 2 can proceed unimpeded by this stable secondary structure once degradation is initiated by the poly(A) tail.

To further check the ability of RNase activity 2 to proceed through RNA secondary structures, we incubated RNase activity 2 with a synthetic RNA substrate derived from nucleotides 3095 to 3230 of the maize rps2A gene (GenBankTM accession number AF273103). This RNA substrate is predicted to fold into a double stem-loop structure (Fig. 4A). As expected, the non-polyadenylated rps2A RNA substrate was stable in the protein extract, whereas the polyadenylated version of this RNA substrate was degraded (Fig. 4B). No major degradation intermediates were observed, confirming the ability of RNase activity 2 to degrade structured RNA substrates.

RNase Activity 1 Is an Endoribonuclease: RNase Activity 2 Is or Contains an Exoribonuclease—The products of the degradation of atp9 RNA substrates by both RNase activities were...
analyzed using polyethyleneimine-cellulose thin layer chromatography plates (Fig. 5). Under the conditions used, only monoribonucleotides and Pi migrate on the plates, whereas oligoribonucleotides remain at the origin. As shown in Fig. 5, no degradation products generated by RNase activity 1 were visible on the chromatogram, demonstrating that RNase activ-

fig. 7 for the poly(A)19 RNA substrate, confirming

that this intermediate is produced by the removal of the poly(A) tails. RNA substrates containing either no poly(A) tail or a poly(A) tail of five residues were not significantly degraded, indicating that five adenosine residues are not sufficient to initiate degradation. In contrast, poly(A) tails of 20 and 27 residues did promote degradation. Surprisingly, we observed a slight but reproducible reduced degradation rate of the poly(A)27 RNA substrate as compared with poly(A)19 and poly(A)20 RNA substrates (Fig. 7A). This result was confirmed when poly(A)19 and poly(A)20 RNA substrates were incubated simultaneously with RNase activity 2 (Fig. 7, B and C). These experiments suggest that at least in vitro there is an optimal length of the poly(A) tail to induce degradation of RNA substrates by RNase activity 2.

DISCUSSION

In this report, we show that atp9 mRNAs may be polyadenylated in potato mitochondria, and that polyadenylation accelerates the degradation rate of mRNA substrates by an exoribonuclease activity termed RNase activity 2. Under our experimental conditions, non-polyadenylated RNA substrates are not significantly degraded after a 1-h incubation in the

FIG. 4. Degradation of non-polyadenylated or polyadenylated maize rps2A RNA substrates by potato RNase activity 2. A, the maize rps2A sequence is shown from nucleotides 3120 to 3196 (GenBank™ accession number AF273103). Potential double stem-loop forming sequences are indicated by arrows. A GAGG box commonly found 3’ to secondary structures present in the 3’-UTR of plant mitochondrial transcripts is shown in bold. B, Uniformly [α-32P]UTP-labeled non-polyadenylated or polyadenylated RNA substrates corresponding to the maize rps2A sequence from nucleotides 3095 to 3230 were incubated with the RNase activity 2. Incubation times are indicated in minutes (min) above each lane.

FIG. 5. Analysis by thin layer chromatography of degradation products generated by RNase activities 1 and 2. Uniformly [α-32P]UTP-labeled RNA substrates are as indicated in Fig. 3. Incubation times are indicated in minutes (min). Aliquots of degradation reactions such as the ones shown in Fig. 3A were spotted onto polyethyleneimine-TLC plates, and the chromatography was developed using 0.25 M KH2PO4. Note that Pi and UMP co-migrate under these chromatographic conditions.

FIG. 6. Influence of the accessibility of poly(A) tails on the degradation rate by RNase activity 2. Uniformly [α-32P]UTP-labeled non-polyadenylated and polyadenylated substrates are as indicated in Fig. 3. Poly(A)20(N)7 and Poly(A)20(N)20 substrates were synthesized as described under “Experimental Procedures.” The sequences (N)7 and (N)20 correspond to the sequences CGGGAUCGAGGGUCGUGG, respectively. RNA substrates were incubated with the RNase activity 2 fraction. Incubation times are indicated in minutes (min) above each lane. The degradation intermediate produced by the removal of the poly(A) tail from the polyadenylated substrate is indicated by a white arrow as in Fig. 3.
phate or diphosphate is produced by this activity, required to ascertain whether either nucleoside monophos-
totivity. However, further purification of RNase activity 2 will be indicated by a
by the removal of the poly(A) tail from the polyadenylated substrate is
strates with poly(A) 20 and poly(A) 55 tails were incubated simulta-
neously with the RNase activity 2 fraction. The size of the poly(A) tail for each RNA substrate is
indicated at the
time point.

In eubacteria, polynucleotide phosphorylase and RNase II are the major 3′- to 5′-exoribonucleases involved in the RNA degradation process (2), and a polynucleotide phosphorylase-like protein has been characterized in chloroplasts (20). It has also been reported that a 5′- to 3′-exoribonucleolytic activity is involved in RNA degradation pathways in *Chlamydomonas* chloroplasts (21). Here, we present two lines of evidence strongly in favor of the involvement of a 3′- to 5′-exonuclease activity in the degradation of plant mitochondrial polyadenylated mRNAs. First, the addition of seven nucleotides (including six non-A nucleotides) 3′ to the poly(A) tail almost completely abolishes the degradation of the polyadenylated substrate (Fig. 6). This finding strongly suggests that the poly(A) tail must be exposed at the 3′ end to recruit an exonuclease activity. However, this hypothesis is tempered by the finding that at least one endonuclease, the bacterial endonuclease RNase E, preferentially binds single-stranded extremities (22). However, our second line of evidence is that the preferential degradation of polyadenylated RNA substrates is accompanied by the release of monoribonucleotides from a [α-32P]UTP-labeled RNA sub-
strate (Fig. 5). Taken together, these results show that RNase activity 2 either is or contains a 3′- to 5′-exoribonuclease activity. However, further purification of RNase activity 2 will be required to ascertain whether either nucleoside monophos-
phate or diphosphate is produced by this activity, i.e. whether RNase activity 2 is a hydrolytic or a phosphorolytic enzyme,
respectively. Alternatively, the RNase activity 2 fraction may contain both a hydrolytic RNase and a phosphorolytic RNase.

The major characteristic of plant mitochondrial mRNA polyadenylation as compared with the situation in chloroplasts is the position of the polyadenylation sites. We have demonstrated here that the majority of poly(A) tails are added at nucleotides that correspond to the mature 3′ ends of *atp9* mRNAs. Only minor polyadenylation sites were detected within the *atp9* ORF or at the level of a stable secondary structure present within the 3′-UTR of *atp9* mRNAs. This secondary structure does not hinder the progression of RNase activity 2 (Fig. 3). Thus, we believe that poly(A) tails added at the mature 3′ ends recruit RNase activity 2, which then proceeds in a 3′ to 5′ direction to degrade the mRNA. This finding is in striking contrast with the results obtained for chloroplast mRNAs in which most of the polyadenylation sites correspond to endoribonucleolytic cleavage sites (5, 6). Mature 3′ ends of chloroplasts mRNAs may also be polyadenylated but with a frequency approximately 50 times less than endoribonucleolytic cleavage products (3, 4). To date, the polyadenylation of only four plant mitochondrial mRNAs has been reported. The 3′ ends of either sunflower *atp1-orf522* (7) or pea *atp9* and *Oenothera atp1* (9) are predicted to fold into a single stem-loop or a double inverted stem-loop structure, respectively. Poly-
adenylation sites were found within or 3′ but not 5′ to these predicted secondary structures. Maize *cox2* mRNAs are the only example so far where several internal polyadenylation sites have been reported (8). However, 42% of the polyadenyl-
ation sites in maize *cox2* mRNAs correspond to mapped 3′-extremities, which far exceeds the proportion of polyadenylated 3′ ends of chloroplast mRNAs investigated to date. Thus, although the polyadenylation/degradation relationship is con-
served between plant mitochondria and chloroplasts, there seems to be a clear difference in the polyadenylation processes in these two plant organelles.

A degradation intermediate corresponding to the removal of the poly(A) tail is observed only in the protein extract corre-
sponding to the 20–40% ammonium sulfate fraction. This interme-

diate was not observed or was present at greatly reduced levels in few experiments (data not shown), when RNase activ-
ity 2 was further purified on an anion-exchange column (Fig. 3C). Thus, during this additional purification step, protein(s) and/or nucleic acids that are responsible for the formation of the intermediate are eliminated. Several hypotheses can be proposed to explain the removal of the poly(A) tail in the 20–40% ammonium sulfate fraction. First, a poly(A)-specific nuclease or a RNase activity that would stall at the junction of the poly(A) tail and of the remainder of the RNA substrate could be present in the fraction. The polyadenylated substrate would be converted into a non-polyadenylated substrate and, hence, accumulate as it is stable in this extract. The poly(A)-specific nuclease activity would be lost during the next step of RNase activity 2 purification. Second, there could be competition between RNase activity 2 and other proteins that are involved in the correct processing of mature 3′ termini. Such a phenomenon has been described for instance in chloroplasts (20) and in yeast mitochondria (23). Third, RNase activity 2 could in some cases dissociate from the substrate at the junction of the poly(A) tail and of the remainder of the RNA sub-
strate as has been observed for the bacterial polynucleotide phosphorylase (24). Endogenous nucleic acids present in the 20–40% ammonium sulfate fraction but removed during the anion-exchange chromatography step could influence the stall-
ing or dissociation of RNase activity 2 after degradation of the poly(A) tail.

We have demonstrated that 7 or 20 nucleotides added at the

-FIG. 7. Influence of the size of the poly(A) tail on the degrada-
tion rate by RNase activity 2. Uniformly [α-32P]UTP-labeled RNA sub-
strates corresponding to the last 138 nucleotides of atp9 transcripts
ending at site i as indicated in Fig. 1C were incubated with RNase activity 2 fraction. The size of the poly(A) tail for each RNA substrate is indicated at the top of each panel. Incubation times are indicated in minutes (min) above each lane. The degradation intermediate produced by the removal of the poly(A) tail from the polyadenylated substrate is indicated by a white arrow as in Fig. 3. A, each RNA substrate was incubated separately with the RNase activity 2 fraction. B, RNA sub-
strates with poly(A) 20 and poly(A) 55 tails were incubated simulta-
neously with the RNase activity 2 fraction. C, radioactive counts corre-
sponding to full-length RNA substrates with poly(A) 20 (solid line) and poly(A) 55 (dotted line) tails were quantitated in three experiments using a PhosphorImager and plotted against time. The upper and lower error bars correspond to the maximal and minimal values obtained at each time point.

protein extracts, whereas the amount of RNA substrates with
20–27 adenosine residues at the 3′ terminus is significantly reduced (Figs. 3, 4, 6, and 7).
3’ end of the poly(A) tail of atp9 RNA substrate efficiently inhibit the in vitro degradation (Fig. 6). Although we cannot exclude that these artificial 3’ extensions would fold into very stable secondary structures, we consider it more likely that the actual nucleotide composition of the tail added at the 3’ ends of mRNAs is a determinant for initiating degradation. RNase activity 2 seems to specifically recognize poly(A) tails that would thus represent a degradation tag rather than be merely an unstructured single-stranded extension required by RNase activity 2 to initiate degradation. We have shown that five adenosine residues are not sufficient to trigger degradation (Fig. 7). A similar result was also observed for pea atp9 mRNA (9). Surprisingly, we found that 55 A-long tails are not as efficient at initiating degradation as are 20 or 27 A-long tails (Fig. 7). These results may give information regarding the actual recognition mechanism by RNase activity 2 of its RNA substrate. An important aspect of our understanding of the RNA degradation process in plant mitochondria will be to determine the actual signal that targets a particular mRNA molecule to be polyadenylated and, thus, degraded.

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