Characterization and Partial Purification of tRNA Processing Activities from Potato Mitochondria

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In plant mitochondria, as in most other genetic systems, several enzymatic processing and modification steps are required to yield mature tRNAs from primary transcripts. Three of the enzymes involved, RNase P, 3'-processing activity, and tRNA nucleotidytransferase, were identified in potato (Solanum tuberosum) mitochondria and have been separated by several purification steps. RNase P was partially purified, with only a few proteins detectable in active fractions after a final glycerol gradient step. A small RNA molecule present in fractions with RNase P activity contains the heptanucleotide conserved in the other known RNase P RNA sequences and may be a fragment of the RNA moiety of the plant mitochondrial RNase P.

Mature functional tRNAs are generated in all genetic systems by several highly specific processing reactions (Altman, 1981). In mitochondria, tRNA processing is, furthermore, one of the essential steps in protein-coding gene expression. In mammals each strand of the mitochondrial genome is transcribed from single promoters into primary transcripts that contain tRNAs located between the various open reading frames and tRNA sequences (Bibb et al., 1981). As one of the first processing steps tRNAs are excised from the large primary transcripts, resulting in usually monocistronic mRNAs and tRNAs (Ojala et al., 1981).

To excise tRNAs from the precursor transcripts, processing at both 5' and 3' termini is required. Processing at the 5' end is performed in all organisms by the enzyme RNase P (Darr et al., 1992). In eukaryotes the tRNA 3' end is generated by a precise endonucleolytic cleavage at the tRNA terminus in a single reaction, whereas in prokaryotes processing at the 3' end requires a two-step mechanism. After an endonucleolytic cut several nucleotides downstream of the 3' end of the tRNA, the mature tRNA is produced by further exonucleolytic digestion (Altman et al., 1982).

Another essential reaction in tRNA maturation is usually the addition of the CCA terminus, which is encoded in the tRNA genes only in Escherichia coli and some other bacteria. In plant mitochondria, as in all other organisms, most or all tRNA genes lack the CCA triplet, which has to be added after 3' processing.

In plant mitochondria, transcription and processing are not yet understood in great detail. The plant mitochondrial genome is 10 to 50 times larger than the mammalian mitochondrial genome, giving ample space to encode more genes than does the mammalian mitochondrial genome. It is unclear whether mitochondrial tRNAs in plants play any role as potential signals in the processing cascade of the different primary transcripts. To investigate tRNA processing in plant mitochondria, in vitro systems have been developed (Hanic-Joyce and Gray, 1990; Marchfelder et al., 1990). Active fractions have been isolated from Oenothera and wheat mitochondria, which efficiently process 5' and 3' termini of tRNA precursors in vitro (Hanic-Joyce and Gray, 1990; Marchfelder et al., 1990). In addition to these, tRNA nucleotidyl transferase activity has been detected in wheat mitochondrial fractions (Hanic-Joyce and Gray, 1990). Here we describe the detection and characterization of the 5' and 3'-processing enzymes and the tRNA nucleotidyl transferase in potato mitochondria and the development of a partial purification procedure for the enzyme RNase P.

MATERIALS AND METHODS

Substrate Preparation

Several of the tRNA genes identified in the dicot Oenothera berteriana (Gottschalk and Brennicke, 1985; Schuster et al., 1988; Binder et al., 1990) were recloned as templates for precursor RNA synthesis (Fig. 1). Expected product sizes were calculated from the relative positions of transcript termini and the tRNA genes. Precursor RNA molecules for tRNA^{Trp} (Schuster et al., 1988) and tRNA^{Gln} (Binder et al., 1990) were synthesized in vitro as run-off products from genes cloned downstream of T7 RNA polymerase promoters up to the XhoI site and were uniformly labeled with incorporated [α-32P]UTP. The precursor for the chloroplast tRNA^{Ala} (Shinozaki et al., 1986) fragment was a chemically synthesized oligoribonucleotide (Fig. 1; kind gift of H. Kössel), which for analysis was labeled at the 5' end with [γ-32P]ATP. Labeled precursor RNAs were purified through a PAA gel.

In Vitro Assays

For each 5' and 3'-processing reaction (for a detailed description, see Marchfelder et al., 1990) aliquots containing 100 μg of protein of mitochondrial lysate were incubated with precursor tRNAs in buffer A (40 mM Tris [pH 7.6], 40...
b. C.

Figure 1. Schematic presentation of the precursors used as substrates for processing assays. a, O. berteriana mitochondrial pseudotRNA\textsuperscript{Pr} and tRNA\textsuperscript{TrP} of plastid origin (Schuster et al., 1988). The pseudotRNA\textsuperscript{Pr} with a 4-nt deletion in the anti-codon loop is not processed by the plant mitochondrial enzymes (Marchfelder et al., 1990; data not shown). b, O. berteriana mitochondrial tRNA\textsuperscript{Ty}' (Binder et al., 1990). c, Nicotiana tabacum chloroplast tRNA\textsuperscript{Ta}' deletion mutant (Shinozaki et al., 1986; H. Kossel, personal communication). The anti-codon arm and the D arm are deleted; only the TYC arm is left. Nt 1 to 15 represent the 5' leader, nt 16 to 47 represent the incomplete tRNA, and nt 48 to 49 represent the 3' trailer.

**Preparation of Mitochondrial Matrix and Intermembrane Space**

Mitochondria were disrupted with three sonication bursts of 10 s each, and the mitochondrial membranes were pelleted at 100,000g for 2 h. The supernatant, i.e. matrix and intermembrane space fraction, was concentrated with an Amicon cell.

**DEAE-Trisacryl Column**

The DEAE-Trisacryl material (Sigma) was packed according to the manufacturer's protocol. The matrix and intermembrane space fraction were loaded onto the column in buffer A without spermidine (40 mM Tris [pH 7.6], 40 mM KCl, 10 mM MgCl\textsubscript{2}, 2 mM DTT). The column was washed with buffer A until no proteins were detectable in the flow-through. Retained proteins were eluted with a KCl step gradient (0.2 and 0.4 M) and collected in 4-mL fractions. Fractions were dialyzed and concentrated with Centricon filtration units. Protein concentrations were determined with a modified Bradford assay (Bradford, 1976) (Bio-Rad), and fractions were tested for tRNA processing activities.

**Cation-Exchange Chromatography**

A BioRex 70 (Bio-Rad) column was packed with buffer A without spermidine following standard protocols. The 0.2 M KCl fraction from the DEAE column was loaded onto the cation-exchange column in the same buffer conditions. Fractions (4 mL) were collected, and protein concentrations were determined. Fractions were dialyzed, concentrated, and tested for activity.

**Glycerol Gradient**

The RNase P-containing fractions of the cation-exchange column were loaded on a continuous glycerol gradient of 15 to 35% preformed in buffer A without spermidine and were centrifuged for 13 h in a Beckmann SW55 Ti at 42,000 rpm (214,000g). Fractions of 300 \( \mu \text{L} \) each were taken from top to bottom, tested for activity, and analyzed on SDS-PAGE gels. The 4.5% PAA stacking gel (0.1% SDS, 0.125 M Tris [pH 6.8]) and 15% PAA resolving gel (0.1% SDS, 0.75 M Tris [pH 8.85]) were run in a buffer containing 0.1% SDS and 0.025 M Tris (pH 8.5).
RNA Sequencing

Enzymatic sequencing of RNA was done with an RNA-sequencing kit (Pharmacia) and analyzed on 8% PAA-sequencing gels (Sambrook et al., 1989).

3' Labeling of RNA in Column Fractions

Fractions were digested with proteinase K to remove proteins. After the samples were extracted with phenol and chloroform, nucleic acids were precipitated and labeled with [32P]-pCp and T4 RNA ligase for 12 h at 4°C.

Quantitation of Processing Activities

To determine relative processing activities, autoradiographs were scanned with a LKB Ultroscan XL laser densitometer.

RESULTS

To analyze enzymatic activities processing tRNA precursors in potato mitochondria, available heterologous substrates from other plant species were tested. RNase P- and 3'-processing activities in mitochondrial lysates from potato tubers were found to correctly recognize and process tRNA substrates from Oenothera mitochondria as judged by the identical gel migration properties of the products generated in both potato and Oenothera lysates (data not shown). These tRNA precursor molecules were, therefore, subsequently used in the activity assays with the potato mitochondrial lysate.

Identification of tRNA Nucleotidyl Transferase

To determine whether a tRNA nucleotidyl transferase activity is detectable in mitochondrial lysates from S. tuberosum, incorporation of CTP into tRNA precursor molecules was investigated. Crude matrix preparations and nucleic acid-depleted fractions eluted from a DEAE-Trisacryl column were incubated with either only [α-32P]CTP or both unlabeled precursor tRNAs and [α-32P]CTP. Three different tRNA precursors were tested as substrates, namely Oenothera mitochondrial tRNAAla and tRNATyr and an incomplete tobacco chloroplast tRNAAla, respectively (Fig. 1). Incubation of matrix fraction with [α-32P]CTP alone yields a large number of labeled RNA species (Fig. 2, lane 1), which obscure the added templates (Fig. 2, lanes 3, 5, and 7). These labeled RNAs are presumably derived from endogenous tRNA molecules present in the crude mitochondrial matrix fraction, since the size distribution of these labeled RNA species corresponds to the pattern of the total mitochondrial tRNA population (S. Binder and A. Brennicke, unpublished results; Gottschalk and Brennicke, 1985; Schuster et al., 1988; Binder et al., 1990). Passage of the mitochondrial lysate through a DEAE-Trisacryl column removes these endogenous mitochondrial tRNAs and reduces the background of tRNA-sized molecules labeled with [α-32P]CTP.

Both precursors of intact tRNAs (tRNAAla and tRNATyr) were processed to mature-sized tRNAs, 77 nt (Fig. 2, lane 4) and 88 nt (Fig. 2, lane 6), respectively, by the DEAE-Trisacryl flow-through fractions. The actual sizes of the RNA molecules do not directly correlate with the gel migration properties, since RNA molecules carrying a deletion at the 3' end migrate differently in sequencing gels (Sambrook et al., 1989).

Figure 2. tRNA nucleotidyl transferase assay. [α-32P]CTP was incubated with different mitochondrial fractions and unlabeled tRNA precursors. Lanes 1, 3, 5, and 7, Incubation with the matrix fraction results in specific labeling of numerous endogenous mitochondrial tRNAs masking the added templates in lanes 3, 5, and 7; lanes 2, 4, 6, and 8, incubation with the DEAE-Trisacryl flow-through fraction shows that most of the endogenous tRNAs have been removed. Lanes 1 and 2, No exogenous tRNA precursor was added; only endogenous tRNAs are labeled. Lanes 3 and 4, Unlabeled precursor tRNAAla was added. Lanes 5 and 6, Unlabeled precursor tRNATyr was added. Lanes 7 and 8, Unlabeled precursor δ-tRNAAla was added (lanes 7 and 8, short exposure to detect the 52-nt labeled δ-tRNAAla, which with longer exposure becomes masked by the endogenous tRNAs in lane 7). Positions of the labeled mature tRNAs are indicated by arrows.

Identification and Separation of 5'- and 3'-Processing Activities

Crude mitochondrial lysates from S. tuberosum are competent in processing precursor tRNA molecules to mature RNA molecules (Shinozaki et al., 1986). Incorporation of CTF into tRNA precursor molecules was, therefore, subsequently used in the activity assays with the potato mitochondrial lysate.

Figure 2 shows the 3'-processing activity in the crude mitochondrial matrix fraction of potato tubers. The labeled product of the precursor containing the deleted tRNA (tRNAAla, Shinozaki et al., 1986) corresponds to the 5'-unprocessed but CCA-loaded tRNA of 52 nt in length (Fig. 2, lane 8). In contrast to the specificity requirements of the 5'-processing activity, the tRNA nucleotidyl transferase appears to accept this incomplete, 5'-unprocessed tRNA-like molecule as substrate.
rconidia were tested, and the mature tRNA products obtained in the potato and *Oenothera* (Marchfelder et al., 1990) mitochondrial lysates were identical in size and gel mobility in all instances (data not shown). Organelle fractionation experiments located the bulk of the processing enzymes in the S100 supernatant, the mitochondrial intermembrane and matrix fraction (not shown).

The mitochondrial matrix and intermembrane space fraction were further purified on a DEAE-Trisacryl column, from which proteins were eluted with a stepwise KCl gradient. The majority of both 5'- and 3'-processing activities were released with 0.2 M KCl (Fig. 3), with about 20% of the 5'-processing activity detected in the pooled flow-through and 80% in the pooled 0.2 M KCl fractions (not shown). This latter eluate was dialyzed, concentrated, and further fractionated on a cation-exchange column with a KCl step gradient. After this column the 3'-processing activity was detected in the flow-through fractions, whereas the 5'-processing activity eluted with 0.4 M KCl (Fig. 4; Marchfelder, 1992); thus, the 5'- and 3'-processing activities were separated. The 0.4 M KCl fraction was dialyzed, concentrated, and loaded onto a glycerol gradient. After centrifugation, gradient fractions were tested for RNase P-processing activity (Fig. 5). The yields of each purification step were estimated from the quantitated tRNA product formation as given in Table I. These estimates are complicated by the instability of the RNase P and its rapid degradation during fractionation, which was also observed during RNase P purification schemes in other organisms, e.g. the yeast mitochondrial enzyme (Morales et al., 1989).

Analysis of aliquots from each glycerol gradient fraction for protein content on SDS gels (Fig. 6) showed only three proteins, about 22, 34, and 47 kD in mass, to be detectable in fractions with the RNase P peak activity in separate gradients centrifuged for different times (data not shown). It remains unclear at present whether any of these three proteins are connected with the RNase P activity, since their abundances do not exactly correlate with activity. Such a direct correlation is complicated by the nature of RNA-protein complexes, in which both moieties are required and need to co-fractionate for activity to be observed. An additional difficulty is the high specific activity of the RNase P, which may result in protein concentrations below detection limits. If fraction 8 is considered a bad data point, the 47-kD polypeptide appears to be the best candidate for a protein connected with the RNase P activity.

**Identification of an RNA Molecule Potentially Associated with RNase P**

Although the main peak of the RNase P activity elutes with 0.2 M KCl from the DEAE-Trisacryl column, a certain amount (20%) of the activity is also detected in the flow-through fraction (data not shown). This separation could be explained by progressive degradation of the RNA in the ribonucleoprotein complex during the first steps of purification. The anion part (i.e. predominantly the RNA) of the

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**Figure 3.** DEAE-Trisacryl chromatography. Proteins were eluted with a KCl step gradient of 40, 200, and 400 mM steps, represented by the horizontal lines. Protein concentrations of the 4-ml fractions are shown for the entire gradient, and the fractions containing tRNA-processing activities are indicated with an arrow. Both RNase P- and 3'-processing activities elute with 0.2 M KCl.

**Figure 4.** Elution profile of the BioRex 70 cation-exchange chromatography. Proteins were eluted with a KCl step gradient (straight lines). Fractions of 4 ml were collected and assayed for protein concentration and tRNA-processing activities. The 3'-processing activity is identified in the flow-through, whereas the RNase P elutes only with 0.4 M KCl.

**Figure 5.** RNase P activity in glycerol gradient fractions. Fractions (300 μL) were taken off the glycerol gradient from top to bottom and assayed for RNase P activity. Fractions 5 to 9, corresponding to a sedimentation coefficient between 4.5S and 11.3S, show significant RNase P activity.
Table I. Purification of RNase P from potato mitochondria

Specific activity was estimated from densitometric quantification of reaction products resolved by gel electrophoresis. Activities are given relative to the highest specific activity after the last purification step.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (mL)</th>
<th>Protein (mg)</th>
<th>Specific Activity (units/mg)</th>
<th>Yield (%)</th>
<th>Purification Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>S100</td>
<td>60</td>
<td>1200</td>
<td>2</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>DEAE-Trisacryl</td>
<td>10</td>
<td>390</td>
<td>5</td>
<td>81.3</td>
<td>2.5</td>
</tr>
<tr>
<td>BioRex 70</td>
<td>0.6</td>
<td>8</td>
<td>12.5</td>
<td>4.2</td>
<td>6.3</td>
</tr>
<tr>
<td>Glycerol gradient</td>
<td>0.04</td>
<td>1</td>
<td>100</td>
<td>4.0</td>
<td>50</td>
</tr>
</tbody>
</table>

complex is reduced by degradation, weakening the interaction with the anion-exchange column. The presence of unbound RNase P activity may then be explained by the observation that parts of the RNA moiety are sufficient for processing, as observed for the E. coli (Guerrier-Takada and Altman, 1986) and yeast mitochondrial RNase P enzymes (Morales et al., 1989; Wise and Martin, 1991). Since the DEAE-Trisacryl flow-through fraction contains no free nucleic acids (which should quantitatively interact with the anion exchanger), the RNA molecules still present in this fraction are presumably masked by proteins.

After these flow-through fractions were depleted of proteins, total RNAs were 3' end labeled and analyzed. Several RNA molecules are detected in this fraction, between about 40 and 80 nt in apparent size (Fig. 7A, lane 1). One prominent RNA species of about 80 nt and two of about 40 nt can be identified. Similar analysis of the RNA molecules contained in the RNase P-active fractions of the 0.4 M KC1 step from the cation-exchange column and the glycerol gradient showed RNA molecules of larger sizes (Fig. 7A, lane 2, and data not shown). RNA molecule species larger than those found in the DEAE-Trisacryl flow-through are expected if RNase P retention on the latter column was due to more
intact RNA moieties with the potential to interact with the DEAE-Trisacryl matrix.

One of these larger RNA molecules was identified by direct sequencing (not shown) as the nuclear encoded 5S rRNA frequently observed in plant mitochondrial RNA preparations. The other RNA molecules detected in the active fractions of the cation-exchange column and after the glycerol gradient were not present in sufficient quantities for further analysis, whereas several RNA molecule species detected in the DEAE-Trisacryl flow-through could be eluted from the analyzing gel and sequenced. Sequences were obtained for the three prominent RNAs (Fig. 7A, lane 1) and two less abundant RNAs (not shown). Four of these RNA sequences show no similarity with any sequence in the data bases and may be derived either from other parts of the RNase P RNA or from different RNA-protein complexes (data not shown).

Alignment of the 41 nt RNA sequence (Fig. 7B) with RNase P RNAs (Fig. 8A) of other species shows that this purified RNA contains the unique sequence element conserved among all RNase P RNAs investigated so far. Scattered similarities are also apparent outside of this highly conserved core sequence, e.g. in the pairwise comparison with the E. coli RNase P RNA (Fig. 8B). These observations suggest that this RNA is possibly part of the plant mitochondrial RNase P RNA moiety.

**DISCUSSION**

**Identification of Potato Mitochondrial tRNA Nucleotidyl Transferase**

Since plant mitochondrial tRNA genes do not encode the terminal triplet CCA, the specific nucleotidyl transferase is an essential enzyme in this organelle. In wheat mitochondria tRNA nucleotidyl transferase activity has been described previously in lysates and was shown to add stepwise the tRNA nucleotidyl transferase activity has been described an essential enzyme in this organelle. In wheat mitochondria thought to be derived from a prokaryotic ancestor, the 3'-end (Chen and Martin, 1988). Although mitochondria are known to be derived from a prokaryotic ancestor, the 3'-end (Chen and Martin, 1988). Although mitochondria are thought to be derived from a prokaryotic ancestor, the 3'-

**Figure 8.** Alignment of RNase P RNA and the isolated plant mitochondrial RNA fragment sequence. A, RNase P RNA sequences of different species contain a conserved heptamer sequence (box) that is also present in the RNA fragment isolated from *S. tuberosum* mitochondria. This suggests that this RNA molecule may be part of the RNase P RNA moiety in potato mitochondria. *S. tub. S. tuberosum; H. vol. Halolerax volcans; S. cer. Saccharomyces cerevisiae; X. lae. Xenopus laevis; mt. mitochondrion. B, Sequences of the E. coli RNase P RNA and of the 41-nt RNA fragment isolated from the DEAE-Trisacryl flow-through fraction also show scattered similarities outside the conserved heptamer sequence (conserved nt are indicated by stars).
tRNA-processing activity seems to resemble more the eukaryotic enzyme.

**Potato Mitochondrial RNase P Consists of a Basic Protein and an RNA Moiety**

The potato mitochondrial RNase P interacts with the DEAE-Trisacryl column similarly to other isolated RNase P enzymes, probably because of the common presence of a highly charged RNA moiety. The fractionation of the RNase P activity on this column resulting in 20% of the activity flowing through the column suggests that plant and yeast mitochondrial RNase P enzymes have similar properties. In yeast mitochondria purification of this enzyme has been hampered by instability of the RNA moiety, which is partially degraded during chromatography and unstable at room temperature (Morales et al., 1989). However, RNase P activity is still observed with fragments of the RNA in yeast mitochondrial preparations, suggesting that the potato mitochondrial RNase P activity is likewise detectable with fragments of the RNA, which may be masked by the protein moiety from binding to the DEAE-Trisacryl matrix. The protein subunit appears to be rather basic, as suggested by its binding to the cation-exchange column, similar to the *E. coli* and *Bacillus subtilis* RNase P protein subunits (Altman, 1981; Darr et al., 1992).

**An RNA Species with Similarity to Other RNase P RNAs**

Since the RNA molecules not retained by the DEAE-Trisacryl column should be masked from interaction, they may be breakdown products of the RNase P RNA moiety or other RNA-protein complexes. Sequence analysis is expected to allow tentative assignment of function to these RNAs. The sequence of one of the isolated RNA species indeed shows promising similarity to RNase P RNAs from other organism (Fig. 8A). The unique, single motif conserved among eukaryotic, archaeabacterial (Darr et al., 1990), bacterial, and yeast mitochondrial RNase P RNAs is also present in this RNA fragment. The presence of this perfectly conserved heptanucleotide suggests that this RNA fragment may be part of the potato mitochondrial RNase P RNA moiety. The fact that the nucleic acid was not retained on the DEAE-Trisacryl column implies that it was protected from interaction with the anion-exchange column, presumably by a protein. If this RNA molecule is indeed part of the RNase P RNA, it should thus be in close contact with the protein subunit and possibly contain the catalytic core. An oligonucleotide with the sequence of this RNA molecule can now be used to search for the gene of this RNA and to investigate whether this sequence is indeed present in the RNase P RNA. It will be of particular interest to determine whether the RNA subunit of the mitochondrial RNase P is encoded in the nucleus or in the mitochondrion. If the RNA is nuclear encoded, it would have to be imported into the mitochondrion. Several tRNAs have been found to be imported into mitochondria in plants, showing that highly charged RNA molecules can indeed pass the hydrophobic mitochondrial membranes (Marechal-Drouard et al., 1988; Sangare et al., 1989; Small et al., 1992). Identification and cloning of the gene encoding the plant mitochondrial RNase P RNA moiety will allow further in vitro studies to determine whether this molecule is also active in tRNA processing in the absence of the protein moiety.

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**LITERATURE CITED**


Binder S, Schuster W, Grienenberger J-M, Weil J-H, Brennicke A (1990) Genes for tRNA <sup>55</sup>, tRNA<sup>57</sup>, tRNA<sup>49</sup>, tRNA<sup>56</sup>, tRNA<sup>57</sup>, tRNA<sup>54</sup> and tRNA<sup>57</sup> are encoded in *Oenothera* mitochondrial DNA. Curr Genet 17: 353–358


