tRNA 3′ Processing in Plants: Nuclear and Mitochondrial Activities Differ[†]

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ABSTRACT: The nuclear tRNA 3′ processing activity from wheat has been characterized and partially purified. Several characteristics of the wheat nuclear 3′ processing enzyme now allow this activity to be distinguished from its mitochondrial counterpart. The nuclear enzyme is an endonuclease, which we termed nuclear RNase Z. The enzyme cleaves at the discriminator base and seems to consist only of protein subunits, since essential RNA subunits could not be detected. RNase Z leaves 5′ terminal phosphoryl and 3′ terminal hydroxyl groups at the processing products. It is a stable enzyme being active over broad temperature and pH ranges, with the highest activity at 35 °C and pH 8.4. The apparent molecular mass according to gel filtration chromatography is 122 kDa. The nuclear RNase Z does process 5′ extended pretRNAs but with a much lower efficiency than 5′ matured pretRNAs. Nuclear intron-containing precursor tRNAs as well as mitochondrial precursor tRNAs are efficiently cleaved by the nuclear RNase Z. Mitochondrial pretRNA^{His} is processed by the nuclear RNase Z, generating a mature tRNA^{His} containing an 8 base pair acceptor stem. The edited mitochondrial pretRNA^{Phe} is cleaved easily, while the unedited version having a mismatch in the acceptor stem is not cleaved. Thus, an intact acceptor stem seems to be required for processing. Experiments with precursors containing mutated tRNAs showed that a completely intact anticodon arm is not necessary for processing by RNase Z. Comparison of the plant nuclear tRNA 3′ processing enzyme with the plant mitochondrial one suggests that both activities are different enzymes.

tRNA 3′ processing is one of the essential steps in tRNA maturation leading to functional tRNA molecules in every organism. In contrast to the 5′ processing event, catalyzed by the ubiquitous RNase P (for a review see refs *1* and *2*), 3′ processing has not been that well characterized hitherto. In the bacterial kingdom only *E. coli* has been thoroughly investigated with regard to tRNA 3′ processing. In *E. coli* a multistep reaction involving endo- and exonucleases generates the mature tRNA 3′ end, and maturation of the 3′ end seems to occur after 5′ processing (*3*). Several exonucleases have been shown to be involved in this reaction, but so far it was not possible to identify a specific tRNA 3′ exonuclease. This result suggests that there may not be a tRNA-specific exonuclease in *E. coli*.

In archaea nothing is known about 3′ processing, beyond the observation that RNase P generates the mature 5′ end prior to tRNA 3′ processing (*4*). Whether endo- and/or exonucleases are involved in the generation of the mature 3′ end is so far not known.

Organelle 3′ processing is expected to be basically similar to bacterial 3′ maturation since organelles are of prokaryotic origin (*5*). However, several studies showed that the generation of the mature tRNA 3′ end in mitochondria (*6*−*10*) as well as in chloroplasts (*11*) is a single-step reaction involving endonucleases, which cut at the tRNA 3′ end. Thus, organelles appear to have lost their original bacterial 3′ processing system and to have developed unique activities possibly adapted from host enzymes.

In contrast to organelle and prokaryotic tRNA genes coding for precursors with long 5′ and 3′ extensions, nuclear tRNA genes are transcribed by polymerase III into precursors containing only short 5′ (about 6−15 nt) and 3′ (about 5−10 nt) extensions. The 5′ extension was shown to be removed by RNase P (*12*). Since the 3′ trailer consists only of a few nucleotides, an exonuclease could theoretically be sufficient to complete tRNA 3′ maturation. Several nuclear 3′ processing enzymes have been investigated (*13*−*24*, *30*, *35*), and the majority of these 3′ processing activites has interestingly been found to be endonucleases cleaving at the tRNA 3′ end, although some studies also reported an involvement of exonucleases (*18*−*22*). In *Saccharomyces cerevisiae* Papadimitriou and Gross (*20*) found exo- as well as endonucleases capable of generating mature tRNA 3′ ends. The observed differences between the nuclear 3′ processing systems may be due to different precursors used. The various degrees of purification of the extracts tested might also have caused the different results reported. Yet another possibility is that both modes of processing can be used as found in yeast. Studies by Yoo and Wolin showed that in *S. cerevisiae* the presence of the Lhp1p protein is essential for endonucleolytic 3′ processing (*22*). In the absence of this protein the tRNA 3′ end is generated by exonucleases.

Hitherto except for the exonucleases from *E. coli* not a single gene for a 3′ processing activity has been isolated. An extensive purification was performed with the *S. cerevisiae* 3′ processing enzymes (*20*), where two endonucleases (45/60 and 55 kDa) and three exonucleases (33, 60, and 70

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kDa) were reported to participate in tRNA 3′ end maturation. Purification of the *Aspergillus* enzyme showed a 160 kDa endonuclease to be involved in 3′ processing (*23*); in *Xenopus* a 97 kDa protein was identified (*24*). Purification of the potato mitochondrial 3′ processing enzyme identified a protein of about 43 kDa as correlated with the activity (*34*).

To clarify the nature of the nuclear 3′ processing enzyme, we initiated the characterization and partial purification of this activity from wheat. Comparison with the potato mitochondrial 3′ processing enzyme, mitochondrial RNase Z, should clarify whether this organelle has adopted the host enzyme after losing its original bacterial activity or whether it developed a unique 3′ processing system.

EXPERIMENTAL PROCEDURES

Materials

Radioactivity was purchased from Amersham, chemicals were purchased from Fisher Scientific, Merck, or Fluka, and enzymes—if not stated otherwise—were purchased from Boehringer Mannheim.

Isolation of tRNA Genes

Isolation of the mitochondrial $tRNA^{His}$ gene from *Oenothera berteriana* was described earlier (*25*). The gene for $tRNA^{Tyr}$ from *Nicotiana rustica* was amplified from total cellular DNA of *N. rustica* using primers cyTyr3 (5′ TAA TAC GAC TCA CTA TAG GAC GAC TTC TAT ATA GGT ATG 3′) and cyTyr2 (5′ GCA TTA AGA AGA TTT TTT GAT G 3′). The resulting PCR[1] product contains the tRNA gene and additional 5′ and 3′ sequences. PCR with primers cyTyr1 (5′ TAA TAC GAC TCA CTA TAC CGA CCT TAG CTC AGT TGG 3′) and cyTyr2 yielded a fragment containing the tRNA gene and the 3′ trailer (no 5′ leader). Both PCR products were cloned into pBluescript (Stratagene), yielding plasmid pcyTyr12 (including 5′ leader) and pcyTyr5 (without 5′ leader).

Preparation of Mutant tRNAs

To remove the intron from the wild-type $tRNA^{Tyr}$ gene, a deletion PCR was performed with primers cyTyr4 (5′ CTA CAG TCC TCC GCT CTA CC 3′) and cyTyr5 (5′ ATC CTT AGG TCA CTG GTT CG 3′), which amplifies the entire clone pcyTyr5 except the intron. The resulting PCR product was ligated, and the new clone was termed pcyTyr7. Sequencing of the ligation products revealed some clones which contained additional deletions which were termed pcyTyr6 (containing a point mutation and missing one nucleotide in the anticodon loop), pcyTyr 8 (missing the whole anticodon loop and one nucleotide of the anticodon stem), and pcyTyr16 (missing the whole anticodon loop, parts of the anticodon stem, and one nucleotide of the variable arm); see also Figure 8.

Substrate Preparation

Templates pCys (for $pretRNA^{Cys}$ from *N. rustica*), pTyrII (for mitochondrial $pretRNA^{Tyr}$ from *O. berteriana*), pPheIa (5′ extended edited form of mitochondrial $pretRNA^{Phe}$ from *O. berteriana*), and pPheIIa and pPheIIb (5′-mature unedited and edited forms, respectively, of mitochondrial $pretRNA^{Phe}$ from *O. berteriana*) were prepared as described (*9*). (I) PCR of $tRNA^{Tyr}$ genes: (a) Template pcyTyrV(for $pretRNA^{Tyr}$ from *N. rustica*) was amplified from plasmid pcyTyr5 with primers cyTyr1 and cyTyr2. The resulting transcript contains a 58 nt long trailer and $tRNA^{Tyr}$ (73 nt) with a 13 nt long intron. (b) Template pcyTyrVII was amplified from plasmid pcyTyr7 using primers cyTyr1 and cyTyr2. The resulting transcript contains a 58 nt long trailer and the intronless $tRNA^{Tyr}$, with 73 nt. (c) Templates pcyTyrVI, -VIII, and -XVI were amplified from plasmids pcyTyr6, -8, and -16 using primers cyTyr1 and cyTyr2. All transcripts contain a 58 nt long trailer and deleted versions of $tRNA^{Tyr}$: pcyTyrVI contains a 64 nt long tRNA, pcyTyrVIII contains a 72 nt long tRNA, and pcyTyrXVI contains a 58 nt long tRNA (see also Figure 7). (d) Template pcyTyrXII was amplified from plasmid pcyTyr12 using primers cyTyr3 and cyTyr2. The resulting transcript contains a 5′ leader (43 nt long), the tRNATyr (73 nt long), and the 3′ trailer (58 nt long). (II) Template pHis (for mitochondrial $pretRNA^{His}$ from *O. berteriana*) was amplified from plasmid 10/83-1 (*25*) with primers AH1 (5′ TAA TAC GAC TCA CTA TAG GCG GAT GTA GCC AAG TGG 3′) and AH2 (5′ ACT TGC AGA AAA AAT GCT TTA ACG 3′). Transcription of this template yields a pretRNA with a 5′ mature $tRNA^{His}$ (76 nt, 8 bp in the acceptor stem) and a trailer of 64 nt length.

Polymerase chain reactions were carried out essentially as described (*9*) using the following conditions: (a) for template pcysTyrV, -VII, -VI, -VIII, -XII, and -XVI, 1 min at 95 °C, 1 min at 50 °C, and 30 s at 72 °C, (b) for pHis, 1 min at 95 °C, 1 min and 30 s at 33 °C, and 30 s at 72 °C. In vitro transcription and purification of transcripts were performed as described (*31*). Transcripts were labeled with [α-^{32}P]UTP if not stated otherwise.

Purification of RNase Z from Wheat

A. Initial Purification Scheme for the Biochemical Characterization of RNase Z. A 125 g sample of wheat germ was repeatedly (3×) treated with 250 mL of carbon tetrachloride and 70 mL of cyclohexane to isolate embryos which float on the organic solvent. To separate the embryos from the solvent, the mixture was filtered and the embryos were left on the filter to dry. Subsequently the embryos were swollen by soaking them in water o/n. Cells were disrupted in a waring blendor using homogenization buffer (50 mM Tris−HCl, pH 8/5% gylcerol/0.1 M KCl/0.2 μM Aprotinin/63 μM EDTA/11 μM Antipaindihydrochlorid/1 μM Leupeptin/1 μM Pepstatin/4.2 μM Chymostatin/0.2 mM Pefabloc SC/5 mM DTT, and 1.5% PVPP). Centrifugation at 11000*g* for 30 min removed cell debris, mitochondria, and proplastids. The resulting supernatant was filtered through one layer of miracloth and concentrated with ultrafiltration cells (Amicon). A high-speed supernatant (S100) was prepared by ultracentrifugation at 100000*g* for 60 min. Protein concentrations were determined using a modified Bradford assay (Roti-Quant, Roth-Germany). *Resource Q column*. A 6 mL prepacked Resource Q column (Pharmacia) was equilibrated with buffer A (40 mM Tris−HCl, pH 8/5 mM $MgCl_2$/5% glycerol/0.05% Nonidet P40/2 mM DTT/0.5 mM PMSF). The column was loaded with 100 mg of S100 and washed

[1] Abbreviations: DTT, dithiothreitol; mt, mitochondrial; nt, nucleotides; MES, 2-(*N*-morpholino)ethanesulfonic acid; Np, 3′-monophosphate nucleoside; pN, 5′-monophosphate nucleoside; NTP, nucleoside triphosphate; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PIPES, piperazine-*N*,*N*-bis(2-ethanesulfonic acid); pretRNA, precursor tRNA.; Tris, tris(hydroymethyl)aminoethane.

with buffer A. Proteins were eluted with a KCl step gradient (0.1, 0.2, and 1.0 M KCl in buffer A), and 2 mL fractions were collected. Fractions were pooled and dialyzed with buffer A. Protein concentrations were determined with a modified Bradford assay (Roti-Quant and Nanoquant from Roth, Germany), and fractions were tested for activity. RNase Z activity eluted with 0.2 M KCl.

B. Purification of RNase Z. Initial purification steps were as described in (A), the only difference being that the first centrifugation was done at 30000g instead of 11000*g*. This S30 extract was filtered through miracloth and fractionated with PEG as follows: A 40% PEG stock solution (40% PEG 6000 in homogenization buffer (w/w)) was slowly added to the S30 fraction under constant stirring until the final PEG concentation was 6% (w/w). After the solution was stirred for another 60 min, the precipitate was pelleted for 30 min at 30000*g*. The resulting pellet was dissolved in buffer A10 (buffer A containing 10% glycerol), and again a 40% PEG stock solution was added to the supernatant to a final concentration of 12% PEG. The precipitate was centrifuged as above, and the resulting pellet (6−12% PEG) was dissolved in buffer A10. This 6−12% PEG fraction contained the RNase Z activity. *Source 30Q column*. The PEG fraction was loaded onto a Source Q (Pharmacia) column (80 mL, packed according to the manufacturer's protocol). The column was washed with buffer A until the flow-through contained no more proteins. Bound proteins were eluted with a KCl step gradient (0.1, 0.2, and 1.0 M KCl in buffer A) and collected in 10 mL fractions. Fractions of each salt step were pooled and concentrated. KCl was removed from the protein fractions using a HiPrep 26/10 desalting column (Pharmacia) according to the manufacturer's protocol. Protein concentrations were determined with a modified Bradford assay (Roti-Quant and Nanoquant from Roth-Germany), and fractions were tested for activity. RNase Z eluted with 0.2 M KCl. *Heparin Sepharose column*. The RNase Z containing activity (0.2 M KCl Source Q) was further fractionated on a 50 mL Heparin Sepharose column (Pharmacia) packed according to the manufacturer's instructions. After the column was equilibrated with buffer A, the dialyzed Source Q fraction was loaded, and proteins were eluted with a KCl step gradient (0.1, 0.2, 0.25, 0.3, and 1.0 M KCl in buffer A), and 10 mL fractions were collected. 3′ tRNA processing activity was eluted with 0.25 M KCl. *Blue column*. A prepacked Hi-Trap Blue column (Pharmacia) was equilibrated with buffer A− (buffer A without Nonidet P40) containing 0.25 M KCl and was loaded with the 0.25 M KCl fraction from the Heparin column. Bound proteins were eluted with a KCl step gradient in buffer A (0.4, 0.6, 0.8, 1.0, and 2.0 M KCl) and collected in 1 mL fractions. RNase Z activity eluted with 0.6 M KCl. This fraction was concentrated and dialyzed. *tRNA affinity column*. Wheat tRNA (Sigma) was coupled to cyanogen bromide activated Sepharose 4B resin (Sigma) according to the manufacturer's protocol. The resulting 5 mL tRNA affinity column was equilibrated with buffer A and subsequently loaded with the fraction from the previous Blue column. A step gradient (0.2, 1.0, and 2.0 M KCl) eluted bound proteins, which were collected in 1 mL fractions, with RNase Z being in the 1.0 M KCl fraction. *Gel filtration*. A Superdex 200 PC gel filtration column (Pharmacia) was equilibrated with buffer A containing 150 mM KCl without Nonidet. Calibration was performed using low and high molecular weight gel filtration calibration kits (Pharmacia). An aliquot of the RNase Z active fraction from the tRNA affinity column was loaded onto the column. According to the size standards RNase Z has an apparent molecular mass of 122 kDa. *SDS/PAGE*. Aliquots from the RNase Z active Heparin, Blue, and tRNA affinity fractions were analyzed with SDS/PAGE as described (*36*).

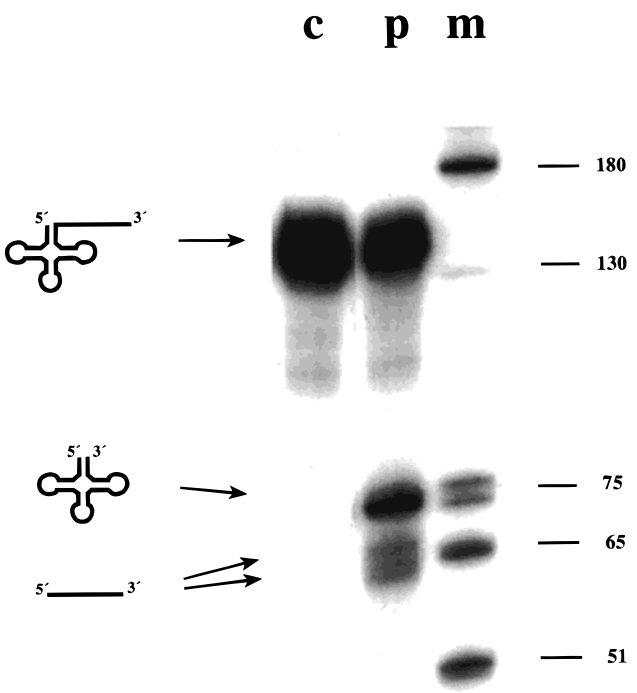

FIGURE 1: In vitro processing of pretRNA^{Cys} with an RNase Z active fraction. The RNase Z active fraction from the Resource Q column was incubated with the pretRNA^{Cys} substrate as detailed in the Experimental Procedures. Lane c: incubation of pretRNA^{Cys} with reaction buffer as control reaction. Lane p: incubation of pretRNA^{Cys} with nuclear RNase Z. At the right a DNA size marker is shown (m), fragments given in nucleotides. Precursor (132 nt) and products (tRNA 72 nt and 3′ trailer 60 nt) are shown schematically at the left. The precursor is cleaved at two sites, yielding two trailer molecules (see also Figure 2).

Optimization of Processing Assays

The initial processing assay was performed as described (*9*). For determination of optimal reaction conditions the RNase Z active fraction (0.2 M KCl) from the Resource Q column (purification scheme A) was used. Each reaction contained 80 μg of protein in a reaction volume of 100 μL in different buffers, depending on the conditions analyzed. All experiments were repeated three times, and the resulting data were averaged. To analyze whether cations are required for the reaction, 10 mM EDTA was added to the protein extract prior to the reaction.

The following buffers were used for pH determination: MES for pH 5.5−6.5, Pipes for pH 7, and Tris for pH 7.5−8.9. In vitro processing reactions were performed as described (*9*). Processing products were quantitated by measuring signal intensities of an exposed X-ray film with a Molecular Dynamics densitometer. After the determination of optimal reaction conditions all processing reactions were carried out with 80 μg of protein (0.2 M KCl Source Q fraction) in 100 μL of buffer ivp (40 mM Tris, pH 8.4, 2 mM DTT) for 25 min at 37 °C if not stated otherwise.

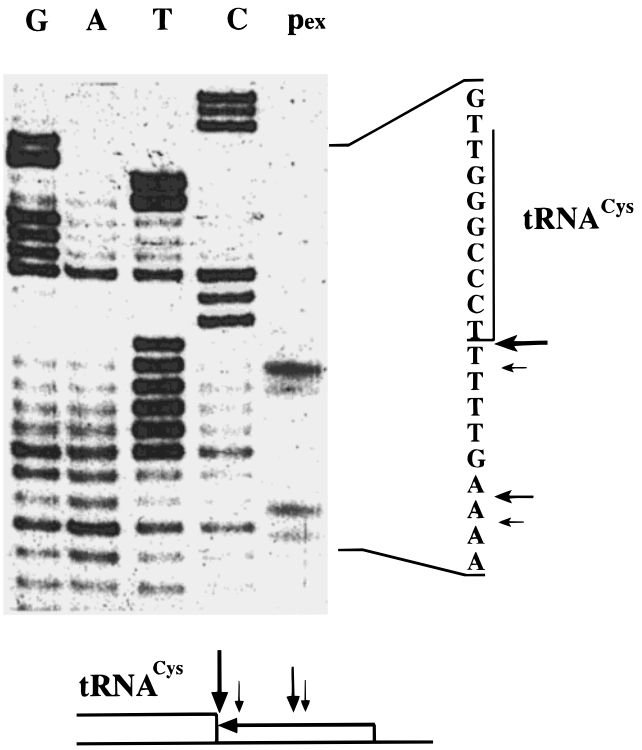

FIGURE 2: Cleavage site determination by primer extension. Sequencing reactions (lanes G, A, T, and C) and primer extension (lane p_{ex}) were started from primer Cys3. The coding sequence of the pCys template is shown at the right. The prominent cleavage site is indicated with a bold arrow; minor cleavage sites are indicated with light arrows. The main processing site lies immediately 3′ to the discriminator. Minor cleavage sites are located one, six, and seven nucleotides downstream of the discriminator.

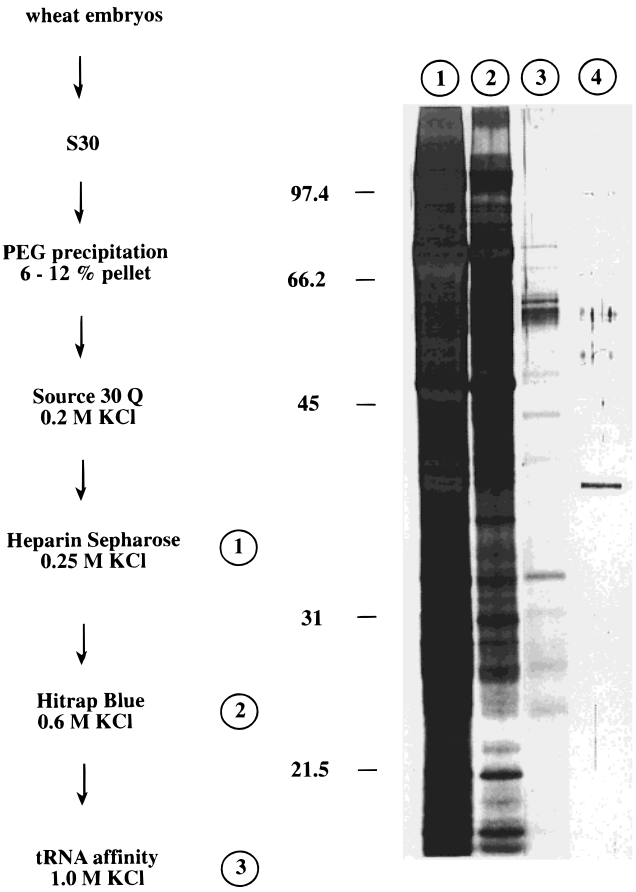

FIGURE 3: SDS/PAGE of RNase Z active fractions. Aliquots of RNase Z active fractions were separated with SDS/PAGE to monitor the progress of purification. Molecules were visualized by silver staining. Lanes 1−3 show aliquots from the nuclear RNase Z active fractions of wheat. Lane 1, 57 μg of the 0.25 M Heparin fraction; lane 2, 10 μg of the 0.6 M Blue fraction; lane 3, 300 ng of the 1 M tRNA affinity fraction. Lane 4 shows the most purified fraction of the mitochondrial RNase Z from potato (approximately 50 ng). At the left a protein size marker is indicated in kilodaltons.

Analysis of RNase Z Composition

To investigate whether the RNase Z activity contains RNA and/or protein subunits, preincubations with proteinase K and RNase A, respectively, were performed as described (*9*). For this analysis the 0.2 M KCl fraction eluted from the Resource Q column was used. To prevent potential inhibition of tRNA processing by RNA masking (*32*), different amounts of RNA ((a) 10 μg of 5S rRNA, (b) 20 μg of 5S rRNA, and (c) 100 μg of polyA) were added to the reaction mix after preincubation and before the addition of precursor tRNA.

Characterization of Processing Products

To analyze the nature of the 5′ and 3′ terminal groups of the processing products, experiments were performed as described (*9*).

Determination of Cleavage Site

The exact cleavage site was determined as described (*9*).

Purification of the Mitochondrial RNase Z from Potato

Mitochondrial RNase Z from potato was purified as described (*9*). The following additional purification steps were used to further purify the enzyme. Heat-treated fractions were loaded onto a Mono Q column and subsequently purified through a Heparin and a Hi-Trap Blue column. RNase Z active fractions from the Blue column were further fractionated on a gel filtration column and a Mini S column. Aliquots from the Mini S column were analyzed on an SDS gel (Figure 3, lane 4). Protein concentrations of the most purified fractions were estimated from silver-stained SDS gels.

RESULTS

Characterization

The tRNA 3′ Processing Activity from Wheat Is an Endonuclease. The precursor $tRNA^{Cys}$ used for characterization of the nuclear RNase Z contains a rather long 3′ trailer (60 nt). The length of this trailer prevents degradation by unspecific exonucleases and thus allows endonucleolytic and exonucleolytic 3′ processing to be distinguished. The 5′ end of the precursor is the mature tRNA 5′ end to take into account that some of the 3′ tRNA endonucleases have been reported to process only 5′ matured tRNA precursors. Incubation of this substrate with a wheat cytoplasmic fraction yielded three products, an RNA of about 72 nt and two fragments of about 60 nt (Figure 1). The short RNA molecules correspond in length to the 3′ trailer, and the longer fragment is as long as the tRNA. Thus, mature tRNA 3′ ends are generated by an endonuclease which cleaves 3′ to the discriminator base or close to it. We termed this activity nuclear RNase Z.

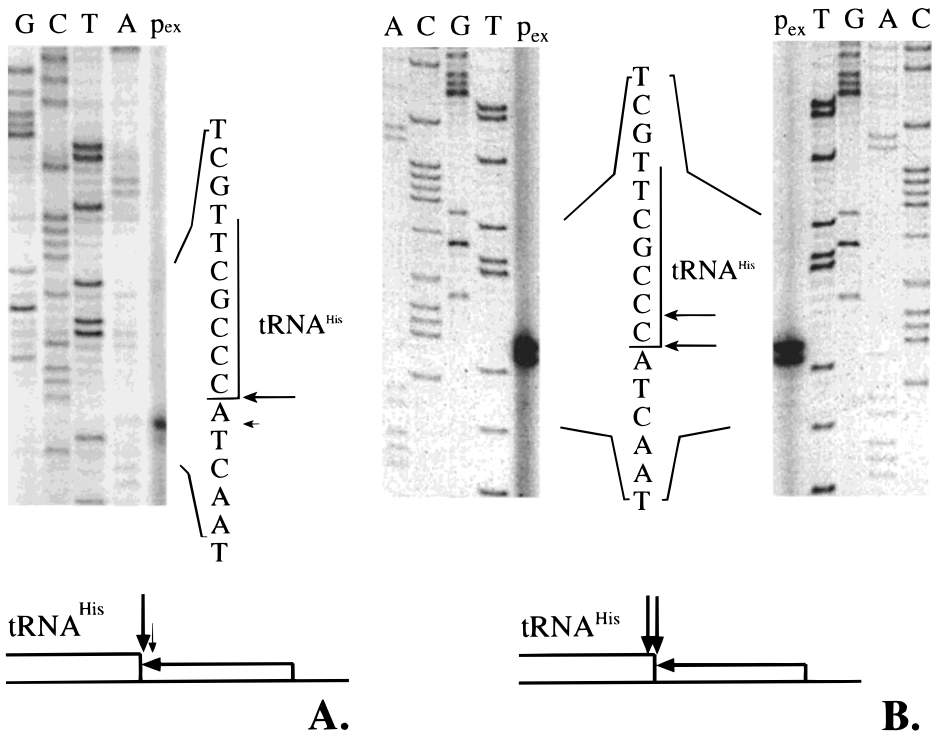

FIGURE 4: Primer extension analyses of processing products of mt pretRNA^{His}. (A) Total mt RNA from *O. berteriana* was used in a primer extension experiment. The coding strand sequence is shown at the right. DNA sequencing and primer extension were both started with primer AH2 within the 3′ trailer. One major cDNA is visible which corresponds to a cleavage directly 3′ to the discriminator base. (B) In vitro processing reactions with mitochondrial (left panel) and nuclear RNase Z (right panel) were performed. The resulting processing products were subjected to a primer extension analysis with primer AH2. The coding strand sequence is shown in the middle. DNA sequencing and primer extension reactions were both started with primer AH2. Two major reaction products are visible corresponding to cleavage 3′ to the discriminator and one nucleotide upstream.

Optimal Reaction Conditions for RNase Z. The influence of varying salt concentrations, pH, and temperature on the 3′ processing reaction was investigated. The cleavage reaction is most efficient at 35 °C and pH 8.4, and addition of Mg^{2+} is not necessary, but if 10 mM EDTA is added to the reaction mix, processing is totally inhibited. If Mg^{2+} is replaced by Ca^{2+}, a concentration of 2 mM achieves optimal processing. Similar observations were made using monovalent cations: addition of K^+ is not necessary, but if K^+ is replaced by NH_4^+, 2 mM yields maximum activity. Higher concentrations of divalent cations (Mg^{2+}, more than 50 mM; Ca^{2+}, more than 20 mM) and monovalent cations (K^+, 150 mM; NH_4^+, 100 mM) inhibit the cleavage reaction.

Enzyme Composition. To investigate whether the wheat 3′ processing activity consists of RNA and protein subunits, we preincubated the protein fractions with either RNase A or proteinase K. Preincubation with proteinase K and RNase A totally abolished the processing activity (data not shown). To prevent substrate masking by RNase A (*32*) and thus cleavage inhibition, additional RNA was added to the reaction mixture. Under these conditions, activity could be restored, suggesting that the nuclear RNase Z is an all-protein enzyme.

Determination of Cleavage Site. To determine the exact cleavage site of the RNase Z enzyme, primer extension analysis was carried out with the 3′ trailer of the nuclear pretRNA^{Cys} generated by incubation with the nuclear RNase Z. The primer extension experiment yielded one major product, which corresponds to cleavage 3′ to the discriminator nucleotide. One minor product was generated, which corresponds to cleavage one nucleotide downstream of the discriminator (Figure 2). Two additional minor cleavage sites lie six and seven nucleotides, respectively, downstream of the major cleavage site.

Characterization of 3′ Processing Products. For the addition of the CCA triplet a 3′ hydroxyl group is required at the tRNA 3′ end. To analyze whether the endonuclease characterized here leaves a 3′ hydroxyl group at the tRNA terminus and therefore allows immediate addition of the CCA sequence, we analyzed the nature of the terminal groups of the processing products. For analysis of the 5′ end of the 3′ trailer precursor, tRNA^{Tyr} was transcribed with [α-^{32}P]CTP to label the α-phosphate of the first nucleotide of the trailer (sequence of the 3′ trailer: 5′ CTTGTTT 3′). This substrate was incubated with the wheat germ fraction, and processing products were separated with PAGE. After elution from the gel the trailer was digested with RNase T2 and RNase A to yield 3′-monophosphate nucleosides (Np). Products of this digestion were separated on two-dimensional thin-layer chromatography (data not shown). Four prominent spots are detectable on the autoradiograph of the 2D-TLC, corresponding in their location to the four expected 3′-monophosphate nucleosides (*33*). A weak fifth spot is also detectable, which corresponds to pCp (*33*). Thus, the nuclear RNase Z leaves a 5′ phosphoryl group at the trailer molecule. The nature of the 3′ group of the tRNA was examined by incubating an unlabeled tRNA precursor with the wheat RNase Z and eluting the resulting tRNA product from a denaturing gel. The tRNA molecules were subsequently incubated with [α-^{32}P]pCp and T4 RNA ligase. Separation of ligation products with PAGE shows a single product of tRNA size (data not shown). Successful labeling of the tRNA 3′ end

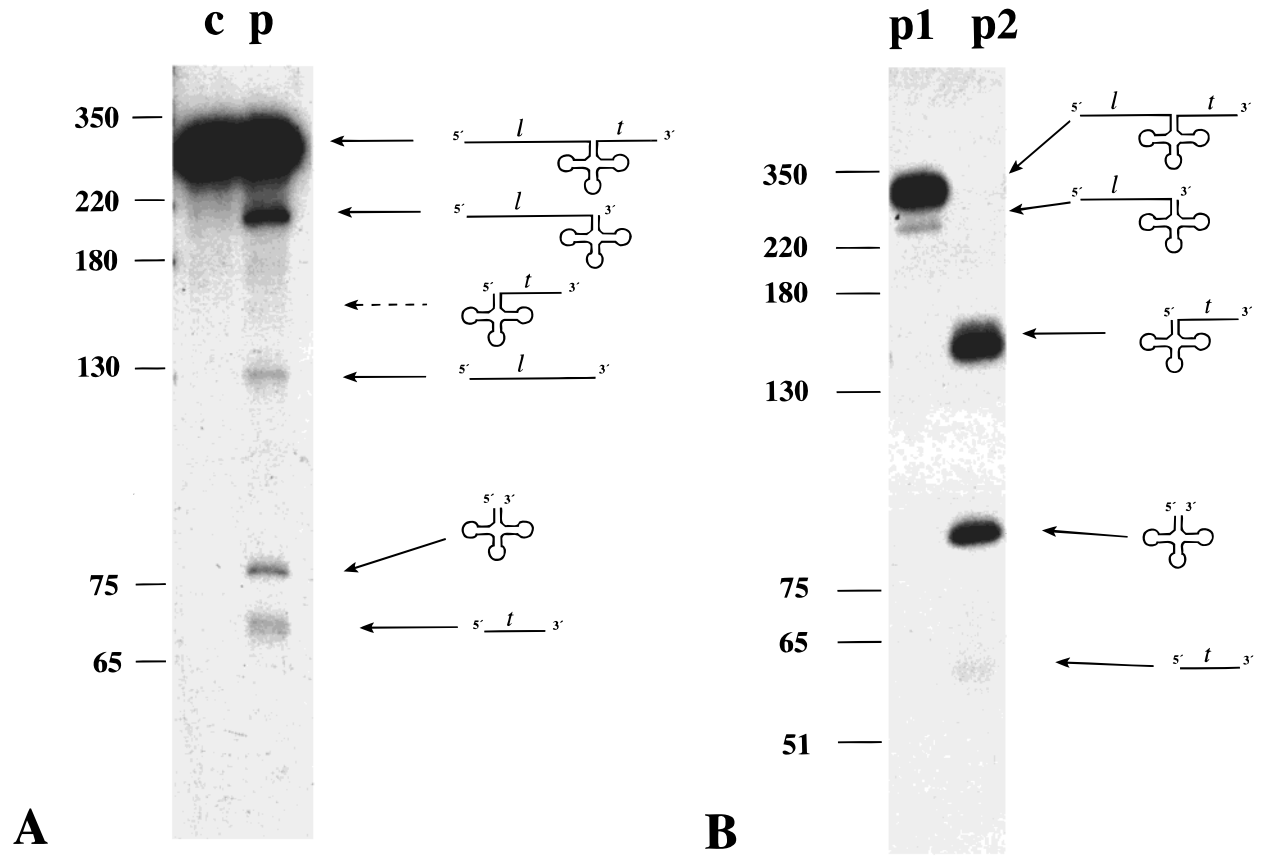

FIGURE 5: Processing of 5′ extended pretRNAs by RNase Z. Precursor tRNAs containing 5′ extensions were incubated with an RNase Z active fraction with (A) and without (B) RNase P activity. (A) Processing of pretRNA^{Phe}. Lane p: incubation with a wheat germ extract. Lane c: control reaction without protein. A DNA size marker is indicated in nts at the left, and expected sizes of the various products are depicted at the right (*l*, 5′ leader; *t*, 3′ trailer). Both products of the 3′ only processing reaction are clearly visible. Just one product of the 5′ only reaction is visible (5′ leader), while the other (tRNA plus 3′ trailer) is not detectable. It is probably immediately processed to yield the mature tRNA and the 3′ trailer. (B) Processing of pretRNA^{Tyr}. Lane p1: incubation of 5′ extended pretRNA^{Tyr} with wheat germ extract. The precursor is processed very inefficiently, and only one of the 3′ only processing products (5′ leader and tRNA) is visible. Lane p2: incubation of 5′ matured pretRNA^{Tyr} with wheat germ extract. The precursor is processed effiently, yielding tRNA and 3′ trailer. A DNA size marker in nts is given at the left, and expected sizes of the various products are depicted at the right (*l*, 5′ leader; *t*, 3′ trailer).

indicates an accessible 3′ hydroxyl group essential for this ligation. RNase Z thus cleaves the phosphodiester bond such that a 3′ hydroxyl group is left at the tRNA terminus and a 5′ phosphoryl group at the trailer molecule.

Partial Purification

For a detailed analysis of the plant nuclear RNase Z, we initiated purification of this enzyme. An S30 fraction from wheat embryos was fractionated by PEG precipitation. The 6−12% PEG cut containing the RNase Z activity was loaded onto a Source Q column, and 3′ processing activity was eluted with 0.2 M KCl. This fraction was further purified on a Heparin column, RNase Z activity eluting with 0.25 M KCl. RNase Z was bound to a subsequent Blue column and eluted with 0.6 M KCl. A final purification step consisted of a tRNA affinity column, leaving only very few proteins in the RNase Z active fraction (Figure 3). To isolate the nuclear RNase Z, we thus expect only a few additional purification steps. Gel filtration chromatography sizes the nuclear RNase Z to an apparent molecular mass 122 kDa.

Substrate Specificity

Processing of a Mitochondrial Precursor. Incubation of the mitochondrial pretRNA^{Tyr} from *O. berteriana* with the nuclear RNase Z yielded two processing products: the mature tRNA and the 3′ trailer (data not shown). Thus, mitochondrial tRNAs are also accepted as substrates for the nuclear RNase Z.

Processing of Mitochondrial pretRNA^{His}. The tRNA acceptor stem usually consists of seven base pairs, exceptions being histidine- and selenocystein-tRNAs with acceptor stems containing eight base pairs. The generation of the longer acceptor stem in the nucleus is different from the corresponding process in organelles and bacteria. In nuclei RNase P processes the histidine precursor at the 5′ end, yielding an acceptor stem with 7 bp, and the additional 5′ nucleotide is subsequently added by guanylyltransferase. The organelle and bacterial RNase P enzymes process the precursor at the 5′ end to generate an 8 bp acceptor stem, thus requiring no additional activity to generate the mature 5′ end. To investigate how the nuclear RNase Z acts upon a mitochondrial 5′ matured precursor with an 8 bp acceptor stem, we incubated the 5′ matured mitochondrial pretRNA^{His} with the wheat nuclear RNase Z. The mitochondrial precursor was processed efficiently, yielding the tRNA and the 3′ trailer (data not shown). The exact cleavage site was determined with primer extension analysis of the trailer molecule (Figure 4B). Of the two major cleavage sites found one generates an acceptor stem with 7 bp and the other a stem with 8 bp.

To compare cleavage site selection of the nuclear RNase Z with that of the mitochondrial RNase Z, we also incubated pretRNA^{His} with the plant mitochondrial RNase Z. The precursor is efficiently processed, and primer extension experiments show that the mitochondrial RNase Z cleaves at exactly the same sites as the nuclear one, generating 7 bp and 8 bp acceptor stems (Figure 4B). To analyze the in vivo situation, we performed a primer extension with mitochondrial RNA. In vivo there is only one major cleavage site which generates an 8 bp acceptor stem (Figure 4A).

5′ Extended Precursors Are Poor Substrates for the Nuclear RNase Z. Since several 3′ processing activities have been described to act only on 5′ matured precursors, we were interested in whether the wheat RNase Z would also process 5′ extended precursors. Incubation of 5′ extended pretRNA^{Phe} with a wheat fraction containing RNase P and RNase Z yielded processing intermediates from the RNase P reaction and from the RNase Z reaction, respectively (Figure 5A), although the reaction efficiency of the 3′ reaction was significantly reduced. Incubation of the same precursor with a fraction containing only RNase Z yielded no processing products (data not shown). Upon incubation of a 5′ extended pretRNA^{Tyr} with a fraction containing only RNase Z, just weak processing could be detected (Figure 5B). A 5′ extended pretRNA^{His} was not processed at all by the nuclear RNase Z (data not shown). All precursors used in these experiments had rather long 5′ extensions (tRNA^{Phe} 117 nt, tRNA^{His} 96 nt, and tRNA^{Tyr} 43 nt) which could be an obstacle for RNase Z processing and could slow the reaction to inhibition.

Processing of a tRNA Precursor Containing a Mismatch in the Acceptor Stem. RNA editing has been shown to have an effect on the processing efficiency of some tRNA precursors in plant mitochondria (*26*, *27*). The unedited form of mitochondrial tRNA^{Phe} from *O. berteriana* contains a mismatch in the acceptor stem (^{4}C*A^{69}), which is restored to a normal Watson−Crick base pair (^{4}U−A^{69}) by RNA editing. We were interested to see whether the mismatch in the acceptor stem of the unedited precursor tRNA^{Phe} has an influence on processing by the nuclear RNase Z. Upon incubation with the wheat RNase Z, only the edited precursor was processed efficiently by the enzyme (Figure 6).

Processing of Intron-Containing Precursor tRNAs. Some of the nuclear pretRNAs contain introns which have to be removed to generate mature tRNAs. To assess whether the endonuclease RNase Z is able to process intron-containing precursors, we incubated the pretRNA^{Tyr} from *N. rustica* with RNase Z, which removes the 3′ trailer efficiently. In addition, this wheat fraction contains the splicing endonuclease which removes the intron from the 3′ matured tRNA (Figure 7). Processing of the same precursor lacking the intron was as efficient as processing of the intron-containing precursor (data not shown).

Processing of Precursors Containing Mutated tRNAs. Precursors of the *N. rustica* tRNA^{Tyr} lacking parts of the anticodon or with point mutations in one of the arms (Figure 8) were incubated with RNase Z. The precursor lacking almost the entire anticodon arm and part of the variable arm was not processed at all, while the precursor missing only the anticodon loop and the last base pair of the stem was processed efficiently. A precursor with only one nucleotide deleted from the anticodon loop was also cleaved efficiently (data not shown).

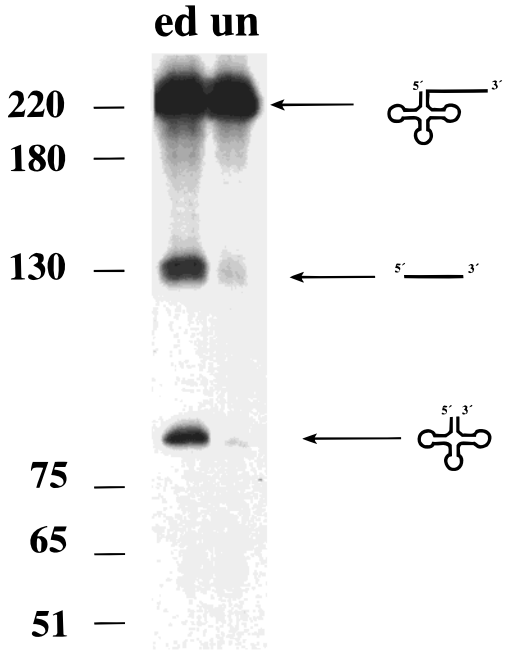

FIGURE 6: A precursor containing a mismatch in the acceptor stem is not processed in vitro. Unedited (lane un) and edited (lane ed) pretRNA^{Phe} were incubated with an RNase Z active fraction. The unedited form of tRNA^{Phe} contains a mismatched ^{4}C*A^{69} base pair in the acceptor stem, while the edited precursor contains a regular Watson−Crick ^{4}U−A^{69} base pair at this position. Sizes of DNA fragments are indicated in nts at the left; precursor and products are shown schematically at the right. While the edited precursor (with an intact acceptor stem) is processed efficiently, the unedited substrate (containing a mismatch in the acceptor stem) is barely processed.

DISCUSSION

For the nuclear RNase Z, tRNA modification and short 3′ trailer sequences do not seem to be necessary for 3′ processing, since the tRNA precursors efficiently cleaved by this enzyme are routinely generated with T7 polymerase and tRNA nucleotides are not modified in these precursor molecules. Furthermore, all precursors tested contained rather long 3′ ends (>50 nt), the in vivo nuclear tRNA 3′ trailers generally being only 3−10 nt long.

Biochemical Characterization

The enzyme preparation used seems to contain enough cations to allow the cleavage reaction to proceed, since additional divalent or monovalent cations are not required. However, cations are essential for the reaction since cleavage is completely inhibited by addition of 10 mM EDTA. Other 3′ processing endonucleases have also been reported to require low $MgCl_2$ concentrations for activity (*9*, *20*, *24*). The 3′ processing activity is rather stable, cleaving efficiently over broad pH and temperature ranges, similar to the *Xenopus* and plant mitochondrial enzymes (*9*, *24*). The apparent molecular mass of the plant nuclear RNase Z is measured to be 122 kDa, smaller than the respective nuclear tRNA 3′ endonucleases from *Aspergillus nidulans*, which runs at a size of 160 kDa (*23*), and potato mitochondria (165 kDa) (34) but larger than the 3′ endonuclease 45/60 from *S. cervisiae*, which appears with a molecular mass of 90 kDa (*20*). Thus, the apparent size of the nuclear tRNA 3′

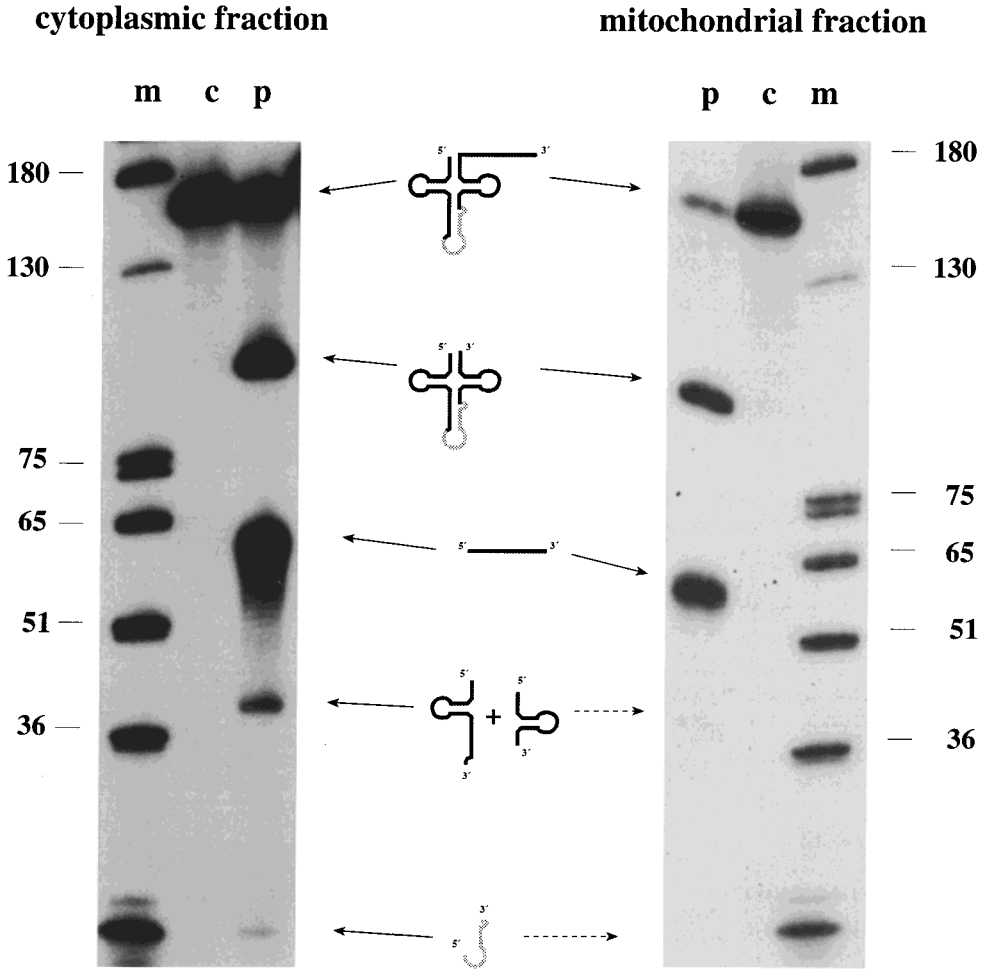

FIGURE 7: In vitro processing of an intron containing pretRNA. PretRNA^{Tyr} from *N. rustica* was processed with RNase Z active fractions from potato mitochondria and wheat (nuclear activity). Left panel: processing with wheat nuclear RNase Z. Right panel: processing with potato mitochondrial RNase Z. Lane p: incubation of pretRNA^{Tyr} with the respective protein extract. Lane c: incubation of pretRNA^{Tyr} with reaction buffer as control. The pretRNA contains a 58 nt long 3′ tailer, tRNA^{Tyr} (73 nt), and an intron (13 nt). Both activities process the precursor efficiently. The wheat extract also removes the intron from the tRNA. A DNA size marker is shown on the left and right, and precursor and products are shown schematically in the middle.

processing endonuclease seems to vary significantly between species. Analysis of the processing products revealed that RNase Z cleaves the precursor, leaving a 5′ phosphoryl group at the trailer and a 3′ hydroxyl group at the tRNA, similar to both the yeast and plant mitochondrial enzymes (*6*, *9*, *10*). The phosphodiester bond is thus cleaved by RNase Z as by other endonucleases such as RNase P, but different from cleavage by, e.g., RNase A.

Enzyme Composition

3′ processing is abolished by treatment with proteinase, suggesting that the 3′ processing activity contains one or more protein subunit(s). Treatment with RNase A also inhibits cleavage, which however can be restored by addition of carrier RNA to the extract. Analysis of RNase P activities from archaea and eukarya has shown that the identification of essential RNA subunits by RNase digests or CsCl centrifugations is not straightforward (*28*, *29*). Only purification of the enzyme to homogeneity will definitively clarify whether this activity also contains RNA subunits. However, the fact that other RNase Z activities purified to date consist of protein subunits only (*9*, *20*, *23*, *24*) supports our results indicating that the nuclear RNase Z in wheat is as well an all protein enzyme.

Cleavage Site Selection

Nuclear pretRNA^{Cys} is cleaved immediately 3′ to the discriminator by wheat RNase Z, allowing the tRNA nucleotidyl transferase to add the CCA triplet directly. This observation is in accordance with the results obtained for tRNA 3′ processing in *Xenopus*, wheat mitochondria, and potato mitochondria (*9*, *10*, *24*). Minor cleavage sites are also detected, two being located six and seven nucleotides downstream from the discriminator. These additional cleavage sites were only observed with the tRNA^{Cys} precursor and thus might be due to secondary structure in the pretRNA^{Cys} 3′ trailer. Other pretRNAs tested are cleaved only next to the discriminator (data not shown).

Substrate Specificity

Cleavage of 5′ Extended Precursors. Oomen et al. observed that in a wheat embryo cytoplasmic extract only 5′ extended precursors were processed, while 5′ matured precursor tRNAs were not cleaved (*35*). We observed with the wheat nuclear RNase Z that 5′ extended pretRNA^{His} is not processed, a 5′ extended pretRNA^{Phe} is only processed at the 3′ end in the presence of RNase P, and a 5′ extended pretRNA^{Tyr} is weakly processed. Thus, a 5′ extension seems to slow the 3′ processing reaction sometimes even all the

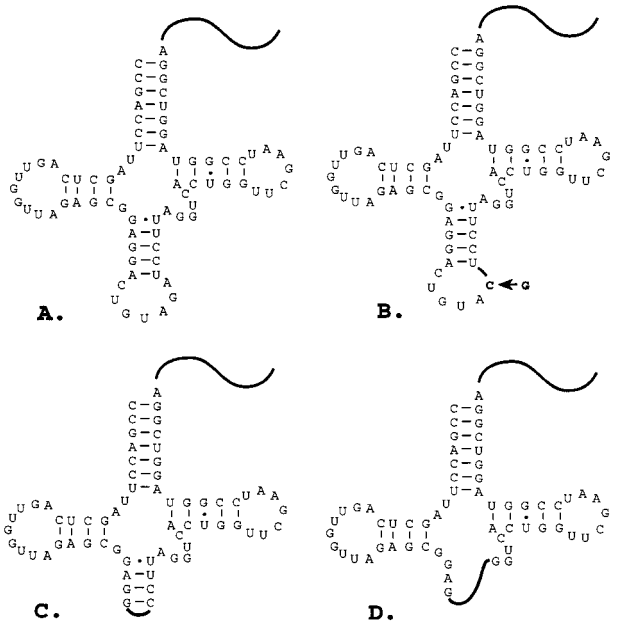

FIGURE 8: Structures of pretRNA^{Tyr} mutants. The secondary structure of pretRNA^{Tyr} mutants used is shown. (A) Wild-type pretRNA^{Tyr}. (B) This mutant contains a point mutation in the anticodon arm (G → C) and one deletion (marked by a line). (C) The whole anticodon loop and one base pair of the stem are deleted. (D) In mutant D almost the entire anticodon arm and part of the variable arm are deleted.

way to inhibition. Nashimoto et al. made similar observations with the mammalian tRNA 3′ processing enzyme (*37*). Other nuclear 3′ processing activities have been reported to prefer or process exclusively 5′ matured precursors (*9*, *23*, *24*).

Processing of a Mitochondrial pretRNA and an Intron-Containing pretRNA. The 5′ matured mitochondrial pretRNA^{Tyr} is as efficiently processed by the nuclear enzyme as a 5′ matured pretRNA^{Tyr} from *N. rustica* containing a 13 nt long intron. Thus, at least in vitro tRNA 3′ end processing in plant nuclei can occur prior to intron removal.

Processing of pretRNA^{His}. The 5′ matured mitochondrial tRNA^{His} precursor was processed efficiently by the nuclear 3′ processing enzyme at two major cleavage sites, one giving rise to a 7 bp acceptor stem and the other to an 8 bp acceptor stem. Identical cleavage sites were observed when the 5′ matured pretRNA^{His} was incubated with the plant mitochondrial RNase Z. Investigation of the in vivo cleavage sites revealed that in mitochondria there is a major cleavage site generating a tRNA with an 8 bp acceptor stem and a minor cleavage site located one nucleotide further downstream. Thus in vivo and in vitro tRNA 3′ processing enzymes generate the functional tRNA^{His} with an 8 base pair acceptor stem. The additional cleavage site found in vitro might be due to the in vitro situation (e.g., missing nucleotide modifications in the T7 transcripts). Thus, the nuclear RNase Z processes the tRNA^{His} precursor such that no additional steps are required at the 3′ end to generate the 8 base pair acceptor stem.

Influence of a Mismatched Acceptor Stem on 3′ Processing by Nuclear RNase Z. Incubation of a tRNA precursor containing a mismatch in the acceptor stem with the wheat nuclear enzyme revealed that the nuclear 3′ processing enzyme prefers pretRNAs with a perfectly paired acceptor stem. This result is in agreement with observations made upon incubation of mutated pretRNAs with *Drosophila* tRNA processing activities. The 3′ processing activity from *Drosophila* does not tolerate mismatches in the acceptor, D, or T stem and requires an intact tRNA structure (*30*).

Table 1: Comparison of the Properties of the Nuclear and Mitochondrial RNase Z Activities with Respect to Several Biochemical Characteristics

characteristic	mitochondrial	nuclear
reaction optima		
pH	8.0	8.4
T °C	30	35
[$MgCl_2$] (mM)	5	0
[KCl] (mM)	30	0
substrates		
precursor with intact tRNA structure	cleaved	cleaved
5′ extended pretRNAs	not cleaved	cleaved poorly
size kDa		
SDS	43	nd
gel filtration	165	122

Processing of Precursors Containing Mutated tRNAs. While mutants lacking parts of the anticodon arm were processed efficiently by the plant RNase Z, only the almost total deletion of the tRNA anticodon arm abolished processing. Studies with the *Drosophila* RNase Z showed that point mutations in the anticodon arm had no effect on processing. Therefore, in contrast to the requirement for a perfectly paired acceptor stem (as seen by the failure of processing unedited tRNA^{Phe}), a completely intact anticodon arm does not seem to be crucial for cleavage by nuclear RNase Z.

Plant Mitochondrial and Nuclear RNase Z Have Different Properties. Plant cells contain three compartments, each having their own set of tRNA processing activities. According to the endosymbiont theory organellar processing enzymes are of bacterial origin and thus differ from those in the nucleus, but it is also possible that the genes encoding the bacterial processing enzymes have been lost and that present day tRNA processing enzymes are adapted host nuclear processing activities. It might even be that organellar activities are derived from bacteriophage enzymes, as has been shown for the organellar RNA polymerases (*38*). Studies with the mitochondrial RNase Z in plants showed that tRNA 3′ maturation in these organelles differs significantly from bacterial 3′ maturation, suggesting that plant mitochondria have lost their original (bacteria-like) processing system (*9*, *10*, *34*). Comparison of the plant mitochondrial and plant nuclear RNase Z should reveal whether mitochondrial and nuclear 3′ processing activities are similar or even identical and encoded by the same gene. Since both enzymes catalyze the same reaction, they of course have similar features. They are both endonucleases consisting of proteins, and they cleave tRNA precursors at the discriminator, leaving 5′ phosphoryl and 3′ hydroxyl groups at the respective processing products (Table 1). Both are rather stable enzymes, active over broad pH and temperature ranges, but with different optimal salt requirements. A clear difference is the exclusive preference of the mt RNase Z for 5′ matured precursors, while the nuclear enzyme accepts 5′ extended precursors as substrates. This observation suggests that mitochondrial and nuclear tRNA 3′ processing activities are different. Nevertheless, it might be possible that the abovementioned differences are due to different posttranslational

modifications or potential cofactors. A further significant distinction between the two enzymes is the size difference observed in gel filtration experiments and denaturing gels. In the most purified nuclear RNase Z fraction (Figure 3, lane 3) no protein with the size of the mitochondrial RNase Z (43 kDa, Figure 3, lane 4) (*34*) is detectable. Gel filtration experiments showed that the mitochondrial RNase Z with an apparent molecular mass of 165 kDa (*34*) is bigger than the nuclear RNase Z with a size of 122 kDa. These observations suggest that nuclear and mitochondrial 3′ processing activities are different enzymes encoded by different nuclear genes, although it cannot be excluded that the differing sizes of the two enzymes might be due to the fact that they were isolated from wheat and potato, two different plant species.

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