The Plant tRNA 3′ Processing Enzyme Has a Broad Substrate Spectrum†

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ABSTRACT: To elucidate the minimal substrate for the plant nuclear tRNA 3′ processing enzyme, we synthesized a set of tRNA variants, which were subsequently incubated with the nuclear tRNA 3′ processing enzyme. Our experiments show that the minimal substrate for the nuclear RNase Z consists of the acceptor stem and T arm. The broad substrate spectrum of the nuclear RNase Z raises the possibility that this enzyme might have additional functions in the nucleus besides tRNA 3′ processing. Incubation of tRNA variants with the plant mitochondrial enzyme revealed that the organellar counterpart of the nuclear enzyme has a much narrower substrate spectrum. The mitochondrial RNase Z only tolerates deletion of anticodon and variable arms and only with a drastic reduction in cleavage efficiency, indicating that the mitochondrial activity can only cleave bona fide tRNA substrates efficiently. Both enzymes prefer precursors containing short 3′ trailers over extended 3′ additional sequences. Determination of cleavage sites showed that the cleavage site is not shifted in any of the tRNA variant precursors.

Generation of functional tRNA molecules from precursor RNAs includes removal of 5′ and 3′ additional sequences in all organisms. Whereas the maturation of the tRNA 5′ end by RNase P has been studied in great detail (for reviews, see refs *1* and *2*), less is known about the activities involved in tRNA 3′ processing. In *Escherichia coli*, several enzymes are involved in the maturation of the tRNA 3′ end (*3*). An endonuclease cleaves the precursor downstream of the tRNA, and exonucleases remove the residual nucleotides up to the CCA triplet which is encoded in almost all bacterial tRNA genes (*4*). Using mutant strains of *E. coli*, it has been shown that several exonucleases are capable of removing the 3′ trailer, suggesting that there is no "tRNA-specific exonuclease" in *E. coli* (*3*). Nothing is known about tRNA 3′ end generation in archaea, beyond the observation that the 5′ end is processed before 3′ end maturation takes place (*5*). It is not clear whether endo- and/or exonucleases are involved in this process in archaea. The general mechanism for yielding the tRNA 3′ end in eukarya seems to be an endonucleolytic cut 3′ to the discriminator (*6*−*10*), although some exonucleolytic processing systems have also been reported (*11*−*14*). Purification of tRNA 3′ processing activities from *Saccharomyces cerevisiae* resulted in the isolation of three exo- and two endonucleases (*13*). Yoo and Wolin made the observation that in *S. cerevisiae* tRNA precursors are cleaved by an endonuclease in the presence of the Lhp1p protein, while in the absence of Lhp1p they are processed by exonucleases (*15*). Thus, in vivo the exonucleolytic pathway may act as backup system for the endonucleolytic processing step. Organellar tRNA precursors are generated by endonucleases which cleave next to the discriminator (*16*−*21*). In this respect, tRNA 3′ processing in these endosymbionts is similar to nuclear tRNA 3′ processing and different from bacterial tRNA 3′ end maturation.

Substrate specificities for the tRNA 5′ processing enzyme, RNase P, have been investigated intensively. The bacterial RNase P has a very broad substrate spectrum, the minimal substrate being a minihelix consisting of solely T and acceptor stems and the 3′ terminal CCA (*22*). In contrast, eukaryotic and organellar RNase P enzymes have a much narrower substrate specificity (*23*−*25*). Substrate specificities of tRNA 3′ processing enzymes have so far only been described in detail for nuclear enzymes from *Drosophila* and mammalia. Both nuclear enzymes process a minimal substrate consisting of the T arm, the acceptor stem, and a short connecting loop as efficiently as the wild-type substrate (*25, 26*). While the mammalian 3′ processing enzyme prefers short 3′ trailers, the *Drosophila* activity is not influenced by 3′ trailer length (*27, 28*).

Here we report the substrate specificity analysis of the tRNA 3′ processing activity from plant nuclei.[1] In addition, we show that plant mitochondrial and nuclear RNase Z have different substrate specificities clearly distinguishing both enzymes. The mitochondrial RNase Z has a very narrow substrate spectrum, while the nuclear RNase Z also accepts several more degenerated substrates. Short 3′ trailers are preferred by both the mitochondrial and nuclear RNase Z.

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[1] We used a cytoplasmic extract from wheat embryos for our studies. Since it was shown previously that tRNA processing occurs in the nucleus and maybe in the nucleolus, we termed the activity nuclear RNase Z.

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In all of the variant substrates, the cleavage site is correctly recognized.

EXPERIMENTAL PROCEDURES

Materials. RNases T1, S1, and V1 were purchased from Amersham Pharmacia, all other enzymes were from Roche. Chemicals were from Fisher Scientific or Merck, and radioactivity was obtained from Amersham Pharmacia.

Synthesis of Mutated tRNA Genes. Variants of the $tRNA^{Tyr}$ were prepared as follows. Clone H12/20 containing wild-type $tRNA^{Tyr}$ from *Oenothera berteriana* mitochondria (*29*) was digested with *Nhe*I and *Pst*I, and the resulting 800 bp fragment was subcloned into pBluescript digested with *Xba*I and *Pst*I to yield the pBlue-Tyr clone. The tRNA gene and surrounding sequences were amplified from this clone with PCR[2] using primers universal[3] and AT1. PCR products were digested with *Pfu*I and *Eco*RI and cloned into pUC. The resulting pUC-Tyr clone contains the $tRNA^{Tyr}$ gene with flanking sequences (a 5′ leader with 110 bp and a 3′ trailer with 51 bp). For variant pUC-T1, inverse PCR[4] was performed with primers T13 and T14 from template pUC-Tyr. All other variants were made accordingly, but with the following primers: variant pUC-T2 with primers T2 and T7, variant pUC-T3 with primers T8 and T9, variant pUC-T4 with primers T10 and T11, variant pUC-T5 with primers T10 and T12, and variant pUC-T6 with primers T8a and T9a. PCR products were ligated and transformed into *E. coli*, and positive transformants were sequenced. All clones contain a 5′ leader (110 bp), the tRNA gene (wild-type or mutated), and a 3′ trailer (51 bp).

Template Preparation. Templates for in vitro transcription were obtained by PCR amplification of plasmids containing the gene for wild-type (pUC-Tyr) and variant tRNAs (pUC-T1−pUC-T6). Templates WT, T1−T3, and T6 were amplified with primer Y1 at the 5′ end; variant T4 was amplified with primer ATM-1 at the 5′ end, and variant T5 was amplified with primer ATM-2. Variant T6a was synthesized using primer OBTT6a at the 5′ end and clone pUC-TyrT3 as template DNA. For all the templates, primer OBTL was used as the 3′ end primer. Products from these PCRs yielded templates containing the tRNA and 51 bp long 3′ trailer sequences. For templates containing 84 bp long trailers, primer RS was used as the 3′ primer in the PCR, and for shorter 3′ trailer variants, primer OBTXX was used as the 3′ primer. For the generation of 3′ mature tRNAs (for structure probing experiments), the following primers were used at the 3′ end: primer OBT1MA for variant T1, primer OBT2MA for variant T2, and primer OBTMA for the wild type and variants T3−T6.

In Vitro Transcription. In vitro transcription reactions and purifications of the resulting transcripts were carried out as described previously (*30*). For structure probing experiments, transcripts were prepared as described previously (*31*) with the following modifications. One microgram of template DNA was used for the in vitro transcription reactions, and the reaction mixture was incubated for 4 h. Following phenol/chloroform extractions, the mixture was desalted on NAP-5 columns (Amersham Pharmacia). Transcripts were purified using 8% PAGE, and the desalting step after ethanol precipitation was omitted.

Structure Probing. Structure probing experiments were performed as described previously (*31*), with the following modifications. Transcripts were dephosphorylated before 5′ labeling using bacterial alkaline phosphatase (BAP). Eight micrograms of RNA in dephosphorylation buffer [10 mM Tris-HCl (pH 8.0)] was heated to 60 °C for 5 min and placed on ice, and 1 unit of BAP was added. After incubation at 37 °C for 15 min, the mixture was heated to 60 °C for an additional 15 min. The solution was extracted with a phenol/chloroform mixture and precipitated. Since we only analyzed 5′-labeled molecules, we cannot rule out the possibility that secondary site cleavages occurred. But since probing data for wild-type and variant RNAs are similar, we assume that secondary site cleavages did not take place.

Determination of Cleavage Efficiency. To determine the cleavage efficiencies, in vitro processing products of internally labeled precursors were separated on polyacrylamide gels. Gels were dried and analyzed using a Fuji BAS 1000 instrument (FujiFilm), and processing products were quantified using the software MacBAS (FujiFilm). All experiments were carried out three times, and the resulting data were averaged.

Isolation of Protein Extracts. Mitochondrial extract from *Solanum tuberosum* was made as described previously (*19*). For in vitro processing reactions, a mitochondrial protein fraction was used, which was purified through four fractionation steps. Fifty-eight micrograms of mitochondrial protein from the fourth purification step, a heat denaturation, was used for each in vitro processing reaction. Cytoplasmic extracts from *Triticum aestivum* were prepared as described previously (*32*), and the nuclear RNase Z was purified with four fractionation steps, the last one being a heparin column. For in vitro processing reactions, 21 μg of protein from the RNase Z active heparin column fractions was used.

In Vitro Processing Reactions. In vitro processing reactions of the mitochondrial and cytoplasmic extracts were performed as described previously (*19*, *32*); reaction mixtures were incubated for 30 min. Mitochondrial and cytoplasmic protein fractions used in in vitro processing experiments were purified through four fractionation steps (see above) representing protein fractions highly enriched in RNase Z.

RESULTS

Substrate Structure. To analyze the substrate specificity of the nuclear tRNA 3′ processing activity, we first synthesized DNA templates for several mutated tRNA precursors. Starting from wild-type mitochondrial $tRNA^{Tyr}$ from *O. berteriana*, we constructed tRNA precursor substrates lacking one or more of the tRNA arms (Figure 1). Wild-type mitochondrial $tRNA^{Tyr}$ is a class II tRNA, containing a rather long variable arm of 14 nucleotides. Class II tRNAs fold into the typical tRNA structure, and the variable arm lies in the plane of the L structure (*33*). According to the structure probing data, the mitochondrial $tRNA^{Tyr}$ higher-order structure strongly resembles the higher-order structure of classic cytosolic tRNAs (Figure 1). The wild-type precursor of this mitochondrial $tRNA^{Tyr}$ is cleaved efficiently by the nuclear

[2] Abbreviations: BAP, bacterial alkaline phosphatase; nt, nucleotide(s); PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; pretRNA, precursor tRNA.

[3] Primer sequences are available upon request.

[4] Conditions for PCRs are available upon request.

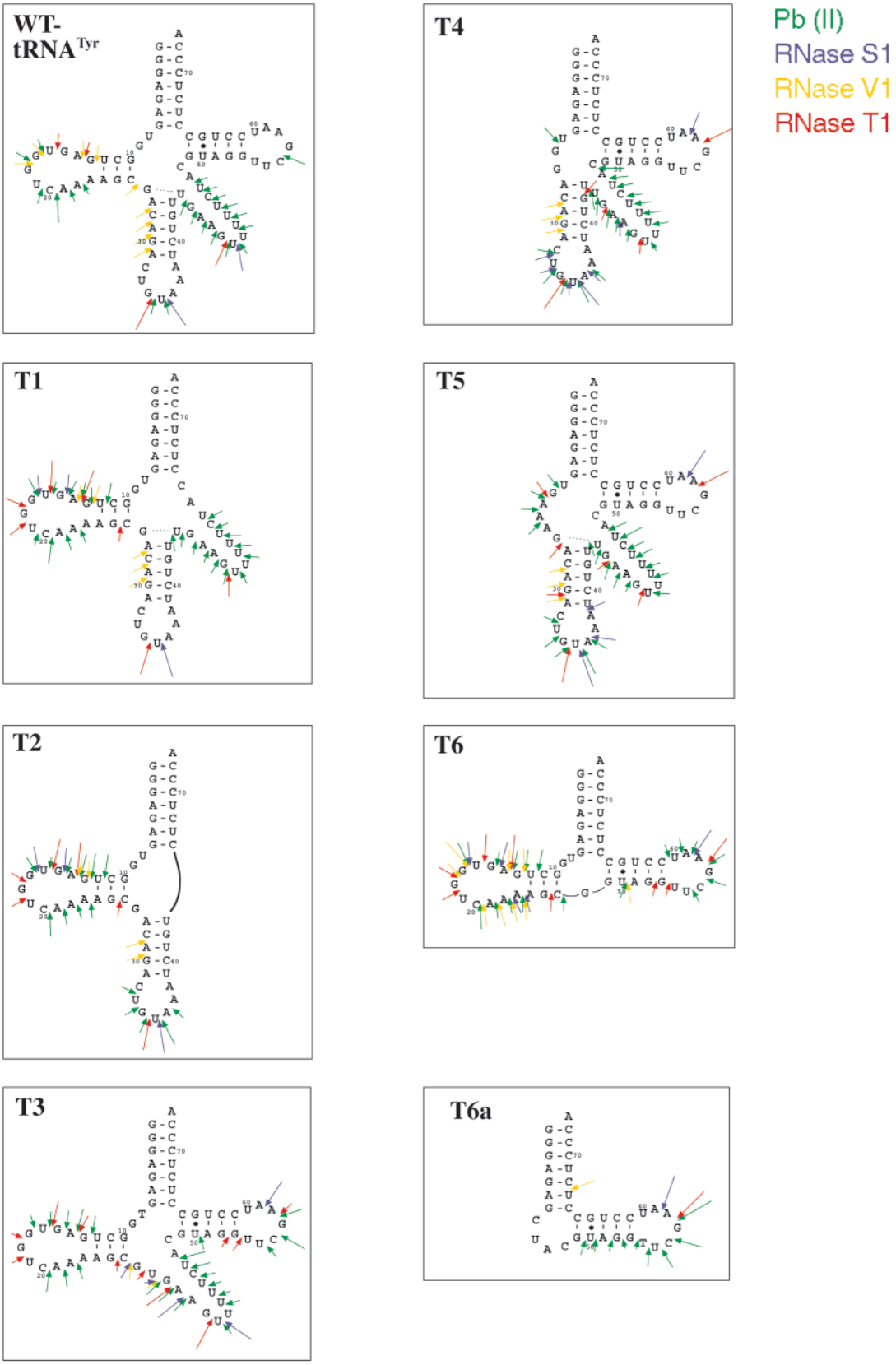

FIGURE 1: Structure probing of $tRNA^{Tyr}$ variants. Genes for wild-type and variant tRNAs were transcribed, 5′ labeled, and subjected to digestion with RNases T1, V1, and S1. In addition, Pb^{2+} cleavage was used to analyze the higher-order structure of the RNA molecules. Cleavage sites of the different probing molecules that were used are shown with colored arrows. Cleavage sites located in the acceptor stem are not indicated since they either could not be resolved by PAGE (3′ end of the acceptor stem) or were subject to secondary cleavages (5′ end of the acceptor stem). Variants T4 and T5 were cleaved with Pb^{2+} in the T loop, but the cleavage sites could not be identified unambiguously and are thus not indicated. Nucleotides are numbered according to the nomenclature of Sprinzl et al. (*45*).

tRNA 3′ processing activity (*32*), and is therefore a suitable substrate for elucidating the structure requirements of this enzyme. One or more of the tRNA arms were successively removed from wild-type $tRNA^{Tyr}$ to yield variants T1−T6a.

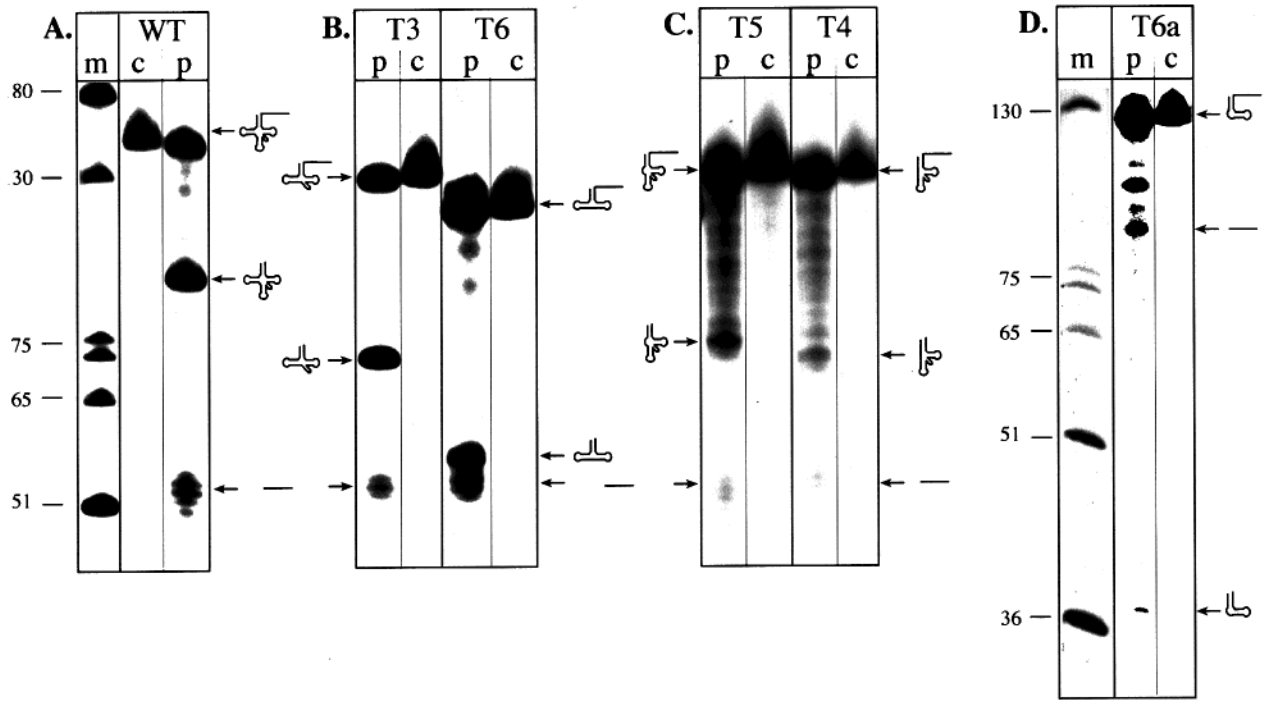

FIGURE 2: In vitro processing of wild-type and variant tRNAs with nuclear RNase Z. Nuclear RNase Z cleaves wild-type and variant precursors T3−T6a. Both processing products, tRNA (WT, 83 nt; T3, 66 nt; T6, 52 nt; T4, 66 nt; T5, 69 nt; T6a, 36 nt) and 3′ trailer (WT and T1−T6, 51 nt; T6a, 84 nt), are detectable. The 3′ trailer is comparatively short and not very stable. Substrates T4 and T5 are likewise rather unstable and are, probably because of the missing D arm, subject to degradation by unspecific nucleases. Additional proof that these substrates are processed correctly is shown in panels D and E of Figure 6. Lane m shows a DNA size standard given in nucleotides. Lanes p show the products of the in vitro processing reactions, and lanes c show control reactions which have been incubated without protein. Lanes WT show incubation of wild-type $tRNA^{Tyr}$ (A), lanes T3 incubation of variant T3, which has the anticodon removed, and lanes T6 incubation of variant T6, which has both the variable arm and anticodon arm deleted (B). In lanes T4 and T5, processing reactions with the respective variants are shown, T4 has the D arm removed, and in variant T5 the D arm has been replaced with a D arm replacement loop (C). In panel D, the in vitro processing reaction with precursor T6a, which consists only of the T arm, the acceptor stem, and a short connecting loop, is shown. The precursor and products are shown schematically next to the respective gel lanes. In vitro processing reactions were performed in buffer ivp-nu [40 mM Tris-HCl (pH 8.4), 2 mM $MgCl_2$, 2 mM KCl, and 2 mM DTT], and reaction products were separated by 8% PAGE.

The predicted secondary structures of these variants are shown in Figure 1. Variant T1 is missing the T arm, thus connecting the variable arm directly to the acceptor stem. In variant T2, the variable arm was removed in addition to the T arm. Removal of the anticodon arm resulted in variant T3, and removal of both anticodon and variable arms yielded variant T6. Variant T4 lacks the D arm, and in variant T5, the D arm was replaced with a D replacement loop, since studies with the 5′ tRNA processing enzyme RNase P have shown that tRNA variants with a D replacement loop are cleaved more readily than are variants in which the D arm has just been deleted (*34*). In addition, variant T5 resembles the naturally occurring tRNAs with unusual structures found in metazoan mitochondria (*35*). Variant T6a consists only of the T arm, the acceptor stem, and a short loop between both (residual nucleotides from the variable arm).

Structure probing data of the mutated molecules generally resemble those of the wild type, showing that the local structure of the tRNA arms is not perturbed by the deletion of other subdomains (Figure 1). However, some differences are detected which can be attributed to changes in the global structure, i.e., the L shape of the tRNA. Because of this, all of the variants appear to be slightly more susceptible to cleavage, and in variants T1−T3, the D arm is more accessible to the probing enzymes, indicating a loss of the classical D loop−T loop interaction. In variants T1 and T2, the D loop cannot interact with the T loop due to the deletion of the T arm. In variant T3, the loss of the D loop−T loop interaction seems to be a result of the removal of the anticodon arm. Taken together, the probing data show that the mutated tRNA molecules as well as the corresponding precursor RNAs indeed fold into the proposed secondary structures, while the tertiary structures might be somewhat compromised.

The tRNA precursors used in this study did not contain 5′ leaders since the mitochondrial and to some extent the nuclear tRNA 3′ processing enzyme do not process 5′ extended pretRNAs (*6*, *9*, *10*, *18*, *21*). Since mitochondrial and nuclear tRNA genes do not encode the 3′ terminal CCA triplet, all precursors that were used do not contain a CCA at the tRNA 3′ end. For each $tRNA^{Tyr}$ variant, we constructed a set of precursors with three different 3′ trailer lengths (20, 51, and 84 nucleotides) to analyze the influence of the trailer length on cleavage efficiency. Structure probing experiments showed that the trailer does not detectably influence folding of the tRNA (data not shown).

Nuclear RNase Z Can Dispense with the Anticodon, Variable, and D Arms. Nuclear tRNA 3′ processing activities from a number of organisms have been described (*6*−*8*, *11*−*14*, *18*, *21*, *36*). The majority of these are endonucleases which cleave the precursor next to the discriminator (*6*, *7*, *9*−*14*, *17*, *18*, *21*, *26*, *37*). The wheat nuclear tRNA 3′ processing activity, nuclear RNase Z, has been characterized previously and was shown to be an endonuclease which

cleaves precursors 3′ to the discriminator (*32*). The mitochondrial pretRNA^{Tyr} is processed very efficiently by the nuclear RNase Z[5] (*32*) (Figure 2A.). The long variable arm does not interfere with processing by the nuclear RNase Z as witnessed by the comparable cleavage efficiencies of nuclear and mitochondrial RNase Z. The nuclear 3′ processing activity still accepts variants T3 and T6 (missing the anticodon arm or anticodon arm and variable arm, respectively) as a substrate, although with reduced cleavage efficiencies (only 59 and 35%, respectively, are cleaved) (Figures 2B and 4). Removal of the D arm (variant T4) is tolerated by the nuclear enzyme, but the cleavage efficiency drops to only 9% (Figures 2C and 4). Replacement of the D arm with a loop (T5) basically has the same effect, resulting in a cleavage efficiency of 14% of that of the wild type (Figures 2C and 4). The nuclear RNase Z surprisingly processes substrate T6a, which has both D and anticodon arms removed, with 13% of the wild-type cleavage efficiency (Figures 2D and 4). Variants T1 and T2 which lack the T arm are not cleaved by the nuclear RNase Z. When all these data are considered in context, the nuclear RNase Z seems to have a comparatively broad substrate spectrum. It even processes a minimal substrate consisting of only the acceptor stem and T arm. The presence of the T arm, however, seems to be essential for substrate recognition by nuclear RNase Z.

Mitochondrial RNase Z Only Tolerates Removal of Anticodon and Variable Arms. The mitochondrial tRNA 3′ processing activity, RNase Z, has been characterized in rats, yeast, and plants (*18*−*21*). In these organisms, the 3′ processing activity is an endonuclease cleaving the precursor at the mature tRNA 3′ end. To compare the substrate requirements of the nuclear RNase Z with those of its mitochondrial counterpart, we also incubated these tRNA variants with the mitochondrial RNase Z. The potato mitochondrial RNase Z[6] used in these experiments processes the wild-type mitochondrial tRNA^{Tyr} precursor very efficiently (Figure 3); both processing products, trailer and tRNA, are easily detectable. Variant T3, which has the anticodon arm removed, is still recognized as substrate by the mitochondrial RNase Z, but the cleavage efficiency is reduced from 100 to 38% (Figures 3 and 4). If in addition to the anticodon arm the variable arm is removed, the precursor (variant T6) is still a substrate for the mitochondrial RNase Z, albeit with a cleavage efficiency (11%) even lower than that of variant T3 (Figures 3 and 4). Deletion of the T arm alone (variant T1) yields a molecule which is not cleaved at all, and deletion of both the T arm and variable arm (variant T2) has the same effect. Variants lacking the D arm (variant T4) are likewise not processed even if the D arm is replaced with a small loop (variant T5). Variants which lack all three arms [D arm, anticodon arm, and variable arm (T6a)] are also not cleaved by the mitochondrial RNase Z. Thus, the mitochondrial RNase Z has a comparatively narrower substrate spectrum and tolerates only the removal of the anticodon and variable arms.

Cleavage Site Selection. To analyze whether the deletion of parts of the tRNA shifts the processing site, we identified

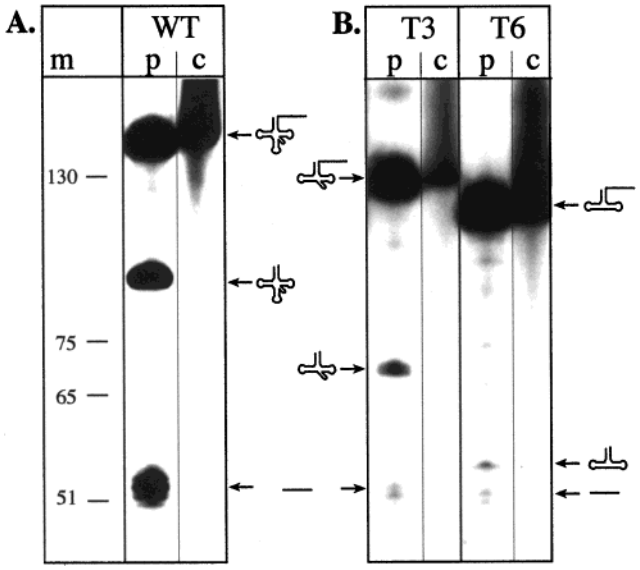

FIGURE 3: In vitro processing of wild-type and variant tRNAs with mitochondrial RNase Z. tRNA precursors were labeled throughout the molecule with [α-^{32}P]UTP. Only wild-type tRNA^{Tyr} and mutated tRNA substrates T3 and T6 are processed by the mitochondrial RNase Z. Both processing products, tRNA (WT, 83 nt; T3, 66; T6, 52) and 3′ trailer (51 nt), are detectable in all three processing reactions. Lane m shows a DNA size standard given in nucleotides. Lanes p show the products of the in vitro processing reactions, and lanes c show control reactions which have been incubated without protein. Lanes WT show incubation of wild-type tRNA^{Tyr} (A), lanes T3 incubation of variant T3, which has the anticodon arm removed, and lanes T6 incubation of variant T6, which has the variable and anticodon arm deleted (B). The precursor and products are shown schematically next to the gel lanes. In vitro processing reactions were performed in buffer ivp-mt [40 mM Tris-HCl (pH 8), 5 mM $MgCl_2$, 30 mM KCl, and 2 mM DTT], and reaction products were separated by 8% PAGE.

the cleavage sites in all tRNA precursors (Figure 5). For this analysis, precursor tRNAs were transcribed in vitro and subsequently labeled at the 5′ end. Processing products were resolved by PAGE, and the cleavage site was located by parallel T1 digests and alkali ladders.

Mitochondrial RNase Z cleaves wild-type and mutant substrates all at the same site between nucleotides 74 and 75 and between nucleotides 75 and 76. The nuclear RNase Z processes wild-type tRNA^{Tyr} close to the discriminator and several nucleotides downstream from the discriminator. The latter cleavage site downstream of the discriminator is only observed with the tRNA^{Tyr} precursor and might be due to local secondary structures formed in the trailer. For analysis of cleavage site selection, we therefore concentrated on the cleavage sites close to the discriminator. Wild-type tRNA and variants T3−T6 are all cleaved between nucleotides 74 and 75 and between nucleotides 75 and 76, but the ratio of cleavage at site 1 (between nucleotides 74 and 75) and site 2 (between nucleotides 75 and 76) varies between the individual variants. Deletions of parts of the tRNA substrates thus influence cleavage efficiencies, but the actual locations of cleavage sites remain the same in the variants and in the wild type.

Effect of 3′ Trailer Length. To analyze whether the length of the 3′ trailer influences cleavage efficiency, we tested each precursor with three different 3′ trailer lengths (Figure 6). In vivo tRNA precursors in plant mitochondria generally have long 3′ trailers (*38*). In vitro the mitochondrial RNase

[5] For all experiments involving the nuclear RNase Z, a highly purified protein fraction was used (see Experimental Procedures).

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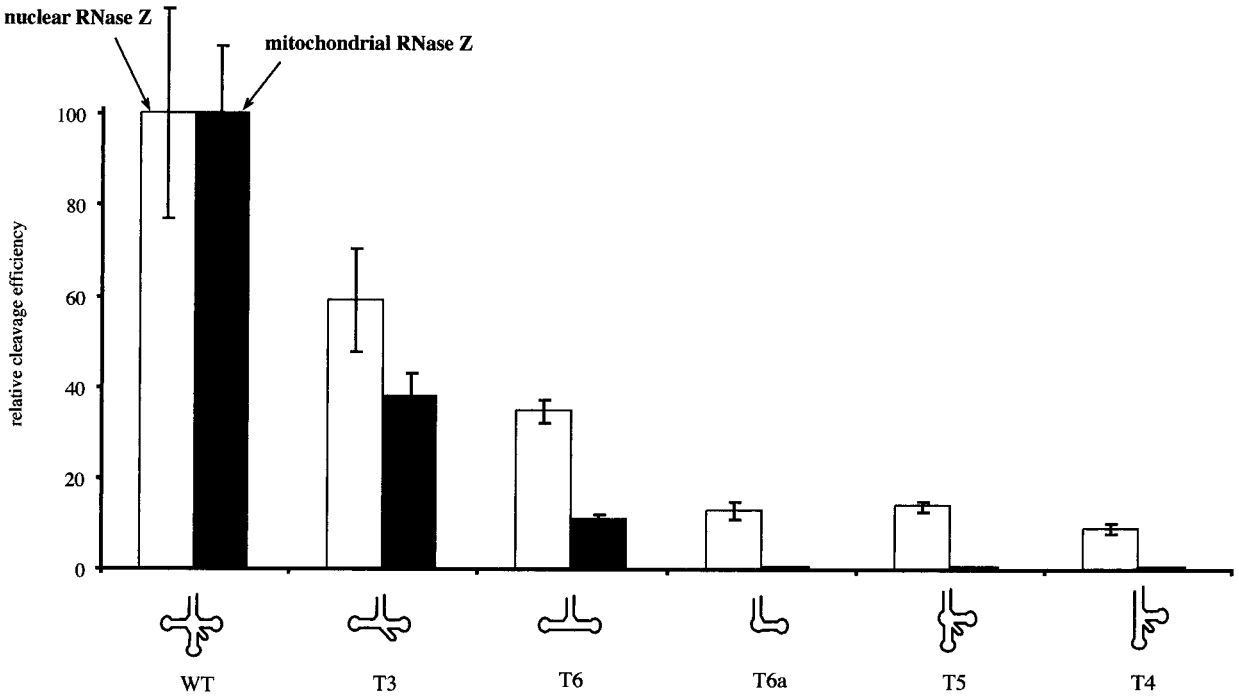

FIGURE 4: Efficiencies of wild-type and variant substrate cleavages by mitochondrial and nuclear RNase Z. Substrates containing a 51 nt trailer were incubated with mitochondrial and nuclear RNase Z. Reactions were repeated three times, and cleavage efficiencies were determined and averaged. The wild-type cleavage efficiency was set to 100%. Wild-type and variant substrates are shown schematically at the *x*-axis. Relative cleavage efficiency is shown at the *y*-axis in percent. Both endonucleases process the wild-type substrate most efficiently. While the nuclear RNase Z cleaves variants T3, T6, and T6a with 59, 35, and 13% cleavage efficiency, respectively, mitochondrial RNase Z cleaves T3 with only 38% efficiency and variant T6 with 11% efficiency, and variant T6a is not cleaved at all. Nuclear RNase Z cleaves variants T4 and T5 with 9 and 14% cleavage efficiency, respectively, while mitochondrial RNase Z does not accept these molecules as substrates at all.

Z seems to prefer shorter trailer sequences, since substrates with 20- and 51-nucleotide trailers are processed more efficiently than a substrate with an 84-nucleotide trailer. Nuclear-encoded pretRNAs have short 3′ trailers ranging from ~6 to 16 nucleotides in length, and shorter trailers are also preferred in vitro by the nuclear RNase Z.

DISCUSSION

In this report, the substrate requirements of the nuclear tRNA 3′ processing enzyme from plants were investigated and compared to the substrate requirements of the mitochondrial counterpart. A set of tRNA variants was constructed for this purpose, and structure probing experiments were employed to confirm the proposed secondary structure for these $tRNA^{Tyr}$ variants (Figure 1).

The Minimal Substrate for the Nuclear RNase Z Consists of the T Arm and the Acceptor Stem. The nuclear RNase Z is found to have a rather broad substrate spectrum. All variants that were tested are cleaved by the nuclear RNase Z, except for the substrates without the T arm. Removal of the anticodon arm reduces the cleavage efficiency of the nuclear RNase Z only by approximately half (Figure 4). The minimal substrate for the nuclear RNase Z consists of the T arm, the acceptor stem, and a short connecting loop. Similar minimal substrates have been reported for the mammalian and *Drosophila* nuclear 3′ processing enzymes (*25*, *26*). Since the nuclear RNase Z also processes RNA molecules smaller than complete tRNAs, it may be possible that this enzyme processes other substrates in the nucleus in addition to tRNA precursors. It has been proposed that in nuclei the tRNA 5′ processing enzyme, RNase P, is involved in the processing of ribosomal RNAs (*39*). These molecules could thus also be candidate substrates for the tRNA 3′ processing enzyme. However, in vitro processing experiments with ribosomal RNA precursors are required to see whether the nuclear RNase Z is capable of cleaving these molecules. The related RNA processing enzymes RNase P and RNase MRP are found in a complex in HeLa cells (*40*), and it would be interesting to investigate whether the 3′ processing activity is also part of that entity.

In vitro the nuclear RNase Z prefers shorter 3′ trailers. This observation is in accordance with the fact that in vivo nuclear tRNA transcripts contain short 5′ and 3′ extensions of ~6−16 nucleotides. The mammalian tRNA 3′ processing enzyme has a similar preference for short trailers (*27*). In contrast, 3′ trailer length has no influence on processing by the *Drosophila* enzyme (*28*).

The Sole Function of Mitochondrial RNase Z Seems To Be tRNA 3′ Processing. Mitochondria are quite special concerning their tRNA population and tRNA genes. Kinetoplastid, yeast, and plant mitochondria have to import tRNAs from the cytoplasma to complete their tRNA set (*41*). Enzymes involved in mitochondrial tRNA maturation thus have to fulfill special requirements. We showed previously that the plant mitochondrial RNase Z is capable of cleaving plastid as well as nuclear tRNA precursors (*42*). Here we report that the plant mitochondrial RNase Z in contrast to the nuclear RNase Z has a rather narrow substrate spectrum since it only cleaves wild-type tRNA substrates and tRNA precursors missing no more than the anticodon and variable arms. Deletion of the anticodon arm reduces the cleavage efficiency of the mitochondrial enzyme more drastically than

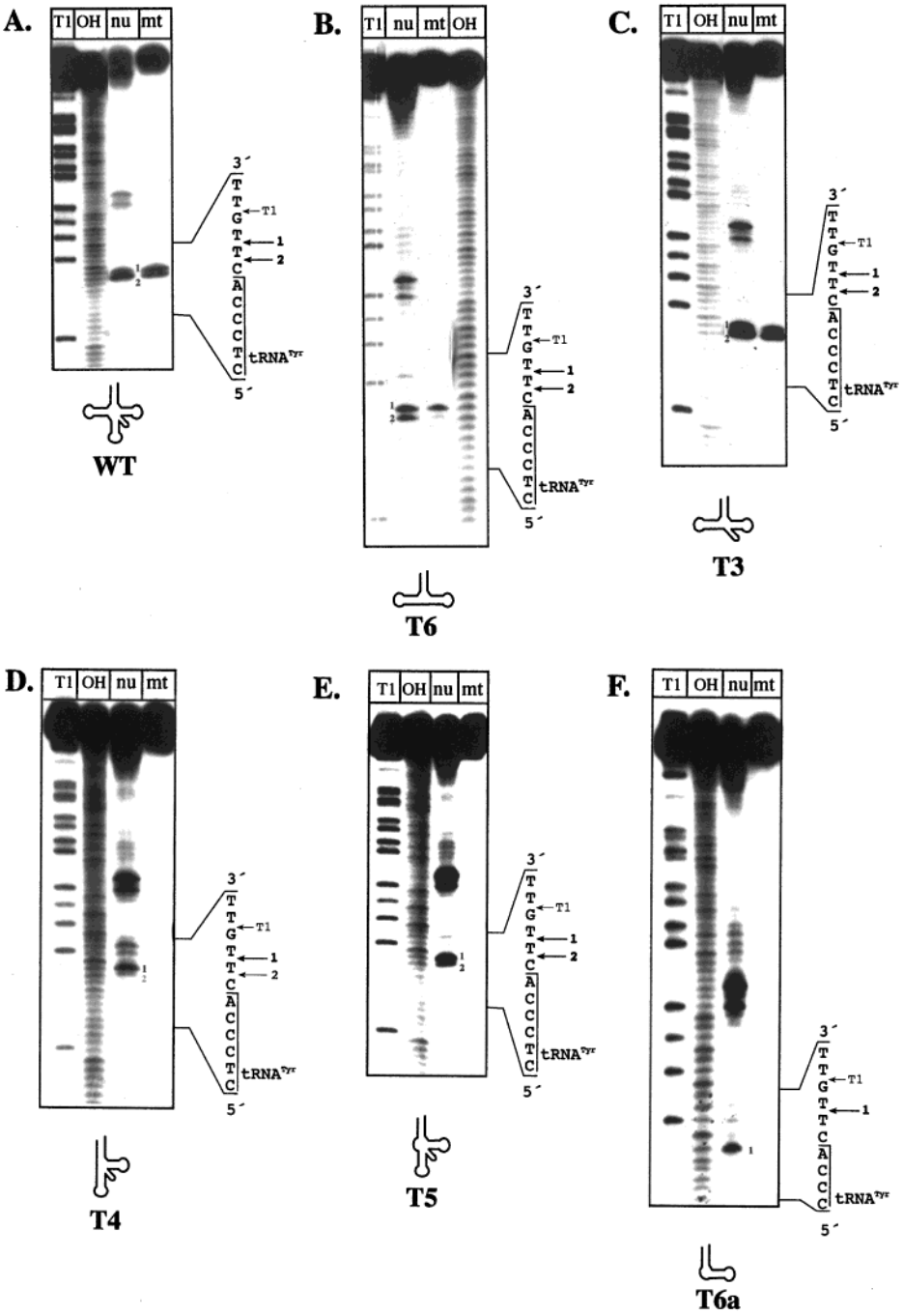

FIGURE 5: Cleavage site identification. To analyze whether the change in tRNA structure has an influence on cleavage site selection, all precursors were 5′ labeled and incubated with the 3′ processing activities. Reaction products were analyzed by PAGE, and cleavage sites were identified with T1 and alkali ladders run in parallel lanes. Analysis of the wild-type precursor (A), variant T6 (B), variant T3 (C), variant T4 (D), variant T5 (E), and variant T6a (F). In lane T1, precursors were partially digested under denaturing conditions with RNase T1. In lane OH, precursors were partially hydrolyzed with alkali. In lane nu, a processing reaction with nuclear RNase Z was carried out, and in lane mt, a processing reaction with mitochondrial RNase Z was carried out. At the right, the sequences of the tRNA and 3′ trailer are shown and cleavage sites are marked with an arrow. The nuclear RNase Z cleaves the tRNA precursors between nucleotides 74 and 75 and between nucleotides 75 and 76. In addition, the enzyme cleaves at a second site about 12 nucleotides downstream of the tRNA 3′ end. This second cleavage site is only detectable with this particular precursor and might be due to secondary structures forming in the 3′ trailer. The mitochondrial RNase Z processes the precursor only between nucleotides 74 and 75 and nucleotides 75 and 76.

that of the nuclear activity, namely, down to $\sim^1/_3$. Additional deletion of the variable arm reduces the efficiency again down to $\sim^1/_{10}$. Thus, substrates for the plant mitochondrial RNase Z have to retain most of the tRNA structure. These results suggest that the tRNA processing activities in plant mitochondria differ from the respective processing activities in mammalian mitochondria, since the latter have to process precursors which lack the D or T arm.

An interesting observation is that in vitro the mitochondrial enzyme prefers shorter 3′ trailers, although in vivo plant mitochondrial tRNA genes are transcribed into precursor molecules containing long 5′ and 3′ extensions (*38*, *43*). Consequently, in vivo substrates for the mitochondrial RNase Z have long 3′ trailers. Maybe 3′ trailers are cleaved in vivo initially by another endonucleolytic activity to yield shorter 3′ trailers which subsequently serve as substrates for the

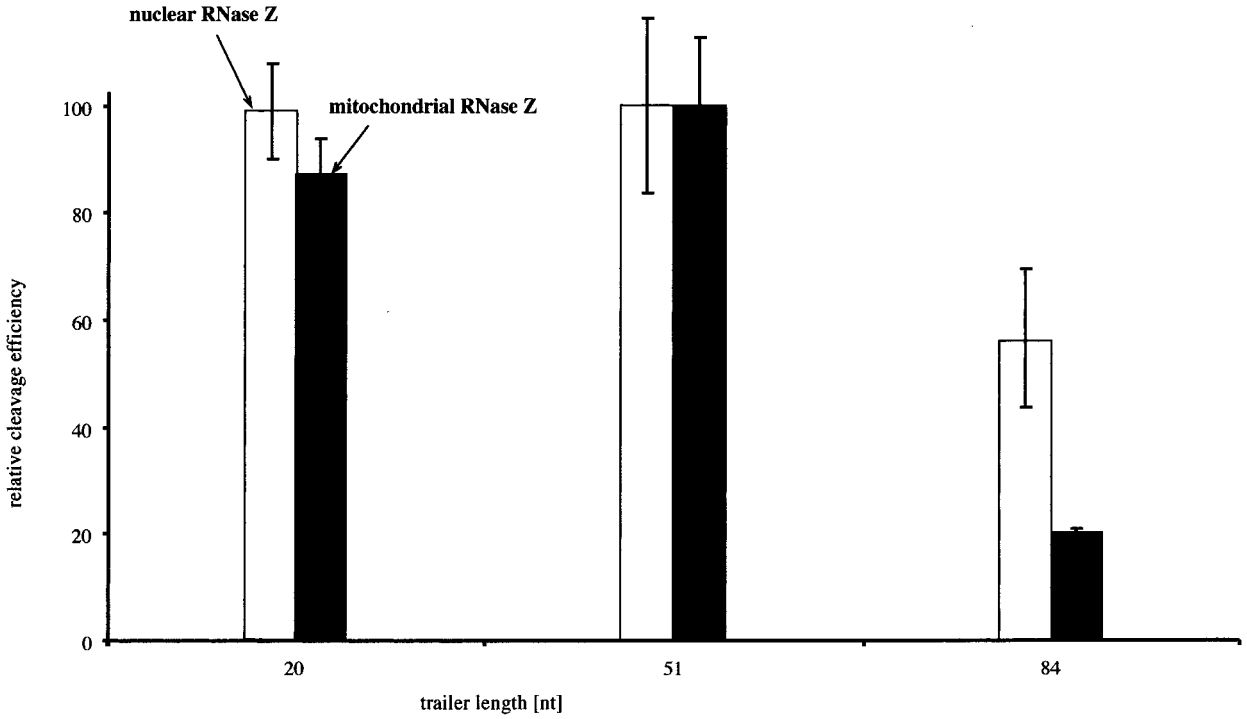

FIGURE 6: Influence of 3′ trailer length on cleavage by 3′ processing enzymes. All substrates were incubated with three different 3′ trailer lengths (20, 51, and 84 nt) with the different 3′ processing activities. Cleavage efficiencies were measured for all substrates. Cleavage efficiency for the precursor containing a 51 nt trailer was taken as 100%. As shown here for the processing of wild-type $tRNA^{Tyr}$, substrates with a shorter trailer (20 and 51 nt) are preferred by the mitochondrial as well as the nuclear enzyme. The *x*-axis shows trailer length and the *y*-axis relative cleavage efficiency.

mitochondrial RNase Z. Another possibility is that processing of precursors occurs cotranscriptionally.

Change in tRNA Structure Has Little Influence on Cleavage Site Selection. Identification of cleavage sites of the nuclear and mitochondrial RNase Z showed that the cleavage site selection remains the same for all substrates. Removal of the anticodon, the variable arm, and the D arm does not shift the cleavage site, but the ratio between cleavages at site 1 (between nucleotides 74 and 75) and site 2 (between nucleotides 75 and 76) changes from substrate to substrate. Thus, determinants for cleavage site selection do not reside in anticodon, variable, or D arms.

Mitochondrial and Nuclear Activities Differ. Plant cells contain three compartments which each harbor their own genetic system, including tRNA processing activities. Enzymes required in all three compartments can be of several origins. Both the endosymbiont and the host may have contributed their own genes and maintained them, or on the other hand, the organellar gene was lost and the nuclear gene acquired the necessary import signals to be able to route the respective protein products also to the organelles, thus effectively creating genes encoding isoenzymes. Another likely scenario is that the nuclear gene was duplicated early and both nuclear copies have since evolved separately. Organellar proteins might even be encoded in the nucleus by phage-derived genes, as has been shown for the plant mitochondrial and plastid RNA polymerases (*44*). Comparison of the mitochondrial and nuclear tRNA 3′ processing enzymes in this report shows that both activities have different substrate requirements. This result suggests either that mitochondrial and nuclear enzymes are encoded by the same nuclear gene and different cofactors specify their substrate spectrum or that alternatively both enzymes are encoded by two different genes. On the other hand, one should be aware that the difference between the two enzymes might as well be due to the fact that the nuclear RNase Z was isolated from wheat and the mitochondrial enzyme from potato.

For the Nuclear RNase Z, the Ancient Part of the tRNA Is Sufficient for Cleavage. For the nuclear RNase Z, the top half of the tRNA (acceptor stem and T arm) is sufficient for substrate recognition. Since this tRNA part is considered to be the ancient part of the tRNA molecule, it seems that substrate requirements of the nuclear RNase Z did not coevolve their specificity with the tRNA from the small ancient molecule to the modern tRNA. In contrast, the plant mitochondrial RNase Z requires one of the modern parts of the tRNA molecule for substrate recognition. In addition to the acceptor stem and T arm, the D arm is essential for cleavage by the mitochondrial RNase Z. Surprisingly, the plant mitochondrial RNase Z thus appears to have increased its specificity requirements during evolution.

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REFERENCES

1. Altman, S., Kirsebom, L., and Talbot, S. (1995) *Recent Studies of RNase P*, American Society for Microbiology, Washington, DC.

2. Frank, D. N., and Pace, N. R. (1998) *Annu. Rev. Biochem. 67*, 153−80.
3. Li, Z., and Deutscher, M. P. (1996) *Cell 86*, 503−12.
4. Deutscher, M. P. (1995) in *tRNA: structure, biosynthesis, and function* (Söll, D., and RajBhandary, U., Eds.) pp 51−65, American Society for Microbiology, Washington, DC.
5. Palmer, J. R., Nieuwlandt, D. T., and Daniels, C. J. (1994) *J. Bacteriol. 176*, 3820−3.
6. Castaño, J. G., Tobian, J. A., and Zasloff, M. (1985) *J. Biol. Chem. 260*, 9002−8.
7. Franklin, S. E., Zwick, M. G., and Johnson, J. D. (1995) *Plant J. 7*, 553−63.
8. Garber, R. L., and Gage, L. P. (1979) *Cell 18*, 817−28.
9. Frendewey, D., Dingermann, T., Cooley, L., and Söll, D. (1985) *J. Biol. Chem. 260*, 449−54.
10. O'Connor, J. P., and Peebles, C. L. (1991) *Mol. Cell. Biol. 11*, 425−39.
11. Garber, R. L., and Altman, S. (1979) *Cell 17*, 389−97.
12. Engelke, D. R., Gegenheimer, P., and Abelson, J. (1985) *J. Biol. Chem. 260*, 1271−9.
13. Papadimitriou, A., and Gross, H. J. (1996) *Eur. J. Biochem. 242*, 747−59.
14. Solari, A., and Deutscher, M. P. (1983) *Mol. Cell. Biol. 3*, 1711−7.
15. Yoo, C. J., and Wolin, S. L. (1997) *Cell 89*, 393−402.
16. Rossmanith, W., Tullo, A., Potuschak, T., Karwan, R., and Sbisa, E. (1995) *J. Biol. Chem. 270*, 12885−91.
17. Wang, M. J., Davis, N. W., and Gegenheimer, P. (1988) *EMBO J. 7*, 1567−74.
18. Manam, S., and Van Tuyle, G. C. (1987) *J. Biol. Chem. 262*, 10272−9.
19. Kunzmann, A., Brennicke, A., and Marchfelder, A. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 108−13.
20. Hanic-Joyce, P. J., and Gray, M. W. (1990) *J. Biol. Chem. 265*, 13782−91.
21. Chen, J. Y., and Martin, N. C. (1988) *J. Biol. Chem. 263*, 13677−82.
22. McClain, W. H., Guerrier-Takada, C., and Altman, S. (1987) *Science 238*, 527−30.
23. Yuan, Y., and Altman, S. (1995) *EMBO J. 14*, 159−68.
24. Marchfelder, A. (1995) *Mol. Biol. Rep. 22*, 151−6.
25. Levinger, L., Bourne, R., Kolla, S., Cylin, E., Russell, K., Wang, X., and Mohan, A. (1998) *J. Biol. Chem. 273*, 1015−25.
26. Nashimoto, M., Tamura, M., and Kaspar, R. L. (1999) *Biochemistry 38*, 12089−96.
27. Nashimoto, M. (1997) *Nucleic Acids Res. 25*, 1148−54.
28. Levinger, L., Vasisht, V., Greene, V., Bourne, R., Birk, A., and Kolla, S. (1995) *J. Biol. Chem. 270*, 18903−9.
29. Binder, S., Schuster, W., Grienenberger, J. M., Weil, J. H., and Brennicke, A. (1990) *Curr. Genet. 17*, 353−8.
30. Marchfelder, A., and Brennicke, A. (1994) *Plant Physiol. 105*, 1247−54.
31. Helm, M., Giegé, R., and Florentz, C. (1999) *Biochemistry 38*, 13338−46.
32. Mayer, M., Schiffer, S., and Marchfelder, A. (2000) *Biochemistry 39*, 2096−105.
33. Dock-Bregeon, A. C., Westhof, E., Giegé, R., and Moras, D. (1989) *J. Mol. Biol. 206*, 707−22.
34. Hardt, W. D., Schlegl, J., Erdmann, V. A., and Hartmann, R. K. (1993) *Biochemistry 32*, 13046−53.
35. Okimoto, R., and Wolstenholme, D. R. (1990) *EMBO J. 9*, 3405−11.
36. Hagenbüchle, O., Larson, D., Hall, G. I., and Sprague, K. U. (1979) *Cell 18*, 1217−29.
37. Yamaguchi-Shinozaki, K., Shinozaki, K., and Sugiura, M. (1987) *FEBS Lett. 215*, 132−6.
38. Binder, S., Marchfelder, A., and Brennicke, A. (1996) *Plant Mol. Biol. 32*, 303−14.
39. Chamberlain, J. R., Pagan, R., Kindelberger, D. W., and Engelke, D. R. (1996) *Nucleic Acids Res. 24*, 3158−66.
40. Lee, B., Matera, A. G., Ward, D. C., and Craft, J. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 11471−6.
41. Hancock, K., LeBlanc, A. J., Donze, D., and Hajduk, S. L. (1992) *J. Biol. Chem. 267*, 23963−71.
42. Marchfelder, A., Schuster, W., and Brennicke, A. (1990) *Nucleic Acids Res. 18*, 1401−6.
43. Binder, S., and Brennicke, A. (1993) *Nucleic Acids Res. 21*, 5012−9.
44. Hedtke, B., Börner, T., and Weihe, A. (1997) *Science 277*, 809−11.
45. Sprinzl, M., Horn, C., Brown, M., Ioudovitch, A., and Steinberg, S. (1998) *Nucleic Acids Res. 26*, 148−53.

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