Naturally Occurring Mutations in Human Mitochondrial Pre-tRNA\textsubscript{Ser(UCN)} Can Affect the Transfer Ribonuclease Z Cleavage Site, Processing Kinetics, and Substrate Secondary Structure*

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Hua Yan\textsuperscript{1}, Neela Zareen\textsuperscript{2}, and Louis Levinger\textsuperscript{3}

From the York College of The City University of New York, Jamaica, New York 11451

Mitochondria, the intracellular organelles responsible for most of a eukaryotic cell energy production, possess their own maternally inherited genome. The circular 16,568-base pair human mitochondrial genome (1) is bidirectionally transcribed into long polycistronic heavy (H)\textsuperscript{1} and light (L)-strand precursor RNAs. Two tRNAs and 11 mRNAs encoding 13 polypeptides (all of them subunits of complexes in the respiratory transport chain) are punctuated by 22 tRNAs (1 tRNA for each of 18 amino acids and 2 each for leucine and serine with different anticodons) with very little intergenic RNA (2, 3). Several thousand additional polypeptides required for mitochondrial metabolism are nuclear-encoded, cytoplasmically translated and imported. Precuror tRNA transcripts have a 5′ end leader and a 3′ end trailer. Eukaryotic nuclear-encoded tRNAs are transcribed from RNA polymerase II promoters with a U\textsubscript{3} tail. The characteristic length and sequence of mitochondrial pre-tRNA leaders and trailers, on the other hand, depend on the setting (S) forms of the tRNase Z gene are present in the human genome. tRNase Z\textsubscript{L} processes a nuclear-encoded pre-tRNA \textsuperscript{1600-fold} more efficiently than tRNase Z\textsuperscript{S} and is predicted to have a strong mitochondrial transport signal. tRNase Z\textsubscript{L} could, thus, process both nuclear- and mitochondrially encoded pre-tRNAs. More than 150 pathogenesis-associated mutations have been found in the mitochondrial genome, most of them in the 22 mitochondrially encoded tRNAs. All the mutations investigated in human mitochondrial tRNA\textsubscript{Ser(UCN)} affect processing efficiency, and some affect the cleavage site and secondary structure. These changes could affect tRNase Z processing of mutant pre-tRNAs, perhaps contributing to mitochondrial disease.

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1 Present address: Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029.

2 Present address: Dept. of Biology, Fairchild Center, Columbia University, NY, New York 10027.

3 To whom correspondence should be addressed: Dept. of Natural Sciences/Biology, York College of The City University of New York, 94-20 Guy R. Brewer Blvd., Jamaica, NY 11451, Tel.: 718-262-2704; Fax: 718-262-2652; E-mail: louie@york.cuny.edu.

4 The abbreviations used are: H, heavy; L, light; nt, nucleotide(s); MOPS, 4-morpholinepropanesulfonic acid; tRNase Z, transfer ribonuclease Z; tRNase Z\textsubscript{L}, transfer ribonuclease Z\textsubscript{L}.

5 More than 150 pathogenesis-associated mutations have been found.
in the mitochondrial genome, ~1/3 of them in tRNA genes (see www.mitomap.org). No nuclear-encoded cytoplasmic tRNAs are known to be imported into human mitochondria, and pathogenesis-associated mutations have been confirmed in most of the mitochondrial encoded tRNAs, suggesting that they are all needed for efficient translation of mitochondrial messages. Pre-tRNA end processing defects could, thus, contribute to mitochondrial diseases (for review, see Ref. 4). tRNA<sup>Ser</sup>(UCN), a mitochondrial L-strand transcript, may be a hot spot for mutations associated with maternally transmitted hearing loss and other symptoms, including epilepsy, ragged red fibers, encephalomyopathy, lactic acidosis, ataxia and myoclonus, and mental retardation (www.mitomap.org). Several naturally occurring mutations in tRNA<sup>Ser</sup>(UCN) are tightly clustered at two locations, on the 5′ side of the acceptor stem and in the pre-tRNA 3′ end trailer immediately after the discriminator, suggestive of possible effects on tRNase Z processing. Wild type pre-tRNA<sup>Ser</sup>(UCN) and these mutants were produced with a mature 5′ end and a 3′ end trailer to investigate their tRNase Z<sup>+</sup> cleavage sites, processing kinetics, and substrate secondary structures. The observed effects could all contribute to mitochondrial pathogenesis.

**MATERIALS AND METHODS**

**Baculovirus Expression of Human tRNase Z<sup>+</sup> and tRNase Z<sup>−</sup>—cDNAs encoding human tRNase Z<sup>+</sup> (also known as ELAC2, a gift of S. Tavtigian (22)) and tRNase Z<sup>−</sup> (also known as ELAC1) were cloned into the baculovirus transfer vector FastBac HTa (Invitrogen), transposed into bacmid DNA, and transfected and expressed in insect Sf9 cells. Full-length 40-kDa (363 amino acid) polypeptide. Expression of soluble active enzymes can depend on the choice of expression system and also on the translation start. tRNase Z<sup>+</sup> and tRNase Z<sup>−</sup> are highly diverged around the amino end. The first conserved residues found (22), the start of a Histidin around r60, suggest that the region on the amino side of r60 is without a specific essential function apart from the putative mitochondrial targeting sequence within the first 25–30 residues. Initiation at Gly-50 produced a soluble enzyme with a high catalytic efficiency. Full-length 40-kDa (363 amino acid) tRNase Z<sup>−</sup> was expressed.

Expressed proteins were affinity-purified by nickel chelate chromatography (Qiagen) and the His tag was removed using TEV protease. Protein concentrations were determined by Bio-Rad protein assay and confirmed using discontinuous SDS-polyacrylamide gels stained with Sypro Orange (Molecular Probes) and scanned with a Storm 840 Imager as in Zareen et al. (25).

**Construction of Pre-tRNA Templates—**An EcoRI-BamHI insert encoding human nuclear-encoded pre-tRNA<sup>AvG</sup> (accession number X64282) consisting of the T7 promoter, strong start, hybridization box, hammerhead ribozyme, 73-nt tRNA, and 18-nt 3′ end trailer with Dral runoff site was constructed by overlap extension and subcloned into the high copy number plasmid pHCl624<sup>+</sup> to ensure efficient transcription and accuracy of 5′ end formation (26–28). Nuclear-encoded tRNA<sup>AvG</sup> consisting of a 73-nt canonical tRNA and a 14-nt 3′ end trailer (Ref. 29; accession number X17514) was constructed using the same methods. Similarly, inserts consisting of the EcoRI site, T7 promoter—strong start, hybridization box—hammerhead ribozyme, 69-nt tRNA<sup>Ser</sup>(UCN), 19-nt 3′ end trailer, hepatitis delta virus ribozyme, and HindIII runoff site were designed to produce an 88-nt pre-tRNA after ribozyme cleavage (27). Complementary primer pairs were synthesized for each tRNA<sup>Ser</sup>(UCN) mutant with one nucleotide mismatch at the position of the mutation. In combination with universal upstream EcoRI-T7 promoter and downstream PsiI-3′ end trailer or HindIII-hepatitis delta virus ribozyme oligonucleotides, overlapping products were joined and amplified by extension PCR (30). Inserts were subcloned, and accuracy of construction was confirmed by sequencing (Herbert Irving Comprehensive Cancer Center, Columbia University). Structure probing of wild type and mutant tRNA<sup>Ser</sup>(UCN) transcripts with RNase T1 under semi-denaturing conditions (see “Results”) confirms the correct location of the G residues. A previous sequence/construction error was corrected (nucleotides AA at positions 37 and 38 on the 3′ side of the anticonodon loop in Fig. 4 is the correct sequence); observations in the present work are generally in agreement with earlier results (27).

**Preparation of Pre-tRNAs—**Wild type and mutant unlabeled pre-tRNAs were prepared by runoff transcription with T7 RNA polymerase. Transcripts were recovered by precipitation, redissolved in 200 µl of deionized water (refolded by heating for 1 min at 90 °C, mixed with an equal volume of 2X ribosome buffer to make 400 µl containing 25 mM Tris-HCl, pH 8.0, 7.5 mM MgCl<sub>2</sub>, and incubated at 60 °C for 30 min). Ribozyme reaction products were precipitated and gel-purified. Expected bands were detected by UV shadowing, and pre-tRNAs were recovered. Concentrations were determined by A<sub>260</sub> and confirmed by gel electrophoresis, staining with Sybr Green II (Molecular Probes), and scanning with a Storm 840 Imager. Pre-tRNAs were 5′ end-labeled with T4 polynucleotide kinase and [γ-<sup>32</sup>P]ATP, gel-purified, and recovered as described (14). Additionally, pre-tRNA<sup>AvG</sup> was 3′ end-labeled with α-<sup>32</sup>P-labeled cytidine 3′-phosphate and T4 RNA ligase (31). Pre-tRNAs were sometimes refolded by heating in water for 1 min at 90 °C, mixing with 2X processing buffer (see below), and cooling to room temperature for 5 min. Refolding did not affect pre-tRNA structure or processing of the tRNA<sup>Ser</sup>(UCN) wild type or two of the most impaired mutants (7445U→C and 7445U→G; data not shown) and was, therefore, not routinely used.

**Optimization of Processing Efficiency for Nuclear-encoded and Mitochondrially Encoded Substrates—**The processing conditions were optimized, including the enzyme (tRNase Z<sup>+</sup> or tRNase Z<sup>−</sup>) and substrates (nuclear encoded pre-tRNA<sup>AvG</sup> or mitochondrial encoded pre-tRNA<sup>Ser</sup>(UCN)), divalent metal ions, monovalent metal ions, and pH. 25-µl reactions using a trace concentration (0.1 nM) of labeled pre-tRNA substrate were sampled after 0, 5, 10, and 15 min of incubation at 37 °C by transferring 5 µl to 2.5 µl of formamide-marker dye mix in a multwell plate on ice and heated to 90 °C. Samples were electrophoresed on 6% denaturing polyacrylamide gels. Dried gels were exposed to a storage phosphor screen, scanned with a Storm 840 imager, and analyzed with ImageQuant (as in Zareen et al. (25)).

Processing of nuclear-encoded pre-tRNA<sup>AvG</sup> with tRNase Z<sup>+</sup> was most efficient using 25 mM K-MOPS, pH 6.75, 2 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 5% glycerol, 4 units/ml RNasin, and 100 µg/ml bovine serum albumin. Processing of pre-tRNA<sup>AvG</sup> with tRNase Z<sup>−</sup> was optimal with 1 mM MnCl<sub>2</sub> in place of MgCl<sub>2</sub> as previously suggested (32, 33). Mitochondrially encoded pre-tRNA<sup>Ser</sup>(UCN) was most efficiently processed with tRNase Z<sup>+</sup> using 2.5 mM CaCl<sub>2</sub> instead of MgCl<sub>2</sub>. Processing of mitochondrial pre-tRNA<sup>Ser</sup>(UCN) with tRNase Z<sup>−</sup> was also optimal with 2.5 mM CaCl<sub>2</sub> and could not be detected with Mn<sup>2+</sup> alone or in a mixture with Ca<sup>2+</sup>. In *vitro* pre-tRNA processing of unmodified transcripts has both advantages and limitations (4). The endonucleolytic end processing reactions may precede most of the nucleoside modifications; even so, the absence of the post-transcriptional nucleoside modifications in tRNA<sup>Ser</sup>(UCN) (e.g. Ψ<sub>28</sub>, m<sub>1</sub>C<sub>32</sub>, ms<sub>2</sub>Ψ<sub>A37</sub>, T<sub>54</sub>, Ψ<sub>55</sub>) could contribute to lower processing efficiency compared with nuclear-encoded pre-tRNA<sup>AvG</sup> (see “Results”).

<sup>5</sup> L. Kallf, unpublished information.
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**Processing Kinetics**—Michaelis-Menten kinetics was performed as previously described (25) under optimal reaction conditions (see above) using constant input-labeled substrate (0.1 nM, more than 10-fold less than the lowest $k_{cat}$ for the enzyme) and varying the concentration of unlabelled substrate over a factor of 20, centered on $k_{m}$. Efficiency and kinetic experiments using nuclear-encoded pre-tRNA<sup>Ser</sup> were performed with a tRNA<sup>3</sup> concentration of 1 pM, ensuring multiple turnover over conditions. In the case of combinations of another enzyme (tRNase ZS) and substrates (mitochondrial pre-tRNAs), when $k_{cat}$ was lower, it was necessary to use a higher concentration of enzyme, as noted in the figures. Experiments with each combination of enzyme and substrate were performed at least twice, with the wild type substrate alongside the mutants each time. S.E. are reported in the second through fourth columns in Tables 1 and 2. Values for “relative to wild type” (the fifth column in Tables 1 and 2) were calculated using the mutant and wild type values from experiments performed the same day.

**Structure Probing**—Secondary structures of 5' end-labeled tRNAs were analyzed by structure probing as previously described (28), including one unincubated pre-tRNA (0), one alkaline ladder, one semi-denaturing ribonuclease T1 reaction (SD-T1), two native T1 reactions (final concentrations 1 and 2.5 $\times$ 10<sup>-3</sup> units/μl; United States Biochemical Corp.), two native S1 reactions (1 and 2.5 units/μl; New England Biolabs), and two native V1 reactions (0.04 and 0.1 units/μl; Pierce). Processing buffers were used for native structure probing but without tRNase Z and with Tris-HCl, pH 7.2, substituted for K-MOPS to avoid electrophoresis artifacts. Samples were split and electrophoresed on a 6% denaturing polyacrylamide gel until the xylene cyanol was 6 cm above the bottom for the highest resolution of the 3' half of the pre-tRNAs and on a 12% gel with the bromphenol blue run to 10 cm above the bottom to analyze the 5' half.

**RESULTS**

Efficiency and Kinetics of Human Nuclear-encoded pre-tRNA<sup>Asp</sup> Processing by tRNase Z<sup>L</sup> and tRNase Z<sup>S</sup>—To choose the best enzyme for analysis of pre-tRNA 3' end processing in vitro, human tRNase Z<sup>L</sup> and tRNase Z<sup>S</sup> were characterized using nuclear-encoded pre-tRNA<sup>Asp</sup> as a substrate under optimized reaction conditions (Fig. 1). Efficiently expressed affinity-purified soluble tRNase Z<sup>L</sup> and tRNase Z<sup>S</sup> display apparent molecular masses just below 90 kDa and around 40 kDa, respectively (Fig. 1A). To obtain a comparable extent of processing of nuclear-encoded pre-tRNA<sup>Asp</sup> (Fig. 1B) based on the reaction time course, 2 pM tRNase Z<sup>L</sup> and 5 nM tRNase Z<sup>S</sup> were used (Fig. 1, C and D), a 2500-fold higher concentration of tRNase Z<sup>S</sup> than tRNase Z<sup>L</sup>. tRNase Z<sup>L</sup> also processed mitochondrially encoded substrates substantially more efficiently than tRNase Z<sup>S</sup> (see below).

Kinetic analyses were performed to determine the relative contributions made by changes in $k_{cat}$ and $k_{m}$ to the more efficient processing of pre-tRNA<sup>Asp</sup> by tRNase Z<sup>L</sup> than by tRNase Z<sup>S</sup> (Fig. 2, Table 1). The processing efficiency ($k_{cat}$/$k_{m}$) with tRNase Z<sup>L</sup> is about 1600 times higher than with tRNase Z<sup>S</sup> (Fig. 2, the 5th column of Table 1) due to a ×250 higher $k_{cat}$ (Table 1, 2nd column) and a ×6 lower $k_{m}$ (Table 1, third column). Based on the much higher processing efficiency and the suggestion that both human and fruit fly tRNase Z<sup>L</sup> have mitochondrial transport signals (24), only tRNase Z<sup>S</sup> was used to analyze processing kinetics with wild type and mutant mitochondrially encoded tRNA<sup>Ser(UCN)</sup>.

**tRNase Z Is an Endonuclease**—Nucleotide-encoded pre-tRNA<sup>Asp</sup> labeled at the 5' and 3' ends was processed with tRNase Z<sup>L</sup> and electrophoresed so as to keep the 3' end trailer on the gel (Fig. 3). The presence of the 18-nt 3' end trailer at the expected position on the gel (Fig. 3B) confirms that tRNase Z is an endonuclease and that the cleavages observed throughout this report are in the expected position on the 3' side of the discriminator.

**Naturally Occurring Mutations in Mitochondrially Encoded tRNA<sup>Ser(UCN)</sup>**—Six mutations have been found in the body of mature tRNA<sup>Ser(UCN)</sup>, and four have been identified in the 3' end trailer (www.mitomap.org; the last of these mutations are entered under “Coding and Control Region Point Mutations,” not under tRNA/mtRNA Point Mutations because they do not occur in the body of the mature tRNA). From the standpoint of 3' end processing, clustering of three
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Kinetic Parameters for Wild Type and Mutant Mitochondrial pre-tRNA\textsuperscript{Ser(UCN)} Processing by tRNase Z\textsuperscript{6}—Wild type mitochondrial tRNA\textsuperscript{Ser(UCN)} is processed by tRNase Z\textsuperscript{6} with an efficiency \((k_{\text{cat}}/k_{m})\) ~16 times lower than that of human nuclear-encoded tRNA\textsuperscript{Ser(UCN)} (4th column of Table 2, cf. 4th column of Table 1). The \(k_{\text{cat}}\) for tRNA\textsuperscript{Ser(UCN)} is reduced ~75-fold, and the \(k_{m}\) is also reduced ~5-fold.

Three mutations on the 5’ side of the acceptor stem (7512A→G, 7511A→G, 7510A→G; Fig. 4) reduce tRNase Z processing efficiency (4th and 5th columns of Table 2) due to an ~33% lower \(k_{\text{cat}}\) (2nd column of Table 2) accompanied in the latter two mutants by an up to 2-fold higher \(k_{m}\) (3rd column of Table 2). Interestingly, the efficiency is progressively more reduced with increasing distance from the end of the acceptor stem and the tRNase Z cleavage site (see “Discussion”). Effects of substitutions in the 3’ end trailer just beyond the normal tRNase Z site (7445U→G, 7445U→G, 7444C→U, 7443U→C) vary widely (Table 2). 7445U→C can be processed with much reduced efficiency compared with wild type (Table 2, 5th column) due to a ~6-fold decrease in \(k_{\text{cat}}\) (2nd column) and a 4-fold increase in \(k_{m}\) (3rd column) but with a cleavage site that is shifted 1 nt in the 3’ direction (Fig. 5, panel B, cf. A).

7445U→C pre-tRNA\textsuperscript{Ser(UCN)} could not previously be processed by tRNase Z from a mitoplast extract (27). The mitoplast extract contains a lower concentration of tRNase Z mixed with many other proteins, and present experiments are performed using baculovirus expressed, affinity-purified tRNase Z\textsuperscript{5} (Fig. 1A) capable of efficient catalysis (Tables 1 and 2). Other proteins and a lower concentration of tRNase Z may have combined to prevent detection of the aberrant cleavage reported here.

Three additional substitutions beyond the body of mature tRNA affect the cleavage site and kinetics of tRNase Z reaction, further illustrating the importance of this region. 7445U→G and 7443U→C both reduce reaction efficiency \((k_{\text{cat}}/k_{m})\) that of Table 2) to ~1/3 of the wild type. In both cases \(k_{\text{cat}}\) is greater than wild type (4–5X and ~3X, respectively), but the increase in \(k_{m}\) is still larger (~20X and ~16X, respectively). 7444C→U, in which \(k_{m}\) more than doubled and \(k_{m}\) increased 1.5-fold (2nd and 3rd columns in Table 2), is the only mutation that causes processing efficiency to increase relative to wild type. The observed processing is due almost entirely to aberrant cleavage 1 nt in the 3’ direction from the normal processing site, as in the 7445U→C mutant (Fig. 5, panel D, cf. B and A).

tRNase Z\textsuperscript{6} Can Process Mitochondrially Encoded Wild Type and Mutant tRNA\textsuperscript{Ser(UCN)} Efficiently. Whereas tRNase Z\textsuperscript{6} Cannot—Wild type, 7445U→C, and 7445U→G pre-tRNA\textsuperscript{Ser(UCN)} were processed in parallel using a wide range of tRNase Z\textsuperscript{6} and tRNase Z\textsuperscript{6} concentrations (Fig. 6). The panels were chosen to illustrate the maximum product observed. Results with tRNase Z\textsuperscript{6} (Fig. 6, panels A–C) are generally in agreement with those reported above (Fig. 5, Table 2). These results confirm that tRNase Z\textsuperscript{6} is capable of processing a substantial proportion (up to ~60%) in the wild type and 7445U→G reactions) of the labeled substrate in the experiments. The concentration of enzyme used in this experiment is substantially higher than that used for the efficiency and kinetic experiments; the most accurate kinetic performance of the minimum concentration of enzyme sufficient to produce an increase in product with increasing reaction time, whereas at the end point of maximum product formation, there is often little increase in product with longer incubation (e.g. lanes 1–3 of Fig. 6, panel A).

Reaction with tRNase Z\textsuperscript{6} requires a much higher concentration of enzyme, and a much lower percentage of product is obtained at the end point of the reactions (Fig. 6, D–F, cf. A–C). The concentration of enzyme required was between 30- and 100-fold higher, and the maximum % product obtained was between 6- and 30-fold lower. These results generally agree with the

FIGURE 2. Michaelis-Menten kinetics of human nuclear-encoded pre-tRNA\textsuperscript{Ser(UCN)} processing by tRNase Z\textsuperscript{6} and tRNase Z\textsuperscript{6}. Processing of pre-tRNA\textsuperscript{Ser(UCN)} was performed using 1.0 pm tRNase Z\textsuperscript{6} and 2.5 nm tRNase Z\textsuperscript{6} at 37°C for 5, 10, and 15 min in lanes 1–3, respectively, in each reaction. Unlabeled substrate was added to a concentration of 2, 5, 10, 20, and 50 × 10\textsuperscript{-8} in reactions 1–5, respectively. C. Eadie-Hofstee plot of the results in A and B. The Y intercept is \(V_{\text{max}}\) and the slope is ~\(k_{m}\).

motions on the 5’ side of the acceptor stem (35–37) and four mutations in the 3’ end trailer immediately after the processing site (38–40) is unusual and suggestive (enclosed in ellipses in Fig. 4; see “Discussion”).

Effect of Substitutions in tRNA\textsuperscript{Ser(UCN)} after the tRNase Z Cleavage Site on Cleavage Site Selection and Processing Efficiency—Cleavage by tRNase Z\textsuperscript{6} is usually precise, but variant sequences in both substrate and enzyme can affect cleavage site selection (42, 43). Two substitutions past the tRNase Z cleavage site in tRNA\textsuperscript{Ser(UCN)}, 7445U→C and 7444C→U (Fig. 4), lead to a predominant tRNase Z\textsuperscript{6} cleavage one nucleotide in the 3’ direction from the normal cleavage site (Fig. 5, panels B and D, indicated with an asterisk; cf. product bands in A, C, and E). tRNA-N ↓ and -N+1 ↓ cleavage products (in which N represents the discriminator) are present in the proportion between 1:10 and 1:5 throughout the reaction time courses. Because the aberrant cleavage products dominate the pattern with these two mutant tRNAs, products were combined to obtain the kinetic parameters (Table 2); processing efficiencies for 7445U→C and 7444C→U would be 5–10-fold lower if the aberrant cleavage products were excluded.

The concentration of tRNase Z\textsuperscript{6} was adjusted to match the overall extent of processing in the time courses and kinetic analyses (Fig. 5). Wild type tRNA\textsuperscript{Ser(UCN)} can be efficiently processed using 12.5 pm tRNase Z\textsuperscript{6}; the 7445U→C mutant requires 100-fold higher enzyme concentration, and 7444C→U reactions can be performed with a 2-fold lower enzyme concentration than with the wild type substrate, but as illustrated above (Fig. 5), the reaction products are aberrant.
Mutations in Mitochondrial tRNA\textsuperscript{Ser(UCN)} Affect Processing

A detailed comparison of nuclear-encoded pre-tRNA\textsuperscript{Arg} processing by tRNase Z\textsuperscript{L} and tRNase Z\textsuperscript{S} (Figs. 1 and 2) and the analysis of mitochondrial pre-tRNA\textsuperscript{Ser(UCN)} (Figs. 1–3 and 5 and Tables 1 and 2), two additional pre-tRNAs were tested (Fig. 7). Pre-tRNA\textsuperscript{Val} is a canonical, nuclear-encoded pre-tRNA with a 14-nt 3' end trailer (Fig. 7B; Ref. 29, accession number X17514). Mitochondrial pre-tRNA\textsuperscript{Ser(UCN)} from human mitochondria has a length of 75 nt to the discriminator and was constructed with a 38-nt 3' end trailer (Fig. 7C; Ref. 44). These two pairs of nuclear-encoded and mitochondrial pre-tRNAs generally gave comparable product end points and time courses when processed with tRNase Z\textsuperscript{L}. Differences between the kinetic results presented here (Fig. 2, Table 1) and those obtained by others using a very similar substrate and enzyme (45), specifically, a 60-fold higher \( k_{\text{cat}} \) and \(~300\)-fold higher \( k_{\text{cat}}/k_{\text{m}} \), which combine to produce a \(~5\)-fold higher \( k_{\text{cat}}/k_{\text{m}} \), could be explained by differences in expression (including post-translational modification and initiation at r50), reaction conditions, and the methods of kinetic analysis.

**TABLE 1**

| Enzyme | \( k_{\text{cat}} \) (min\(^{-1}\)) | \( k_{\text{m}} \) (\( \times 10^{-8} \) M\(^{-1}\) s\(^{-1}\)) | \( k_{\text{cat}}/k_{\text{m}} \) | Re \( k_{\text{cat}}/k_{\text{m}} \) (n-fold reduction) |
|--------|-----------------|-----------------|-----------------|-----------------|
| tRNase Z\textsuperscript{L} | 371 \( \pm \) 151 | 2.2 \( \pm \) 0.5 | 160 \( \pm \) 32 | 1 |
| tRNase Z\textsuperscript{S} | 1.24 \( \pm \) 0.24 | 13.2 \( \pm \) 3.9 | 0.097 \( \pm \) 0.010 | 6.08 \( \pm \) 1.9 E-04 |

\( ^{*} \) Re \( k_{\text{cat}}/k_{\text{m}} \) (n-fold reduction) refers to the relative ratio of processing efficiencies; \( k_{\text{cat}}/k_{\text{m}} \) (tRNase Z\textsuperscript{L})/\( k_{\text{cat}}/k_{\text{m}} \) (tRNase Z\textsuperscript{S}) in parallel kinetic experiments.
TABLE 2

Kinetics of tRNase Z+ processing with the human mitochondrial tRNA\(^{\text{Ser}(UCN)}\) wild type and seven mutant substrates

Averages are presented of two Michaelis-Menten experiments with each of the mutants; wild type experiments performed in parallel with the mutant experiments were performed eight times. The relative \(k_{cat}/k_m\) for the mutants (fifth column; \(n\)-fold reduction relative to wild type) indicates that all of the naturally occurring mutations in tRNA\(^{\text{Ser}(UCN)}\) that were analyzed reduce the tRNase Z+ processing efficiency except for 7444C→U, which causes a change in cleavage site. \(k_{cat}/k_m\) and relative \(k_{cat}/k_m\) (Re WT) do not always agree precisely because values reported in the second through fourth columns are the means of all experiments, and values in the fifth column are the ratio of mutant/wild type \(k_{cat}/k_m\) from experiments performed on the same day.

| tRNA\(^{\text{Ser}(UCN)}\) | \(k_{cat}\) | \(k_m\) | \(k_{cat}/k_m\) | Re WT* |
|--------------------------|----------|----------|----------------|--------|
| Wild type                | 4.4 ± 0.9 | 0.48 ± 0.11 | 10.1 ± 1.7 | 1 |
| 7512A→G                  | 2.7 ± 0.5 | 0.80 ± 0.11 | 6.7 ± 1.2 | 0.68 ± 0.09 |
| 7511A→G                  | 3.2 ± 0.3 | 0.76 ± 0.19 | 4.6 ± 1.6 | 0.45 ± 0.04 |
| 7510A→G                  | 2.9 ± 0.8 | 1.08 ± 0.47 | 3.1 ± 0.7 | 0.34 ± 0.10 |
| 7445U→G                  | 0.76 ± 0.17 | 1.97 ± 0.49 | 0.40 ± 0.03 | 0.06 ± 0.003 |
| 7445U→G                  | 20.7 ± 2.1 | 8.55 ± 0.12 | 2.4 ± 0.09 | 0.35 ± 0.06 |
| 7444C→G                  | 9.2 ± 4.2 | 0.69 ± 0.11 | 12.7 ± 4.1 | 1.66 ± 0.39 |
| 7443U→G                  | 13.9 ± 4.1 | 6.33 ± 0.43 | 2.2 ± 0.5 | 0.29 ± 0.04 |

* Re WT (\(n\)-fold reduction re wild type) refers to the ratio of processing efficiencies; the mean \(k_{cat}/k_m\) (mutant) /\(k_{cat}/k_m\) (wild type) in parallel kinetic experiments.

**FIGURE 6.** tRNase Z+ can efficiently process wild type and mutant mitochondrial tRNA\(^{\text{Ser}(UCN)}\) substrates whereas tRNase Z+ cannot. tRNase Z+ was used to process mitochondrial tRNA\(^{\text{Ser}(UCN)}\) wild type (WT), 7445U→G, and 7445U→G in panels A–C, respectively. M, marker lane. Lanes 0, 1, 2, and 3 show sampling at 0, 5, 10, and 15 min. The parentheses signify spurious nonspecific RNA degradation. The maximum % product obtained and the concentration of tRNase Z+ are shown below the panels. D–F are the same as A–C except that tRNase Z+ used at the higher concentrations is indicated below the panels instead of tRNase Z+. The maximum percentage of product obtained was much lower, as indicated.

**AFFECTING PROCESSING**

**Mutations in Mitochondrial tRNA\(^{\text{Ser}(UCN)}\) Affect Processing**

**Effects of Mutations on Pre-tRNA\(^{\text{Ser}(UCN)}\) Secondary Structure**—Canonical tRNAs possess a cloverleaf secondary structure consisting of the acceptor stem and the D arm, anticodon arm and T arm, the last three comprised of a stem and closing loop. Sequence- and structure-specific ribonucleases are useful probes for RNA secondary structure (46); RNase T1 cleaves specifically after G residues and under native conditions, G residues in unstructured regions are relatively susceptible. S1 and V1 cleave single-stranded unstructured regions of RNA and duplex, structured regions, respectively, producing alternating patterns corresponding to the stem/loop/stem/loop structure. These methods were used to investigate the effect of naturally occurring mutations on tRNA\(^{\text{Ser}(UCN)}\) secondary structure (Figs. 4, 8, and 9; cf. Refs. 27 and 34).

Despite a weaker acceptor stem consisting of \(+\text{GAAAAAG}\) paired with \(\text{U}\text{UUUUUUCG}\), wild type tRNA\(^{\text{Ser}(UCN)}\) possess the canonical secondary structure, providing a base line for comparison to assess the structural effects of naturally occurring mutations.

Structure changes were observed with the acceptor stem mutants 7512A→G, 7511A→G, and 7510A→G (Fig. 8). A “missing tooth” is seen in the V1 patterns on the 3′ side of the acceptor stem (Fig. 8; the bracket at the right of panel A and the arrow at the right of panels B–D) that corresponds with the U opposite the substituted G: 7512A→G = G3/U70; 7511A→G = G4/U69; 7510A→G = G5/U68. The presence of G opposite U in the acceptor stem locally reduces the secondary structure (see the interpretative secondary structure diagram in Fig. 8F, based on data panels A–D and traces in E), providing a structural basis for the effects on processing efficiency and kinetics, which increase as A→G substitutions move down the acceptor stem. For technical reasons, the structural effects of the mutations at +3, 4, and 5 could not be directly visualized on the 5′ side of the acceptor stem.

The 7445U→G substitution one nucleotide beyond the body of mature tRNA\(^{\text{Ser}(UCN)}\) produces a different structure from the wild type (Fig. 9B, cf. A), confined almost entirely to the 3′ side of the acceptor stem (nt 67–72) and the first few nt of the 3′ end trailer (especially U76). U67–U71 become less sensitive to V1 (the dashed line to the right of Fig. 9A and B); the acceptor stem is, thus, less structured. The V1 pattern is practically unaffected from C66 to the bottom of the gel, suggesting that the G7/C66 base pair at the base of the acceptor stem can isolate a rearranged acceptor stem from the rest of the tRNA. Increases in V1 sensitivity are observed at U72 and U76 in the 7445U→G mutant (the solid line to the right of Fig. 9B; second trace in panel C). S1 sensitivity in the 3′ end trailer of 7445U→G pre-tRNA\(^{\text{Ser}(UCN)}\) correspondingly decreased (S1 lanes in Fig. 9B, cf. A; bottom traces in Fig. 9C), confirming that this region became more structured as a result of the substitution. These more structured U residues, symmetrically placed on the 5′ side of the 7445U→G (G74) substitution, suggest that an alternative structure can replace the canonical acceptor stem.

**DISCUSSION**

In organisms such as Homo sapiens in which tRNase Z+ and tRNase Z− are both present, it has not been established which one is principally...
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Involvement of mitochondrial tRNAs in pre-tRNA 3′ end processing. Human tRNase Z\textsuperscript{1} and tRNase Z\textsuperscript{2} have been expressed and compared before (32, 33) but with less detailed analysis of their differences in processing efficiency and kinetics. Mitochondrial tRNAs generally depart from the canonical tRNA structure (47) and might, thus, be predicted to be poorer tRNase Zs. Mitochondrial tRNAs generally depart from the canonical tRNA structure (47) and might, thus, be predicted to be poorer tRNase ZS have been expressed and compared before (32, 33) but with less detailed analysis of their differences in processing efficiency and kinetics. Mitochondrial tRNAs generally depart from the canonical tRNA structure (47) and might, thus, be predicted to be poorer tRNase Zs. Mitochondrial tRNAs generally depart from the canonical tRNA structure (47) and might, thus, be predicted to be poorer tRNase ZS have been expressed and compared before (32, 33) but with less detailed analysis of their differences in processing efficiency and kinetics. Mitochondrial tRNAs generally depart from the canonical tRNA structure (47) and might, thus, be predicted to be poorer tRNase Zs. Mitochondrial tRNAs generally depart from the canonical tRNA structure (47) and might, thus, be predicted to be poorer tRNase Zs. Mitochondrial tRNAs generally depart from the canonical tRNA structure (47) and might, thus, be predicted to be poorer tRNase Zs. Mitochondrial tRNAs generally depart from the canonical tRNA structure (47) and might, thus, be predicted to be poorer tRNase Zs.

Comparison of kinetic parameters for tRNase Z\textsuperscript{1} processing of nuclear-encoded and mitochondrial substrates suggests tighter binding and less efficient chemistry of catalysis for the mitochondrial substrates (Table 2, cf. Table 1). This is the first report of $k_{cat}$ for tRNase Z with a mitochondrial substrate, but $k_{cat}$ values have previously been found to be on the same order as those reported here for tRNase Z reactions using mitoplast extracts with mitochondrial pre-tRNA substrates (27, 28, 44).

Like several other mitochondrial tRNAs, tRNA\textsuperscript{Ser(UCN)} appears to be a hot spot for naturally occurring mutations (Fig. 4, cf. www.mitomap.org). No obvious rules govern which mutations are pathogenesis-associated and which are neutral polymorphisms (48) nor the association between mutations, specific tRNAs, and particular pathologies. Interesting generalizations nonetheless emerge from examination of the mutations in tRNA\textsuperscript{Ser(UCN)} tRNase Z\textsuperscript{Ser(UCN)} could be susceptible to slippage and possible rearrangements due to a weak acceptor stem consisting of a 1⁄72 GU pair followed by five AU pairs (Fig. 4), perhaps contributing to the effects of substitutions in the acceptor stem and beyond the tRNase Z processing site. Three of the mutations on the 5′ side of the mid-acceptor stem arise from A → G substitutions (7512A→G, 7511A→G, 7510A→G), which could produce GU pairs with two H bonds. The GU pair would not be expected to be isosteric with AU (49), and local structural effects of these substitutions on the 3′ side of the acceptor stem opposite the substitutions were observed (Fig. 8), but interestingly, these structural effects do not appear to propagate from the position of the mutation. 7510A→G, the most impairing of the three substitutions, is separated from the processing site by 5 base pairs, which could correspond to a 1⁄2 turn of the duplex; because the processing site is on the 3′ side, 7510A→G and the cleavage site might face the same direction, perhaps augmenting the slight effect on chemistry of catalysis by increasing the difficulty of fitting the mutant substrate into the active site. Pathogenesis-associated mutations in human mitochondrial tRNA\textsuperscript{Ser(UCN)} on the 3′ side of the acceptor stem affect tRNase Z processing (44), and effects of acceptor stem substitutions on tRNase Z processing have also been observed with a mitochondrially encoded potato tRNA (50) and a nuclear-encoded fruit fly tRNA (51).

After the tRNase Z cleavage site, 7445U→C has been independently confirmed by several groups to be associated with non-syndromic deafness (38, 39). This nucleotide would not ordinarily be present in mature tRNA, suggesting that the molecular basis for pathogenesis is a defect in pre-tRNA maturation (38, 52), perhaps due to the introduction of CC, a tRNase Z anti-determinant (27). Interestingly, three other naturally occurring mutations after the discriminator (7445U→C, 7444C→U, 7443U→C) were discovered among Mongolian students in a school for the deaf. These mutations were associated with the 1555A→G polymorphism in small tRNA that causes deafness with use of aminoglycoside antibiotics (53). Although they have not been confirmed to be pathogenesis-associated, the 7444C→U (7444G→A) substitution was suggested several years earlier to lead to translational read-through of the COX1 mRNA on the complementary H strand transcript (54). These mutations affect the tRNase Z reaction (Fig. 4, Table 2), demonstrating sensitivity of the enzyme to the sequence immediately after the processing site.

Proximity of the substitutions to the processing site and a weak acceptor stem could contribute to the observed shift in cleavage site and changes in kinetic parameters (Fig. 5, Table 2). Interestingly, consequences of these aberrant cleavages in vitro would be different; the 7445U→C substitution produces the first and second C residues of the CCA at the 3′ end of mature tRNA, and if the aberrant cleavage occurred between them, maturation of the tRNA could easily be completed by the CCA-adding enzyme. 7444C→U, on the other hand, produces a cleavage product that would have one U protruding beyond the discriminator. This protruding U could be removed by tRNase Z (15), but this type of polishing by tRNase Z appears to be inefficient (Fig. 5, panel D). Trimming by a 3′ exonuclease is in the maturation pathway of Escherichia coli tRNAs (7, 8); results from several laboratories suggest that a 3′-exonuclease can rescue defective endonu-
FIGURE 8. Secondary structure probing of human mitochondrial wild type tRNA<sup>Ser(UCN)</sup> and the acceptor stem substitutions. A–D, the structure probing was performed using the wild type (WT) and mutant pre-tRNAs as designated above the panels. The lanes are a marker (M), untreated 5' end labeled tRNA (0), an alkaline ladder (AL), semi-denaturing T1 (SD T1), two lanes of native T1 (N-T1), two lanes of nuclease S1 (S1), and two lanes of nuclease V1 (V1) as designated below the panels in lanes 1–9, respectively. E, traces from the V1 lanes. F, interpretive secondary structure diagrams of the acceptor stem. In all panels, the bracket signifies the region from nt 67 to 71 on the 3' side of the acceptor stem, and the arrowheads indicate the effect of the acceptor stem substitutions on the V1 pattern and the secondary structure. Nucleotide numbers 7512–7510 are based on the Cambridge numbering of nucleotides in the human mitochondrial genome, and the other numbers used (e.g. 66–72 in the secondary structure diagram) are based on the canonical numbering scheme for tRNAs.
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...cleolytic pre-tRNA 3′ end processing in yeast (23, 55), but this has not been demonstrated directly.

Mature tRNA is not a substrate for 3′ end processing (15–17); indeed, CCA of mature tRNA functions as a tRNase Z processing anti-determinant (but see Schiffer et al. (18) for opposite results). The mechanism of CCA anti-determination has not been ascertained, but resides around the tRNase Z active site may form a rigid binding and exit channel for the tRNA 3′ end trailer, cooperating with nucleotides directly after the discriminator to regulate pre-tRNA catalysis (20). In the case of the 7445U→C substitution suggested to produce the all-important CCA of a CCA anti-determinant (27), an unfavorable interaction with CCA could be reduced, allowing some cleavage, if the 3′ end trailer does not penetrate as deeply into the active site as normal. Additionally, a structural rearrangement arises from the 7445U→C substitution after the discriminator (Fig. 9). Secondary structure probing is not sufficient to solve the alternate folding but might explain the high \( k_{\text{on}} \) of 7445U→G tRNA\textsuperscript{Ser(UCN)} since a structured 3′ end trailer directly after the processing site could sterically interfere with correct placement of the scissile bond in the active site of tRNase Z.

Future Prospects—These results illustrate the importance of naturally occurring mutations in the acceptor stem and immediately after the body of mature tRNA for tRNase Z processing as it may relate to mitochondrial pathogenesis, but significant questions remain unanswered. The 16-fold lower processing efficiency of mitochondrial compared with nuclear-encoded pre-tRNAs by tRNase Z\textsuperscript{L} and the 1600-fold lower processing efficiency of nuclear-encoded pre-tRNA\textsuperscript{ZS} by tRNase Z\textsuperscript{S} than by tRNase Z\textsuperscript{L} have not been completely explained. Persistence of tRNase Z\textsuperscript{S} in the human genome and proteome, perhaps analogous to the retention of the corresponding tRNase Z\textsuperscript{L} in \textit{E. coli} without an established function (56), remains enigmatic. tRNase Z\textsuperscript{S} could be involved in tRNA maturation, which is a complex process, perhaps in a redundant system in which an RNA 3′-exonuclease also participates. The tRNase Z\textsuperscript{S} gene could also function as a reservoir for the evolution of other His domain (metal binding) proteins. Interestingly, tRNase ZL and tRNase ZS exhibit complementary patterns of expression in mouse stem cells; tRNase ZL is expressed most heavily in embryonic stem cells, and tRNase ZS is most heavily expressed in adult stem cells (57).

CPSF73 (a protein in a neighboring branch of the \( \beta \)-lactamase family of metal-dependent hydrolases) has been recently suggested to be the long-sought endonuclease involved in pre-mRNA (including pre-histone mRNA) 3′ end maturation (58, 59), and the Mediator protein, a CPSF73 homolog, has been suggested to be involved in small nuclear RNA 3′ end processing (60, 61). By analogy, human tRNase Z\textsuperscript{S} may yet be assigned a function in RNA metabolism. Finally, interaction between the pre-tRNA 3′ end trailer and the CCA anti-determinant domain of tRNase Z is complex, as suggested by the effects of substitutions just beyond the normal processing site on cleavage site selection, processing kinetics and pre-tRNA structure.

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