Matrices of Paired Substitutions Show the Effects of tRNA D/T Loop Sequence on Drosophila RNase P and 3'-tRNase Processing*

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Drosophila RNase P and 3'-tRNase endonucleolytically process the 5' and 3' ends of tRNA precursors. We examined the processing kinetics of normal substrates and the inhibitory effect of the tRNA product on both processing reactions. The product is not a good RNase P inhibitor, with a K<sub>i</sub> approximately 7 times greater than the substrate K<sub>M</sub> of ~200 nM and is a better inhibitor of 3'-tRNase, with a K<sub>i</sub> approximately two times the K<sub>M</sub> of ~80 nM.

We generated matrices of substitutions at positions G<sup>291</sup>/G<sup>292</sup> and G<sup>55</sup>/C<sup>56</sup> (two contiguous universally conserved D/T loop base pairs) in Drosophila tRNA<sup>His</sup> precursors. More than half the variants display a significant reduction in their ability to be processed by RNase P and 3'-tRNase. Minimal substrates with deleted D and anticodon stems could be processed by RNase P and 3'-tRNase much like full-length substrates, indicating that D/T loop contacts and D and/or anticodon contacts are not required by either enzyme.

Selected tRNAs that were poor substrates for one or both enzymes were further analyzed using Michaelis-Menten kinetics and by structure probing. Processing reductions arise principally due to an increase in K<sub>M</sub> with relatively little change in V<sub>max</sub> consistent with the remote location of the sequence and structure changes from the processing site for both enzymes. Local changes in variant tRNA susceptibility to RNase T1 and RNase A did not coincide with processing disabilities.

tRNAs are small (~76 nt)<sup>3</sup> molecules that possess a conserved cloverleaf secondary structure (1) and a compact L-shaped tertiary structure stabilized by numerous contacts (2). tRNA precursors undergo endonucleolytic end processing and modification before their aminoacylation and use in translation (for reviews, see Ref. 3). An additional G is added to the 5' end of tRNA<sup>His</sup> (4), and CCA is added to the 3' end of all eukaryotic tRNAs (5) following the RNase P and 3'-tRNase reactions in vivo.

Several post-transcriptional tRNA processing reactions have been used to evaluate the relation between primary, secondary, and tertiary structure in recognition and catalysis. RNase P, a ribozyme (6), recognizes internal tRNA tertiary structure; enzyme-substrate base pairs which are important in RNase P catalysis have also been demonstrated between tRNA nucleotides C<sup>74</sup> and C<sup>75</sup> and Escherichia coli M1 RNA nucleotides G<sup>289</sup> and G<sup>292</sup> (7). Although not directly relevant to eukaryotic tRNA end processing reactions (eukaryotic tRNA genes lack a transcriptionally encoded 3'-CCA), the observation of base pairing between enzyme and substrate nonetheless raises the possibility of direct readout of internal substrate sequence, either by RNA or protein in the processing enzymes.

None of the positions where tRNA sequence varies (8), however, could be sequence-specific substrate determinants for recognition or catalysis by general tRNA processing enzymes such as RNase P and 3'-tRNase. The universally conserved, paired nucleotides G<sup>289</sup>/C<sup>290</sup>, G<sup>55</sup>/C<sup>56</sup> in the tRNA D and T loops are good candidates for direct readout. These base pairs also stabilize the tRNA tertiary fold. Herein, we examine the effects of substitutions in these D and T loop nucleotides.

tRNA tertiary contacts have recently been analyzed using substitutions in studies of aminoacylation (9–11) and of suppressor tRNA function (12). Interestingly, the first C of the 3'-CCA pairs with conserved C<sup>252</sup> in the peptidyltransferase center of 23 S ribosomal RNA, as proven by partial matrix analysis (13).

We have studied the 5' and 3' end endonucleolytic processing of Drosophila tRNA<sup>His</sup> precursors (14). In the present report, we investigate processing kinetics and the inhibitory effect of the tRNA product on the RNase P and 3'-tRNase reactions. We generated matrices of substitutions in the conserved nucleotides that stabilize the D/T loop tertiary interaction; most of these variants display reduced processing by RNase P and/or 3'-tRNase. Mini-tRNAs with deleted D and anticodon arms could be processed much like wild type tRNAs by both RNase P and 3'-tRNase, demonstrating the absence of required substrate contacts by either enzyme within these regions. By structure probing, we establish that substrate defects do not generally coincide with the disruption of tertiary contacts. Structure modeling the D and T loop substitutions suggests that local perturbations may have occurred in the variants.

** EXPERIMENTAL PROCEDURES

Cell Culture, Extract Preparation, and Fractionation of RNase P from 3'-tRNase—Drosophila Kc<sub>2</sub> cells adapted to serumless growth were cultured and harvested, and S100 extracts were prepared as described previously (15). RNase P and 3'-tRNase in the S100 were separated by S-Sepharose column chromatography (14). The 50 ml KCl S-Sepharose
small high copy number vector PHC624 (16) for propagation and into the tRNAPhe gene. The template for transcription was obtained by digestion with NcoI (New England Biolabs) to obtain an RNase P substrate or with DraI (Amersham Corp.) to obtain a 3′-tRNase substrate. Runoff transcripts were gel-purified, extracted by a cut-and-crush procedure, and recovered for processing reactions.

Unlabeled RNAs were prepared using 1.25 mM each of four NTPs and a 5-fold molar excess of GMP to obtain a monophosphate at +1 (18). The monophosphate at +1 could be important, both for substrate and for the study of inhibition by product, due to a large local charge difference compared with a triphosphate in the vicinity of the active site for both enzymes. T7 RNA polymerase with a 6-residue N-terminal His tag was prepared using Ni²⁺-NTA-Sepharose (Qiagen). Unlabeled RNAs were detected by UV shadowing, recovered using the Elutrap (Schleicher and Schuell), and concentrations determined by absorbance.

5′-End-labeled RNAs were prepared as described previously (14) by treating unlabeled T7 transcripts with shrimp alkaline phosphatase followed by incubation with [γ-32P]ATP and polynucleotide kinase. 3′-tRNase substrates were 3′-end-labeled for structure probing by preparing 3′P-Cp using [γ-32P]ATP and unlabeled Cp (Sigma) with polynucleotide kinase (the mutant from Boehringer Mannheim lacking 3′-phosphatase activity, as recommended by R. Gumpert). The 32P-Cp was attached to the RNA 3′ end using T4 RNA ligase (19; a gift of R. Gumpert or from Amersham Corp.). End-labeled tRNAs were gel-purified.

Plasmid DNAs were extracted using Wizard minipreps (Promega). All mutants were confirmed by supercoil DNA sequencing using Sequenase version 2.0 (Amersham Corp.). Plasmid DNAs were purified on a larger scale by CsCl ethidium bromide banding.

Preparation of RNAs for Processing and Structure Probing—Internally labeled RNAs were prepared by transcription with T7 RNA polymerase using [α-32P]UTP and three unlabeled NTPs (15). Templates for transcription were obtained by digestion with NsiI (New England Biolabs) to obtain an RNA 5′ end or with DraI (Amersham Corp.) to obtain a 3′-tRNase substrate. Runoff transcripts were gel-purified, extracted by a cut-and-crush procedure, and recovered for processing reactions.

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Processing Reactions—RNase P reactions were performed using a 1/10 dilution of the S-Sepharose 200 mx KCl fraction incubated with the internally labeled RNase P substrate (Fig. 1B) in a volume of 60 μl containing 30 m pot K-Hepes, pH 8, 175 m KCl, 3 m MgCl₂, 3 m dithiothreitol, 0.1 m phenylmethylsulfonyl fluoride, 0.1% Tween 20, 10% glycerol, and 1 μl of RNase P (40 units; Promega) at 28 °C. Reactions were sampled after 0, 15, 30, 60, and 120 min, recovered, and analyzed on 6% denaturing polyacrylamide gels. Results were obtained by autoradiography. 3′-tRNase reactions were performed similarly except that the S-Sepharose flow-through was the source of the enzyme; the 3′-tRNase substrate (Fig. 1C) was processed, and the reaction buffer contained 50 m KCl.

Quantitation—Microdensitometry was performed on all lanes of the autoradiograms using an LKB XL laser microdensitometer. The processing product of both RNase P and 3′-tRNase is a 72-nt tRNA which runs from G¹ to A¹⁸ (Figs. 1–4). Bands above and below the 72-nt product were not scored. These quantitation procedures using a limiting amount of labeled substrate (14) yielded first-order processing rate constants (Vmax/KM) relative to wild type (bottom of Figs. 3 and 4); processing reactions with variants and wild type were performed in parallel on the same day to correct for possible day to day differences in enzyme activity or in the preparation of processing buffer. The lower limit for detection of processing using these methods was less than 1% of the wild type.

For KM and Vmax determinations, we used lower enzyme concentrations with shorter reaction times to obtain product yields that are close to linear, and we performed quantitation using a Storm 840 PhosphorImager and ImageQuant software (Molecular Dynamics). Because the enzymes were not purified to homogeneity, we did not determine kcat but, instead, normalized all Vmax values. The first-order rate constants (Vmax/KM) relative to wild type do not agree precisely with results presented in the processing matrices, perhaps due to differences between these two types of processing experiments.

Structure Probing—Recovered end-labeled tRNAs were heated to 70 °C for 5 min in distilled H₂O and reformed by cooling to room temperature for 10 min in 15 mK-MOPS, pH 7, 175 mKCl, and 3 m MgCl₂ containing 1.25 μg of unlabeled tRNA per 5-μl reaction. Samples were incubated with RNase T1 at 0.4 or 1 unit/ml or with RNase A at 1.5 or 4 × 10⁻³ units/ml, deproteinized, recovered, and analyzed on 10% polyacrylamide denaturing gels.

Structure Modeling—BioSym software was used on a Silicon Graphics Indigo² Extreme to obtain wild type and variant tRNA structure models. A yeast tRNA²⁵⁰ structure from the Brookhaven Protein Data Bank was stripped of modifications and Mg²⁺ so that potentials could be recognized by the software (following instructions provided by J. Stuart, with technical support by E. VanRiper). The structures were minimized by 100 iterations of steepest descent.

RESULTS

The Numbering and Sequence Conservation of Drosophila tRNAHis. We have renumbered Drosophila tRNAHis to match yeast tRNA²⁵⁰ (Fig. 1; 20) by omitting nt 17 and including nt 20a in the D loop and by omitting nt 46 in the V loop. Five of the eight tertiary contacts in yeast tRNA²⁵⁰ (8–14, 15–48, 18–55, 19–56, 22–46, 23–9, 26–44, and 54–58; see Ref. 21), are conserved in Drosophila tRNAHis (bold), based on its sequence and presumed secondary structure (dashed lines in Fig. 1A; green and blue bases in Fig. 1F).

Unlinking the Substrates for RNase P and 3′-tRNase—The Drosophila tRNAHis precursor has a 5′ end leader and a 3′ end trailer which are endonucleolytically removed by RNase P and 3′-tRNase, respectively (Fig. 1A; Refs. 14 and 22); both enzymes share the same internal substrate. We simplified the substrates from the in vivo precursor to minimize possible interference between the 5′ end leader and 3′ end trailer in RNase P refolding and to avoid any problem with reaction order, which appears to be first 5′ end, then 3′ end, processing in vitro (22, 23). The RNase P substrate has a 16-nt 5′ end leader and a 3′ end at A³⁸ (Fig. 1B). The 3′-tRNase substrate has a mature 5′ end and a 36-nt 3′ end trailer (Fig. 1C). The product of both reactions is thus a 72-nt tRNA (nt 1–73). We have used this 72-nt tRNA to determine product KM values for both RNase P and 3′-tRNase (Fig. 2).

Drosophila in Vitro tRNA End Processing Reactions—Pro-

![Figure 2. tRNA end processing kinetics. Panel A, wild type tRNA with a 36-nt 3′ end trailer was processed with 3′-tRNase for 10, 20, and 30 min in lanes 1–3, respectively. The ³²P-tRNA was maintained constant, and unlabeled substrate was added to a final concentration of 0, 2.5, 5, 12.5, 25, 50, and 100 × 10⁻⁷ M in parts A–G, respectively. Panel 2, reactions were the same as in panel 1 but with the 72-nt product of the 3′-tRNase reaction added to a final concentration of 5 × 10⁻⁷ M. Panel 3, Edie-Hofstee analysis of the 3′-tRNase reactions using reaction velocities determined from parts E–G of panels 1 and 2 in the absence (±Prod; solid line) or presence (±Prod; dashed line) of product, respectively. Panel 4, the KM and product K were determined from the data in panels 1 and 2, and in similar RNase P reactions (not shown), using Edie-Hofstee analysis to determine KM and K (slopes in panel 3), and the formula K = (V)/[K] to solve for K.

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FIG. 3. RNase P and 3′-tRNase 18/55 processing matrices. All possible variants at nts 18 and 55 were constructed and tested as substrates in RNase P (left, panels 1–5) and 3′-tRNase (right, panels 6–10) processing reactions. Variants tested are identified below each panel. The A parts of each panel are wild type; B, C, and D parts are variants as noted below each panel. Processing reactions were sampled and analyzed after 0,
Fig. 4. RNase P and 3′-tRNase 19/56 processing matrices. Same as Fig. 3, except that all possible pairwise substitutions at nts 19 and 56 were analyzed.

15, 30, 60, and 120 min of incubation (lanes 1–5 of each set, respectively). Processing efficiencies relative to wild type are indicated in the matrices at the bottom of each figure. Each matrix value is a proportion of wild type processing rate (bold; 1.00). Dashed rectangles and circles highlighting specific matrix values are explained in the text.
karyotic RNase P is subject to product inhibition, especially when its catalytic RNA component is used for processing (24–26). Fierke and co-workers (27) have found that under physiological conditions, however, the affinity of RNase P RNA for substrate and product decreases significantly due to an increase in the dissociation rate constant, thereby also lessening inhibition by product.

We investigated the kinetics of the fly RNase P and 3′-tRNase reactions (Fig. 2 and data not shown) by varying substrate concentration with a constant amount of input [32P]-tRNA, in the presence or absence of a possible inhibitor, the 72-nt product of these reactions. Panel 1 of Fig. 2 shows a time course (10–20-30-min incubations; no burst of processing activity was observed with short reactions) for the 3′-tRNase reaction at seven different concentrations of substrate (A-G).

Panel 2 shows the same substrate concentration series in the presence of 5 × 10⁻⁷ m 72-nt tRNA product. Edie-Hofstee plots used for the determination of $K_M$, $V_{max}$, and $K_I$ are shown in panel 1 of Fig. 2. The ~3-fold increase in slope in the presence of product (+Prod.; - -) with a very similar y intercept is consistent with competitive inhibition.

Fly RNase P displays a $K_M$ of 220 nm (panel 4 of Fig. 2), similar to that for other eukaryotic RNase Ps (e.g. Ref. 28), and $K_I$ for 3′-tRNase is 80 nm. $K_I$ for the 72-nt tRNA product was $1.6 \times 10^{-4}$ M for RNase P and $1.5 \times 10^{-7}$ M for 3′-tRNase. Added product is thus a poor inhibitor of RNase P with a $K_I$ approximately 7 times wild type $K_M$, product is, however, a better inhibitor of 3′-tRNase, with a $K_I$ approximately 2 times the $K_M$ for wild type substrate (Fig. 2C).

Purity of the Enzymes—Before examining the effects of internal substrate sequence on RNase P and 3′-tRNase processing, we consider the purity of enzymes used for the analysis and the possible effects of endogenous inhibitors. Fractions from early stages of enzyme purification might remove endogenous inhibitors and thereby reduce $K_M$. We repeated the kinetic analysis using wild type substrates and enzyme fractions that had been further fractionated by anion exchange chromatography (MonoQ; data not shown), which greatly reduces the concentration of endogenous RNA. We found no decrease in $K_M$, and the case of 3′-tRNase, $K_I$ increased with purification.

Matrix Analysis—The D/T loop tertiary contacts are illustrated schematically in Fig. 1D, and in green in the tRNA*^the tertiary structure (Fig. 1E). We made all 15 possible pairwise substitutions at 18/55 and 19/56 (Fig. 1D) in both RNase P and 3′-tRNase substrates (Fig. 1, B and C) and tested their ability to be processed (Figs. 3 and 4). Relative processing rates are presented in the 16-place matrices (below the processing autoradiograms in Figs. 3 and 4), in which the rows represent the indicated D loop and the columns represent the T loop substitutions. A row in the matrices with reduced processing values suggests that a D loop substitution disables processing; similarly, a column of reduced values implies a processing defect caused by the T loop substitution.

U55A Severely Reduces Processing by Both RNase P and 3′-tRNase, Regardless of the Substitution at nt 18—There are four combinations in the matrices with the substitution U55A as follows: U55A which is wild type at G18 (Fig. 3, C parts of panels 2 and 7), G18C/U55A (D parts of panels 2 and 7), G18A/U55A (B parts of panels 5 and 10), and G18U/U55A (C parts of panels 5 and 10). The RNase P substrates are poorer than the 3′-tRNase substrates in the cases of G18A/U55A and G18C/U55A. The left-most columns in the matrices at the bottom of Fig. 3 (enclosed in dashed vertical rectangles) show that the inhibitory effect of the U55A substitution on both RNase P (left) and 3′-tRNase (right) cannot be compensated by changes at nt 18.

G18C Substitutions Reduce Processing—The four G18C substitutions, which include G18C with a wild type U at nt 55 (Fig. 3, panel 2B), G18C/U55A (panel 2D), G18C/U55G (panel 4B), and G18C/U55G (panel 4C), all severely inhibit RNase P, 3′-tRNase, on the other hand, is not inhibited by G18C (Fig. 3, panel 7B) and G18C/U55G (panel 9C). The quantitative results are enclosed in dashed horizontal rectangles in the third row of the matrices at the bottom of Fig. 3.

Some 18/55 Substitutions Inhibit 3′-tRNase More Than RNase P—In contrast to the above and our previous observations (14) that RNase P tends to be more sensitive to substrate changes than 3′-tRNase, we found three instances in the 18/55 matrix in which 3′-tRNase is more severely inhibited than RNase P (indicated by the dashed circles in the matrices at the bottom of Fig. 3); G18A/U55C (Fig. 3, D parts of panels 3 and 8), U55C (C parts of panels 3 and 8), and U55G (C parts of panels 1 and 6).

Substitutions at G19 and C56 Interfere Differently with Processing by RNase P and 3′-tRNase—G19/C56 is the only Watson-Crick base pair among the tRNA tertiary contacts. There are four C56A substitutions as follows: C56A (Fig. 4, panel 4D), C56A/C56U (Fig. 5A, panel 4D), G19U/C56A (panel 4C), and G19C/C56A (panel 4B), all severely inhibit RNase P, 3′-tRNase (on the left), whereas C56A (Fig. 4, panel 7D) and G19U/C56A (panel 9C) do not. Three of the C56A substitutions inhibit processing by both RNase P and 3′-tRNase (vertical rectangle on the left of the matrices at the bottom of Fig. 4), whereas C56A with a wild type G19 (enclosed by a dashed square in the matrices at the bottom left of Fig. 4) inhibits RNase P processing less severely.

Three 19/56 substitutions in addition to wild type hardly impair RNase P processing (dashed squares in the matrix at the bottom of Fig. 4): G19A/C56U (Fig. 4, panel 2D), G19C/C56G (Fig. 4, 1D), and C56A (Fig. 4, 3C). Interestingly, three of the four apopositions that are processed well are on the Watson-Crick diagonal (G19A/C56U, G19/U/C56 wild type, and G19C/C56G); the fourth member of the Watson-Crick set (G19U/C56A) is poorly processed by both RNase P and 3′-tRNase.

The 19/56 variants that are processed well by 3′-tRNase (dashed squares in the matrix at the bottom right of Fig. 4) are G19C (Fig. 4, panel 6B), C56G (Fig. 4, 6C), G19C/C56G (Fig. 4, 6D), and C56U (Fig. 4, 7C). Combinations which are processed
at an intermediate level include G19A/C56U and G19U (dashed circles). The other nine appositions interfere with 3'-tRNase activity. Seven of the 19/56 appositions are poorly processed by both RNase P and 3'-tRNase as follows: G19A/C56A, G19C/C56A, G19U/C56A, G19A/C56G, G19A, G19C/C56U, and G19U/C56U.

Processing Kinetics of Selected Variants—We prepared unlabeled selected variant RNAs (G18C, U55A, and C56A), mixed them in varying amounts with a fixed amount of internally labeled substrate, and performed Michaelis-Menten experiments to determine $K_M$ and $V_{max}$ for both RNase P and 3'-tRNase reactions (Table I). These variants were selected for kinetic analysis because each one was a poor substrate for either RNase P (G18C) or 3'-tRNase (C56A) or both (U55A). Reduced processing was due principally to increases in $K_M$, with relatively little effect on $V_{max}$, perhaps due to impaired substrate recognition and binding. Using the formula $\Delta G^\circ = -RT \ln(1/K_d)$ and setting $K_M$ equivalent to $K_d$, we can convert the ~75-fold increase in $K_M$ for U55A processed either with 3'-tRNase to a $\Delta G^\circ$ of 2.6, approximately equivalent to breaking 1–2 H bonds that could be involved in stabilizing the E–S complex (Ref. 29; see “Discussion”).

Deleted Substrates for RNase P and 3'-tRNase Processing—The D arm and anticodon arm were established to be dispensable.

Fig. 5. Minimal substrates can be processed by both RNase P and 3'-tRNase. The deleted substrates A11–42 (bottom of figure) were processed with RNase P (A) or with 3'-tRNase (B). Designations at left, marker nucleotide lengths. Designations at right, S is the substrate (56 nt for RNase P; 76 nt for 3'-tRNase); I is the 40-nt internal tRNA product; 5'EL is the 16-nt 5' end leader produced by RNase P, and 3'ET is the 36-nt 3' end trailer produced by 3'-tRNase. A, lanes 1 and 2, 0 and 60 min. RNase P reactions with undeleted RNase P substrate (Fig. 1B); lanes 3–6, 60-min RNase P reactions with deleted 56-nt mini-tRNA in standard RNase P reaction condition except using 250 mM KCl and MgCl$_2$ at 4.5, 3, 1.5, and 0.3 mM, respectively; B, lanes 1–4, 0, 15, 30, and 60 min. 3'-tRNase reactions with undeleted 3'-tRNase substrate (Fig. 1C) under standard 3'-tRNase reaction conditions, except using 0.3 mM MgCl$_2$. Lanes 5–8 are the same as lanes 1–4 except using the 76-nt deleted 3'-tRNase substrate.
sible for RNase P processing by M1 RNA in E. coli (30) and by RNase P holoenzyme in toad (31) and human (32). On the other hand, all four G18C substitutions are poor substrates for RNase P (Fig. 3) and the D stem substitution U11A is a poor substrate for both RNase P and 3'-tRNase (14). We thus investigated the possible functional importance of the D arm for the RNase P and/or the 3'-tRNase reaction by making fly mini-tRNAs consisting of the acceptor stem, T arm, and variable loop (Fig. 5). Both RNase P and 3'-tRNase can efficiently process the mini-tRNAs, as indicated by the 40-nt labeled internal product (I) at the predicted position on the gel, as well as the 16-nt 5'-end leader (5'EL, Fig. 5A) and 36 nt 3'-end trailer (3'ET, Fig. 5B) characteristic of these reactions.

In the process of optimizing mini-tRNA processing, we determined that 3'-tRNase works efficiently at submillimolar MgCl₂ as well as at 3 mM MgCl₂, although the processing is prevented by excess EDTA (data not shown), in agreement with the characterization of these eukaryotic 3'-tRNases as low [Mg²⁺] endonucleases (5).

Structure Changes Revealed by Nuclease Susceptibility Do Not Coincide with Processing Reductions—In the case of variants that gave different processing results for RNase P and 3'-tRNase, the possibility of differential misfolding arising from the internal substitutions was addressed by structure probing both 3'-end-labeled 3'-tRNA substrates and 5'-end-labeled RNase P substrates (Figs. 6 and 7). These variants include G18U (RNase P worse than 3'-tRNase), U55C (RNase P better than 3'-tRNase), G18C/U55G (RNase P worse than 3'-tRNase), illustrated in Fig. 3, and C56A and G19A/C56U (RNase P better than 3'-tRNase in both cases; Fig. 4). The most prominent RNase T1 site in wild type tRNA is at G₃₄ in the anticodon loop; D and T loop nucleotides can become susceptible to T1 as a result of D/T loop substitutions (arrowheads in the structure diagrams at left in Fig. 6 and Ref. 14).

The G19A/C56U RNase P substrate labeled at its 5'-end displayed increased T1 sensitivity at G₃₄ and G₅₈ compared with wild type (Fig. 6A, part F; cf. part A). Using 3'-end-labeled 3'-tRNA substrates (Fig. 6B), we observed an increase in D loop T1 sensitivity at G¹⁵ G¹⁸ and/or G¹⁹ in G18U, G18C/ U55G, and C56A (Fig. 6B, parts D and E) and an increase in T loop T1 sensitivity at G¹⁷ in G18C/U55G and in C56A (Fig. 6B, parts D and E). Some variants display greater T1 sensitivity of G¹⁸ and G⁵₈, suggesting that in these cases the D and T loop substitutions can weaken tertiary structure in the elbow.
Effects of D/T Loop Sequence on tRNA End Processing

region. We previously found the same increases in D and T loop T1 sensitivity arising from substitutions elsewhere in tRNA, however (14), and even occasionally in the wild type tRNA, perhaps due to random variations in tRNA refolding.

Next, we examined the folding of 5′-end-labeled U55A RNase P substrates (Fig. 7), which are all processed poorly by both RNase P and 3′-tRNase (Fig. 3). U55A substitutions generally displayed increased D and T loop sensitivity relative to wild type (A), G18A/U55A (B), U55A (C), G18C/U55A (D), and G18U/U55A (E).

**DISCUSSION**

Some Processing Determinants Are Shared between RNase P and 3′-tRNase, While Others Are Different—Eukaryotic 3′-tRNase has been little studied, and the contacts this processing endonuclease makes with tRNA have not been extensively mapped. Castaño et al. (23) suggested that Xenopus 3′-tRNase is a polypeptide enzyme. Zenarro et al. (33) found a temperature-sensitive lethal substitution (C81U) at the T stem-loop boundary in yeast mitochondrial tRNA and which abolished 3′-tRNase catalysis. T arm sequence may thus be important for 3′-tRNase, as it is for RNase P. Nashimoto (34, 35) determined that annealed half-tRNAs without an intact anticodon loop could be good 3′-tRNase substrates and that tRNA with CCA at its 3′ end is a poor substrate for 3′-tRNase.

We previously found acceptor (G4C), D (U11A), and T stem (U63A) single substitutions that inhibit both RNase P and 3′-tRNase (14). Our present work establishes that RNase P and 3′-tRNase do not recognize substrate determinants in the T loop or the tertiary fold in precisely the same way. Some variants are processed better by RNase P than by 3′-tRNase and some the other way around (see circled matrix values for U55G, U55C, G18C/U55G, and G18C for prominent examples in Fig. 3, and C56A and G19A/C56U in Fig. 4). These variants presumably have the same internal structure; altered loop structure, tertiary folding, or local contacts may thus be recognized differently by these two enzymes.

Substitution of T loop pyrimidines with As at either nt 55 or 56 inhibits both RNase P and 3′-tRNase in almost all cases (vertical dashed rectangles in the matrices in Figs. 3 and 4). There are several plausible explanations. First, U55A could disrupt the T loop U-turn (a suggestion of E. Westhof; see structure modeling in Fig. 8, A and B). Second, the bulky purines could simply take up too much room for a properly folded T loop (but note that substitution of a G at either position does not inhibit processing as severely). Finally, nt 55 and 56 could be important base-specific contacts for both enzymes.

**Influence of the D Arm on Processing Endonuclease Recognition**—The D arm contributes to Drosophila RNase P processing, based on the following results. First, U11A, a single substitution in a D stem base pair, abolishes processing (14). Second, D loop substitutions, especially G18C (Fig. 3), severely inhibit RNase P. Human RNase P is significantly inhibited by deletion of the tRNA D arm but not the anticodon arm (36). The tRNA contact surface with RNase P observed in prokaryotes, toad, and human (30–32) may thus be stabilized by a wild type D arm, perhaps due to an indirect effect on the spatial arrangement of the stacked acceptor stem and T arm. Conversely, the required substrate conformation could be destabilized by unfavorable D arm substitutions. By deleting the entire D and anticodon arms (Fig. 5), we produced RNase P and 3′-tRNase substrates that are processed much like full-length tRNAs. This approach is fundamentally different from the analysis of single or double substitutions; D arm substitutions might interfere with T loop structure and acceptor stem-T loop stacking in ways that a short linker would not.

**Variant Processing Kinetics**—The processing changes that arise from D/T loop substitutions are due largely to increases in $K_{p}$ (Table I), with relatively little change in $V_{max}$. The reduced processing rate arising from D/T loop substitutions would not be expected to affect catalysis, these positions being remote from the processing sites at the end of the acceptor stem. They might, on the other hand, be expected to affect substrate recognition and binding, since the T arm has important RNase P recognition elements (37, 38). Others (e.g. Refs. 7, 29, 39, and 40) have examined the effects of substrate changes in the neighborhood of the mature tRNA 5′ and 3′ ends on RNase P kinetics and, in some cases, found significant reductions in $V_{max}$ (due to effects on the chemistry of catalysis).

**Tertiary Folding**—Gs atnts 14, 18, and 19 in the D loop and at 57 in the T loop are protected from RNase T1 in wild type tRNA and can be exposed as a result of D/T loop substitutions (Figs. 6 and 7 and Ref. 14). In our previous report, based on analysis of three D/T loop substitutions (G19C, C56G, and G19C/C56G), we suggested that disruption of the D/T contact in C56G (revealed by structure probing) causes the inability of the tRNA to be processed. Herein, by analyzing a larger set of D/T loop substitutions, we find that the disruption of contacts revealed by structure probing does not generally coincide with reduced ability of the substrate to be processed.

**Structure Modeling**—We modeled the G18C, U55A, and C56A substitutions (Fig. 8). Interestingly, energy minimization buckles the substituted base in the G18C (Fig. 8C) and C56A (Fig. 8D) variants, perhaps to reduce steric clash. Another possibility is that the phosphate on U54 which stabilizes the T
loop U-turn could be pushed aside by the U55A substitution. The modeling tools we used (Fig. 8) are not powerful enough, however, to elucidate the substrate structure changes responsible for reduced processing.

Matrix methods do not necessarily map the processing effects of substrate sequence changes to the level of a single functional group or nucleotide. Some processing defects could result from intraloop propagation (U55A being the most probable example; Fig. 3), and others could result from loop-loop tertiary contacts (G18C being the most probable example; Fig. 3). The T loop is stabilized by a tertiary base pair, a U-turn phosphate contact, a 2'-OH contact, and others (2). T and D loop substitutions could affect any one of these contacts; the rearrangement required to reduce steric clash or to strengthen one contact could weaken others. Direct enzyme-substrate contacts, indirect substrate effects transmitted from one part of the substrate to another which is a contact position or region, or regional changes in substrate shape could explain the KM increases (Table I). This type of modeling demonstrates the need for more direct analysis of the structure of the enzymes and their contacts with tRNA.

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FIG. 8. Modeling D/T loop substitutions using yeast tRNA^Phe. Green, base contacts investigated in this report. Blue, substitutions. Red, the phosphate involved in the T loop U-turn. A, wild type yeast tRNA^Phe; B, U55A; C, G18C; D, C56A.
Effects of D/T Loop Sequence on tRNA End Processing

Matrices of Paired Substitutions Show the Effects of tRNA D/T Loop Sequence on Drosophila RNase P and 3'-tRNase Processing
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