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The 3′ end CCA of mature tRNA is an antideterminant for eukaryotic 3′-tRNase

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ABSTRACT

Cytoplasmic tRNAs undergo posttranscriptional 5′ and 3′ end processing in the eukaryotic nucleus, and CCA (which forms the mature 3′ end of all tRNAs) must be added by tRNA nucleotidyl transferase before tRNA can be aminoacylated and utilized in translation. Eukaryotic 3′-tRNase can endonucleolytically remove a 3′ end trailer by cleaving on the 3′ side of the discriminator base (the unpaired nucleotide 3′ of the last base pair of the acceptor stem). This reaction proceeds despite a wide range in length and sequence of the 3′ end trailer, except that mature tRNA containing the 3′ terminal CCA is not a substrate for mouse 3′-tRNase (Nashimoto, 1997, *Nucleic Acids Res 25*:1148–1154). Herein, we extend this result with *Drosophila* and pig 3′-tRNase, using *Drosophila melanogaster* tRNA^{His} as substrate. Mature tRNA is thus prevented from recycling through 3′ end processing.

We also tested a series of tRNAs ending at the discriminator base (–), with one C added (+C), two Cs added (+CC), and CCA added (+CCA) as 3′-tRNase inhibitors. Inhibition was competitive with both *Drosophila* and pig 3′-tRNase. The product of the 3′-tRNase reaction (–) is a good 3′-tRNase inhibitor, with a K_I approximately two times K_M for the normal 3′-tRNase substrate. K_I increases with each nucleotide added beyond the discriminator base, until when tRNA+CCA is used as inhibitor, K_I is approximately forty times the substrate K_M. The 3′-tRNase can thus remain free to process precursors with 3′ end trailers because it is barely inhibited by tRNA+CCA, ensuring that tRNA can progress to aminoacylation. The active site of 3′-tRNase may have evolved to make an especially poor fit with tRNA+CCA.

Keywords: eukaryotic 3′-tRNase; mature tRNA; tRNA end-processing kinetics

INTRODUCTION

tRNA 3′ end processing

Prokaryotic tRNAs are generally transcribed as precursors with a 5′ end leader and a 3′ end trailer. The 3′ ends of prokaryotic tRNAs are removed by a redundant family of 3′-exonucleases (reviewed by Deutscher, 1995). Because CCA is transcriptionally encoded in prokaryotes, the 3′-exonucleases which remove the 3′ end trailer can produce a mature tRNA ready for aminoacylation. The CCA-adding enzyme, tRNA nucleotidyl transferase (tNtase), principally performs a repair function in prokaryotes and is not required for viability (Deutscher, 1995).

Eukaryotic 3′ end processing of tRNA (Fig. 1B → D) differs from prokaryotic in two ways. First, in eukaryotes, the 3′ end trailer can typically be removed by a 3′ end endonuclease (3′-tRNase) which cleaves immediately following the first unpaired nucleotide on the 3′ side of the acceptor stem (the discriminator base; Solari & Deutscher, 1983; Castaño et al., 1985; Frendewey et al., 1985), although 3′ exonucleases may sometimes be involved (Furter et al., 1992; Yoo & Wollin, 1997). Second, because CCA is not transcriptionally encoded in eukaryotic tRNA genes (Sprinzl et al., 1998), it must be added by tNtase, an essential eukaryotic enzyme (Aebi et al., 1990).

Order of 3′ end processing reactions

The reaction order depicted in Figure 1, which is supported by biochemical experiments with *Xenopus* and *Drosophila* enzymes (Castaño et al., 1985; Frendewey et al., 1985), is clearly correct in B → D; the 3′-tRNase

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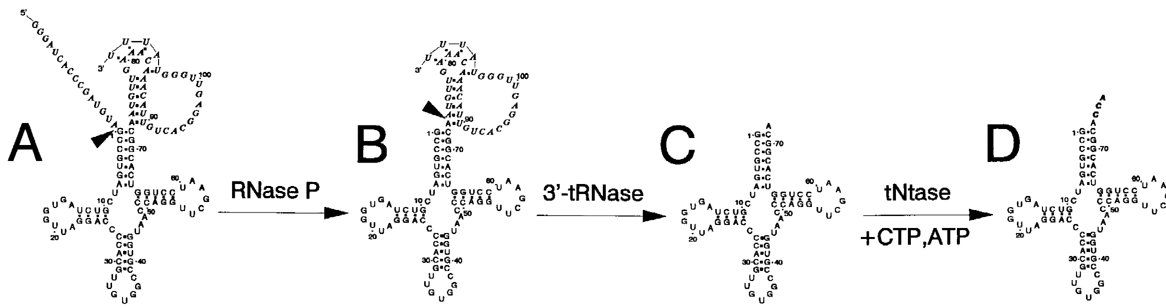


FIGURE 1. Substrates and products in the eukaryotic tRNA end-processing pathway. **A**: Precursor *Drosophila* $tRNA^{His}$ (redrawn from Frendewey et al., 1985) has a 16-nt 5′ end leader and a 36-nt 3′ end trailer. **B**: The 5′ end leader has been endonucleolytically removed by RNase P, producing the 3′-tRNase substrate. **C**: The 3′ end trailer has been endonucleolytically removed by 3′-tRNase, producing the tNtase substrate. **D**: tNtase has added CCA to the 3′ end of substrate tRNA, using CTP and ATP as additional substrates.

reaction must precede the tNtase reaction. tRNA 3′ end trailers vary widely in length and sequence (Fig. 9A; Sprinzl et al., 1998), suggesting that 3′-tRNase can broadly recognize precursor tRNAs with 3′ end trailers. If mature tRNA with CCA at its 3′ end were either a substrate for 3′-tRNase or a good competitive inhibitor, the reaction series depicted in Figure 1 (B → C → D) could become an endless cycle or might be subject to end-product inhibition, either of which would be costly to the cell. tRNA localization, aminoacylation, and transport could, however, eventually end such a loop (see Discussion).

Herein, we establish that mature tRNA (3′+CCA) is a poor substrate for *Drosophila* and pig 3′-tRNase, as was found with mouse 3′-tRNase (Nashimoto, 1997). Furthermore, tRNAs ending successively at the discriminator base (−), with one C added (+C), two Cs added (+CC) or with CCA added (+CCA) become poorer competitive inhibitors of both *Drosophila* and pig 3′-tRNase with each nucleotide added, ending with a K_I 40 times K_M for normal substrate. 3′-tRNase has apparently evolved so that its active site can recognize, bind, and cleave a wide variety of sequences *except* tRNA with CCA at its 3′ end.

Specific and general tRNA identity and an antideterminant for 3′-end processing

tRNA identity refers to a sequence element required for accurate aminoacylation of a particular tRNA by its aminoacyl tRNA synthetase (Schulman, 1991). Determinants are sometimes distant from the anticodon, as in $tRNA^{His}$, in which a minihelix or microhelix can be efficiently and specifically aminoacylated (Francklyn et al., 1992). The term antideterminant refers to a specific sequence which discourages aminoacylation (Frugier et al., 1998); such negative determinants may be included in the identity set. Internal contacts required for folding and long-range order in specific tRNAs are also part of the identity set (Hamann & Hou, 1997).

tRNAs have a generalized identity for enzymes that presumably recognize all tRNAs, such as RNase P, 3′-tRNase, and tNtase. Generalized tRNA identity has been suggested to reside in the coaxially stacked acceptor stem and T arms (McClain et al., 1987; Maizels & Weiner, 1994), although the required tertiary structure may be stabilized by D/T loop interactions (Hardt et al., 1993; Levinger et al., 1998). Here, we use the term antideterminant to describe a characteristic sequence (3′+CCA) common to all mature tRNAs that evidently excludes them from the active site of eukaryotic 3′-tRNase, unlike the general class of precursor tRNAs with 3′ end trailers of mixed sequence.

RESULTS

A tRNA end-processing pathway

Figure 1 illustrates the sequence and presumed secondary structure of *Drosophila melanogaster* $tRNA^{His}$ substrates and products in a series of tRNA end-processing reactions. RNase P endonucleolytically removes the 16-nt 5′ end leader (arrow above +1 in Fig. 1A). 3′-tRNase endonucleolytically removes the 36-nt 3′ end trailer (arrow above the discriminator base in Fig. 1B). The length and possible secondary structure of the 3′ end trailer of $tRNA^{His}$ (Fig. 1A,B; Frendewey et al., 1985) is unimportant for 3′-tRNase activity (see Fig. 9A); this 3′ end trailer is, however, stabilized against nucleolytic degradation, easing the demonstration that the 3′ end processing is endonucleolytic (Frendewey et al., 1985; Levinger et al., 1995, 1998). tNtase adds CCA to the 3′-tRNase product (Fig. 1C) to produce a mature tRNA (+CCA; Fig. 1D).

The numerous modifications of tRNA nucleotides (Bjork, 1995) do not appear to be required for the reactions illustrated in Figure 1, which proceed efficiently in vitro with (presumably unmodified) T7 transcripts. The splicing that some tRNAs require (Westaway & Abelson, 1995) is also omitted from this diagram for

simplicity, and because *Drosophila* $tRNA^{His}$ does not have an intron. Interestingly, eukaryotic $tRNA^{His}$ also has a G added posttranscriptionally to its 5′ end by tRNA Guanylyl transferase (tGtase; Cooley et al., 1982), which is also not depicted.

The reaction order presented in Figure 1 is supported by biochemical evidence (Castaño et al., 1985; Frendewey et al., 1985; Nashimoto, unpubl.). Specifically, the intermediate arising from removal of the 5′ end leader by RNase P (Fig. 1B) was observed on processing gels (Frendewey et al., 1985), whereas the intermediate that would be produced by a 3′-tRNase reaction on the primary transcript (Fig. 1A) was not. On the other hand, human tRNAs are apparently processed first at their 3′ ends using an extract from HeLa cells (Thomann et al., 1989). Independent of the relative order of 5′ end processing, the 3′ end must be processed to the discriminator base before CCA addition can occur. Interestingly, there is little interplay, either as substrate or inhibitor, between the product of the tNtase reaction (Fig. 1D) and the 3′-tRNase which catalyzes the reaction shown in Figure 1B → C (see below).

Mature tRNA appears to be a stable product in the simultaneous presence of 3′-tRNase and tNtase

We directly followed the cleavage by 3′-tRNase (Fig. 1B → C) with addition of CCA by tNtase (Fig. 1C → D), as shown in Figure 2. This was possible because the same enzyme fraction which contains 3′-tRNase also has tNtase activity. Efficient addition of CCA to the tRNA 3′ end produced by 3′-tRNase (Fig. 2, lane 1) was achieved by including 1 mM CTP and ATP in the reaction mix (Fig. 2, lane 2; bracketed arrow pointing up).

We interpret that the new band present in Figure 2, lane 2, results from addition of CCA to the 3′ end of tRNA by tNtase, and not from some other tRNA processing or modification product, for the following reasons:

1. Based on markers and the migration of tRNAs of known size, the tRNA appears to have increased in length by 3 nt.
2. The reaction illustrated in Figure 2, lane 2, requires the addition of CTP and ATP.
3. A reaction performed as in Figure 2, lane 2, but using unlabeled tRNA ending at the discriminator base, unlabeled CTP, and α-^{32}P-ATP, followed by gel purification of the labeled product tRNA and analysis with RNase T1, produced a pattern consistent with 3′ end labeling of the tRNA (data not shown).

3′-tRNase may be unable to remove the three added nucleotides from the tNtase product (right of Fig. 2; bracketed arrow pointing down with an X through it).

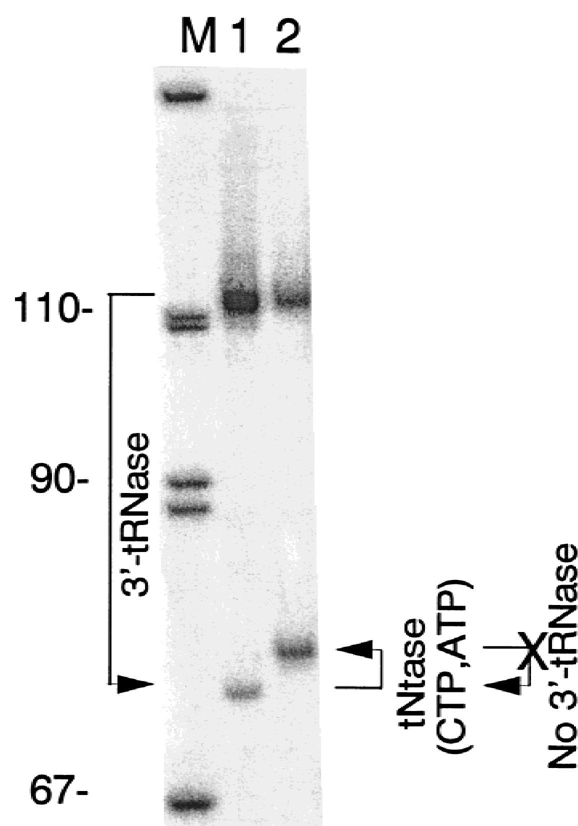


FIGURE 2. The product of CCA addition is not a 3′-tRNase substrate. Lane 1: the internally labeled 3′-tRNase substrate with a 36-nt 3′ end trailer (Fig. 1B) was incubated with fly 3′-tRNase for 60 min at 28 °C. Lane 2: 1 mM ATP and 1 mM CTP were added to activate tNtase present in the 3′-tRNase fraction. Numbers at left indicate marker sizes. Bracket and arrow at left indicate the 3′-tRNase substrate and product. First bracket and arrow at right designate the tNtase substrate and product. Second bracket and arrow at right with X indicate that 3′-tRNase present along with tNtase is unable to cleave the tNtase product.

Alternatively, the tNtase activity may exceed that of 3′-tRNase when both enzymes are active in a mixture. We investigated the former hypothesis by testing tRNAs with C, CC, and CCA added to the discriminator base as 3′-tRNase substrates and inhibitors (see below).

Relative efficiency of 3′-tRNase reactions performed using tRNAs with short 3′ end extensions

To extend these and earlier results (Nashimoto, 1997), we constructed a series of tRNAs with C, CC, and CCA added to the 3′ ends (see Materials and Methods). These labeled tRNAs varying in length by 1 nt (Figs. 3A and 3B, parts b–d) were incubated with *Drosophila* and pig 3′-tRNase (Figs. 3A and 3B, respectively), using the full length 3′-tRNase substrate (Fig. 1B) as a positive control (Figs. 3A and 3B, part a). Because all the short substrates tested here were expected to be poorer substrates than full length tRNA,

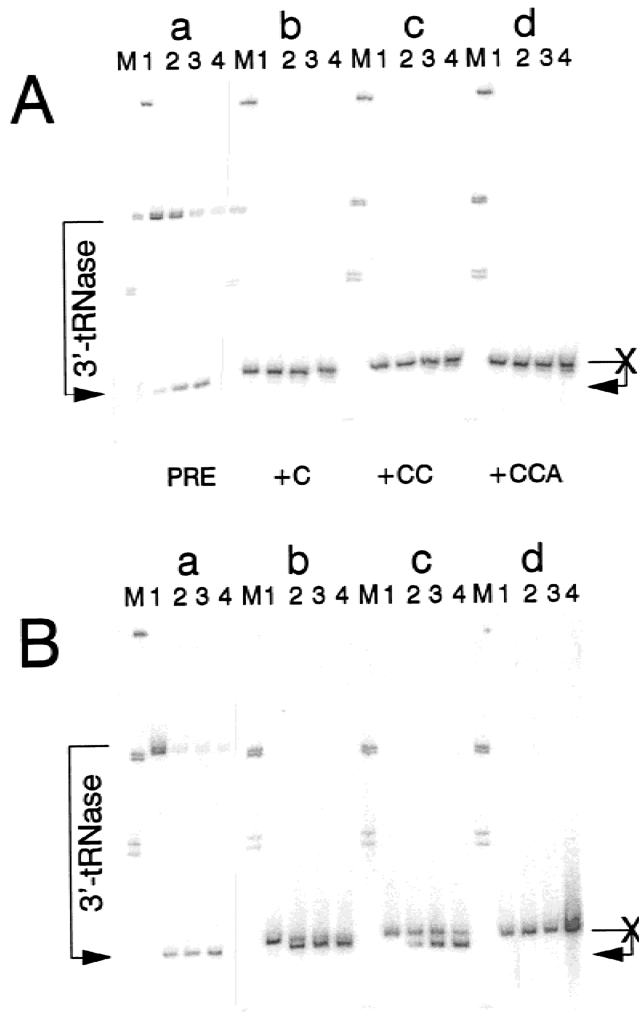


FIGURE 3. 3′-tRNase activity observed with a series of tRNAs. **A**: Internally labeled tRNAs were incubated with *Drosophila* 3′-tRNase for 0 (lane 1), 15 (lane 2), 30 (lane 3), or 60 min (lane 4). Reactions in (a) were performed with the same 3′-tRNase substrate as in Figure 2. Reaction (b): tRNA with one C added to the discriminator base (+C). Reaction (c): CC added to the discriminator (+CC). Reaction (d): CCA added to the discriminator (+CCA; same as tNtase product illustrated in Fig. 1D). Bracket and arrow at left of **A** indicate the 3′-tRNase substrate and product. Bracket and arrow with X at right of **A** indicate the absence of 3′-tRNase product with incubations of tRNA+CCA in (d). **B**: same as **A**, except that pig 3′-tRNase was used and reactions were performed at 37 °C.

based on earlier results (Nashimoto, 1997), we used a fourfold higher concentration of *Drosophila* 3′-tRNase (Fig. 3A, b–d) and a fivefold higher concentration of pig 3′-tRNase (Fig. 3B, b–d) than with the wild-type 3′-tRNase substrate (Figs. 3A and 3B, part a). The *Drosophila* 3′-tRNase reaction using wild-type substrate progresses with increasing incubation time (Fig. 3A, part a, lanes 1–4), and none of the short tRNAs (+C, +CC, +CCA; Fig. 3A, parts b–d) were detectably processed (bracket with X and arrow to the right of Panel A), even with 60 min of incubation, although a fourfold higher enzyme concentration was used than with the wild-type substrate (Fig. 3A, part a). In other experiments (not shown), we found that 3′+C could be a weak substrate for *Drosophila* 3′-tRNase, as previously observed with mouse 3′-tRNase (Nashimoto, 1997).

The activity observed using pig 3′-tRNase on the wild-type substrate (Fig. 3B, part a) was much higher than with *Drosophila* 3′-tRNase (Fig. 3A, part a). Using five times more enzyme, 3′-tRNase activity was observed on the intermediate tRNAs with C and CC added to the discriminator base (Fig. 3B, parts b and c, respectively). On the other hand, no 3′-tRNase activity was observed with either *Drosophila* or pig 3′-tRNase when tRNA-3′+CCA was incubated in these reaction mixtures (Figs. 3A and 3B, part d). tRNA 3′-exonuclease activity was undetectable in these preparations. One, two, or three nucleotide 3′ end trailers other than C, CC, and CCA can be efficiently removed by mouse 3′-tRNase (Nashimoto, 1997). These findings extend the result that tRNA-3′+CCA is not a substrate for mouse 3′-tRNase (Nashimoto, 1997), using a different tRNA and two different 3′-tRNase enzymes.

Optimization of 3′-tRNase reactions

To study 3′-tRNase kinetics (below), it was first necessary to optimize reaction conditions. The concentration of $MgCl_2$ used in 3′-tRNase reactions could be especially important, because low salt (monovalents and divalents) appears to favor the formation of a productive enzyme-substrate complex (Levinger et al., 1995), while $MgCl_2$ can also be required for proper tRNA folding. We investigated a low range of $MgCl_2$ concentrations (millimolar and submillimolar) because Deutscher (1990) has described the eukaryotic 3′-tRNases as being low-$MgCl_2$ endoribonucleases. In addition, Nashimoto (1995) has found mammalian 3′-tRNase to be activated by spermidine.

We found a $MgCl_2$ optimum for fly 3′-tRNase between 0.3–1 mM (Fig. 4A1, parts b and c) and therefore conducted kinetic analysis at 0.3 mM $MgCl_2$. The highest reaction velocity with pig 3′-tRNase was obtained at 3 mM $MgCl_2$, higher than with *Drosophila* 3′-tRNase (Fig. 4B1, part a). All kinetic experiments with pig 3′-tRNase were thus performed at 3 mM $MgCl_2$. An artifactual band (identified with * to the right of Fig. 4B) was produced using pig 3′-tRNase under suboptimal conditions, but is not generally observed (Fig. 3B, part a; Fig. 5B). Interestingly, spermidine could not substitute for $MgCl_2$ with either fly or pig 3′-tRNase (Figs. 4A2, parts b–d and 4B2, parts a–c), and pig 3′-tRNase activity was observed even in the absence of $MgCl_2$ with 1 mM EDTA added (Fig. 4B2, part d). Indeed, the enzyme may not require $MgCl_2$, but tRNAs require $MgCl_2$ for proper folding, and spermidine could effectively substitute for $MgCl_2$ in the folding of certain

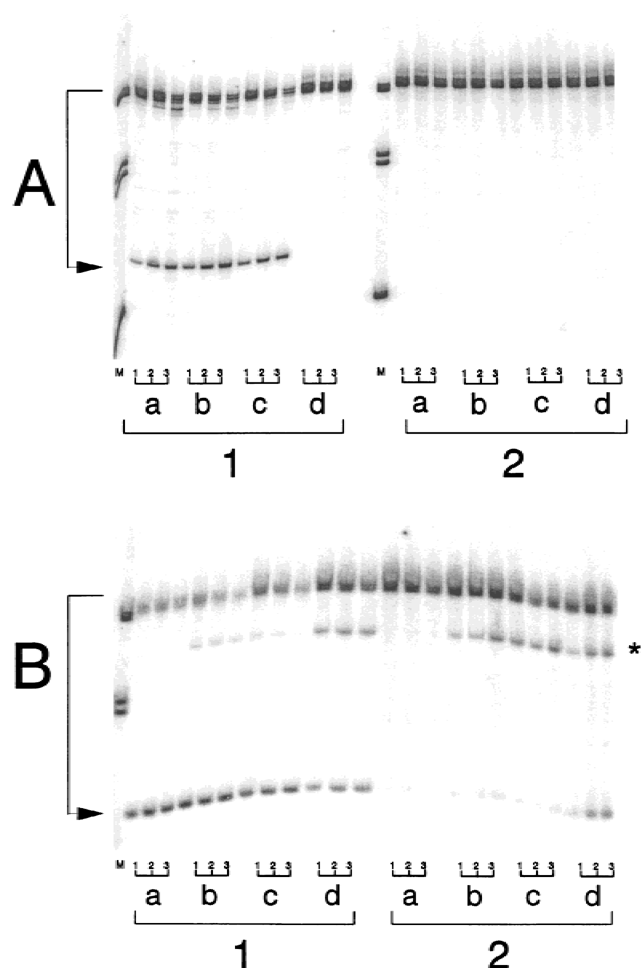


FIGURE 4. Mg^{++} optimum of *Drosophila* and pig 3′-tRNase. **A**: Fly 3′-tRNase. Reactions were sampled at 5 (1), 10 (2), and 15 (3) min of reaction at 28 °C. In part 1, reactions were performed with 3 mM (a), 1 mM (b), 0.3 mM (c), and no added $MgCl_2$ (d), respectively. In part 2, no $MgCl_2$ was added, 1 mM EDTA was added, and spermidine was added to 0 (a), 0.3 (b), 1.0 (c), and 3.0 mM (d). These and other results (not shown) demonstrate that the optimal concentration of $MgCl_2$ for the *Drosophila* 3′-tRNase is just below 1 mM and that spermidine cannot substitute for $MgCl_2$. **B**: Similar to **A** except that pig 3′-tRNase was used. In part 1, $MgCl_2$ was added to 3 (a), 1 (b), and 0.3 mM (c), or no $MgCl_2$ was added (d). In part 2, no $MgCl_2$ was added, EDTA was added to 1 mM, and spermidine was added to 3 (a), 1 (b), and 0.3 mM (c), or no spermidine was added (d). * at right of **B** identifies an artefact produced in some pig 3′-tRNase reactions under suboptimal conditions. The results in **B**, together with data not shown, demonstrate a Mg^{++} optimum for pig 3′-tRNase of 3 mM. Although a small amount of activity was observed with the pig 3′-tRNase in the presence of EDTA without $MgCl_2$ (part 2, reaction d), added spermidine inhibited the reaction.

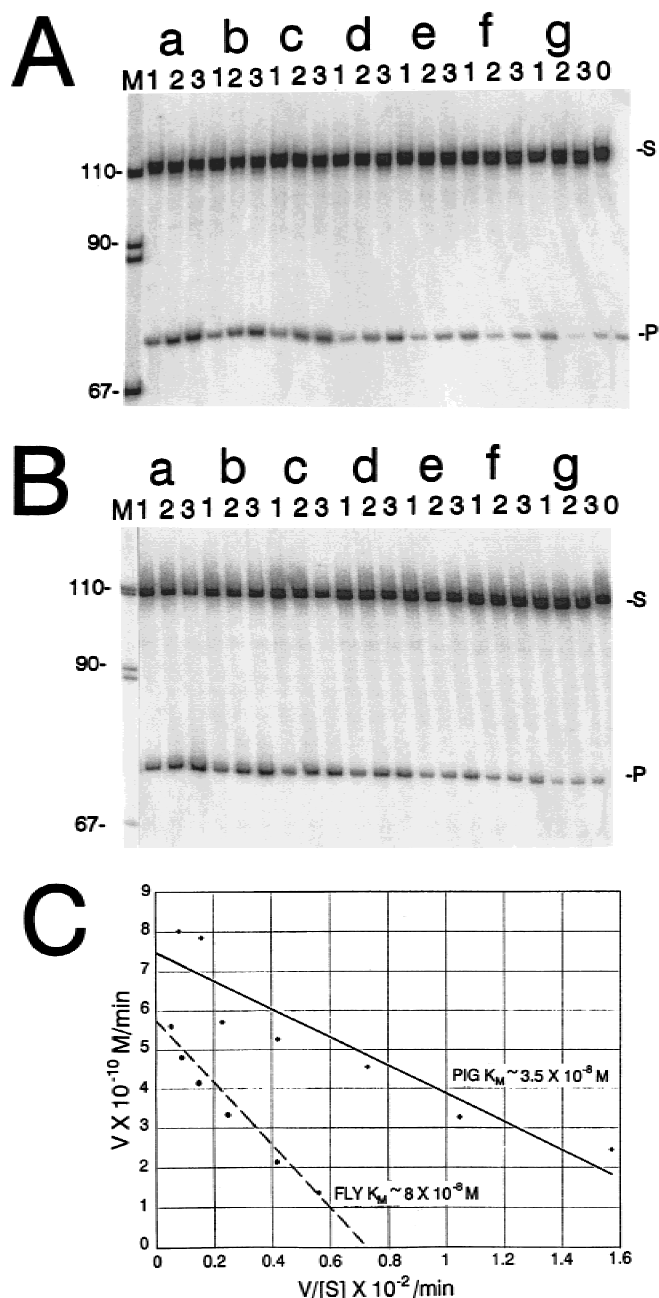


FIGURE 5. K_M determination for *Drosophila* and pig 3′-tRNase. **A**: Wild-type $tRNA^{His}$ with a 36-nt 3′ end trailer was processed with *Drosophila* 3′-tRNase at 0.3 mM $MgCl_2$ for 10, 20, and 30 min in lanes 1–3, respectively. The ^{32}P-tRNA was maintained constant, and unlabeled substrate was added to a final concentration of 0, 2.5, 5, 12.5, 25, 50, and 100 $\times 10^{-8}$ M in parts a–g, respectively. **B**: Reactions were the same as in **A**, except that pig 3′-tRNase was used, with $MgCl_2$ at 3 mM, and a reaction using 1.25×10^{-8} M substrate was included. **C**: The Eadie–Hofstee analysis, in which V is plotted versus V/[S]. Dashed line: data from **A** (*Drosophila* 3′-tRNase); solid line: data from **B** (pig 3′-tRNase). The slope is equal to $-K_M$, leading to K_M values of 80 nM for fly 3′-tRNase and 35 nM for pig 3′-tRNase.

tRNAs, but not with *Drosophila* $tRNA^{His48}$, the tRNA used in these experiments.

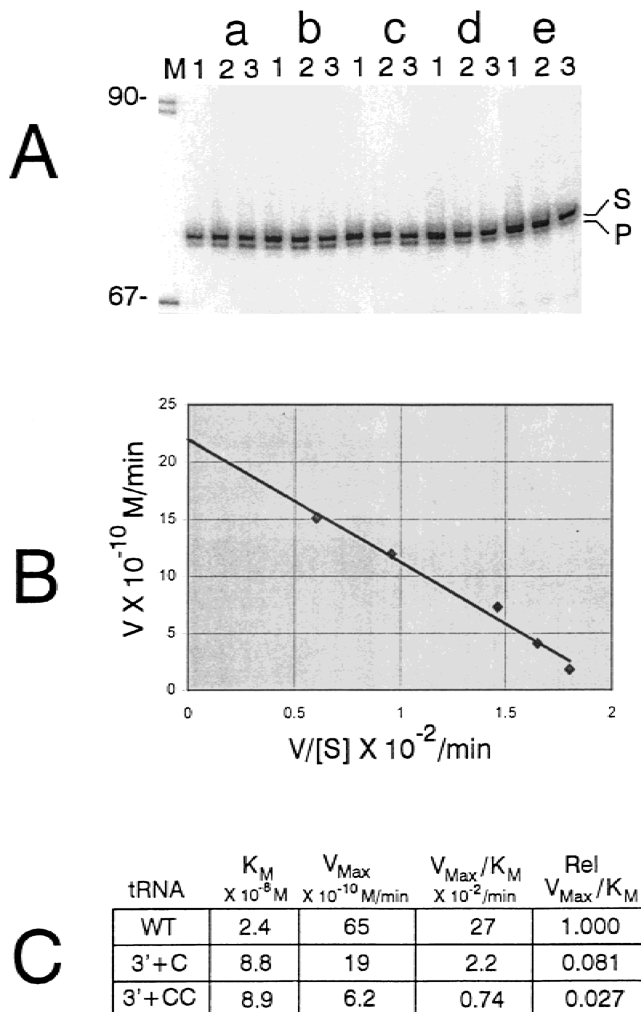
Determination of K_M for *Drosophila* and pig 3′-tRNase

Because of changes in reaction conditions (optimization of $MgCl_2$ and omission of polyamines) and the use of a heterologous substrate (*Drosophila* $tRNA^{His}$) with pig 3′-tRNase, we determined the wild-type substrate K_M for fly and pig 3′-tRNase. Michaelis–Menten experiments were performed at 28 °C (Fig. 5A,B) by varying [S] at constant [E] (see Materials and Methods). K_Ms

were determined using Eadie–Hofstee plots of V versus V/[S]; the rearranged Michaelis–Menten equation gives slope $= -K_M$. Under these reaction conditions, K_M for *Drosophila* 3′-tRNase is 80 nM and K_M for pig 3′-tRNase is 35 nM (Fig. 5C).

Kinetics of pig 3′-tRNase processing using substrates with short 3′ end trailers

We determined K_M and V_{Max} for tRNA-3′+C and +CC using pig 3′-tRNase (Fig. 6). Data quality is illustrated by the gel image of 3′+C tRNA (Fig. 6A) and the Eadie–Hofstee plot (Fig. 6B). K_M and V_{Max} (the average obtained from several experiments) are presented in Figure 6C.



tRNA	K_M X 10^{-8} M	V_{Max} X 10^{-10} M/min	V_{Max}/K_M X 10^{-2}/min	Rel V_{Max}/K_M
WT	2.4	65	27	1.000
3′+C	8.8	19	2.2	0.081
3′+CC	8.9	6.2	0.74	0.027

FIGURE 6. Determination of K_M and V_{Max} for pig 3′-tRNase using tRNAs with the 3′ end trailers 3′+C and 3′+CC. **A**: ^{32}P-tRNA-3′+C was incubated at 37 °C with pig 3′-tRNase at 1/100 of the final reaction mix, varying the concentration of unlabeled 3′+C tRNA as in Figure 5. The substrate concentrations used were 10, 25, 50, 125, and 250 nM (parts a–e, respectively). Reactions were sampled after 10, 20, and 30 min of reaction (1–3 of each set, respectively). **B**: Eadie–Hofstee plot of data from **A**, used to obtain K_M (− slope) and V_{Max} (Y-intercept). **C**: Comparison of K_M, V_{Max}, V_{Max}/K_M and V_{Max}/K_M for 3′+C and 3′+CC relative to the wild type. Wild-type V_{Max} determined using pig 3′-tRNase at 1/500 of final reaction mix was normalized to 1/100 used for 3′+C and 3′+CC.

The K_M and V_{Max} reported for wild-type tRNA^{His} using pig 3′-tRNase in Figure 6C are different from those shown in Figure 5C. The experiments in Figure 5 were performed at 28 °C, the optimum for *Drosophila* 3′-tRNase; later experiments (Figs. 6–8) with pig 3′-tRNase were performed at 37 °C, the temperature optimum for this enzyme (Nashimoto, 1995), resulting in a decrease in K_M (from 35 to 24 nM) and increase in V_{Max}. In addition, wild-type V_{Max} in Figure 6C is normalized for a dilution of pig 3′-tRNase to 1/100 of the final reaction mix.

tRNA-3′+C and 3′+CC have a K_M approximately 3.5 times higher than the wild-type substrate (Fig. 1B). V_{Max} decreases progressively by slightly more than 3 times with each C addition. In combination, catalytic efficiency (V_{Max}/K_M) is reduced to ~1/12 and to ~1/37 that of the wild type with the 3′+C and with the 3′+CC substrates, respectively.

We can also specify from the combination of pig 3′-tRNase concentration and reaction time used with tRNA-3′+CCA (Fig. 3B, part d, lane 4) that the V_{Max}/K_M for this tRNA must be <1/500 that of the wild type, because no product was detected in an assay that is capable of resolving <1% conversion of substrate to product. Kinetics was not performed with the *Drosophila* 3′-tRNase using 3′+C and 3′+CC tRNAs because the reaction products were barely detectable (Fig. 3A, parts b and c).

Competitive inhibition of 3′-tRNase becomes weaker with sequential addition of C-C-A to the 3′ end of tRNA

The product of the 3′-tRNase reaction (Fig. 1C) is a competitive inhibitor of 3′-tRNase with a K_I of 1.5×10^{-7} M, approximately two times the substrate K_M (Levinger et al., 1998), low enough to suggest that inhibition by product could influence the overall reaction rate constant. Here, we used kinetic analysis to directly compare the K_I of the 3′-tRNase product (3′-; Fig. 7A) with that of the tNtase product (3′+CCA; Fig. 7B). We confirmed the 3′-tRNase product (3′-) K_I value of 1.5×10^{-7} M (Fig. 7A; cf. Levinger et al., 1998), determined the tRNA-3′+CCA K_I to be 33×10^{-7} M (Fig. 7B), and established the inhibition to be competitive.

We further investigated the effect on K_I of each added nucleotide in both *Drosophila* and pig 3′-tRNase reactions (Fig. 8). K_I for fly 3′-tRNase increases steeply to 30×10^{-7} M with addition of the second C, extending the earlier finding that mouse 3′-tRNase can utilize tRNA-3′+C as a substrate, but not +CC (Nashimoto, 1997). Substantially the same curve was obtained when these four K_Is were determined for pig 3′-tRNase. The K_Is for pig 3′-tRNase are lower than those for *Drosophila*

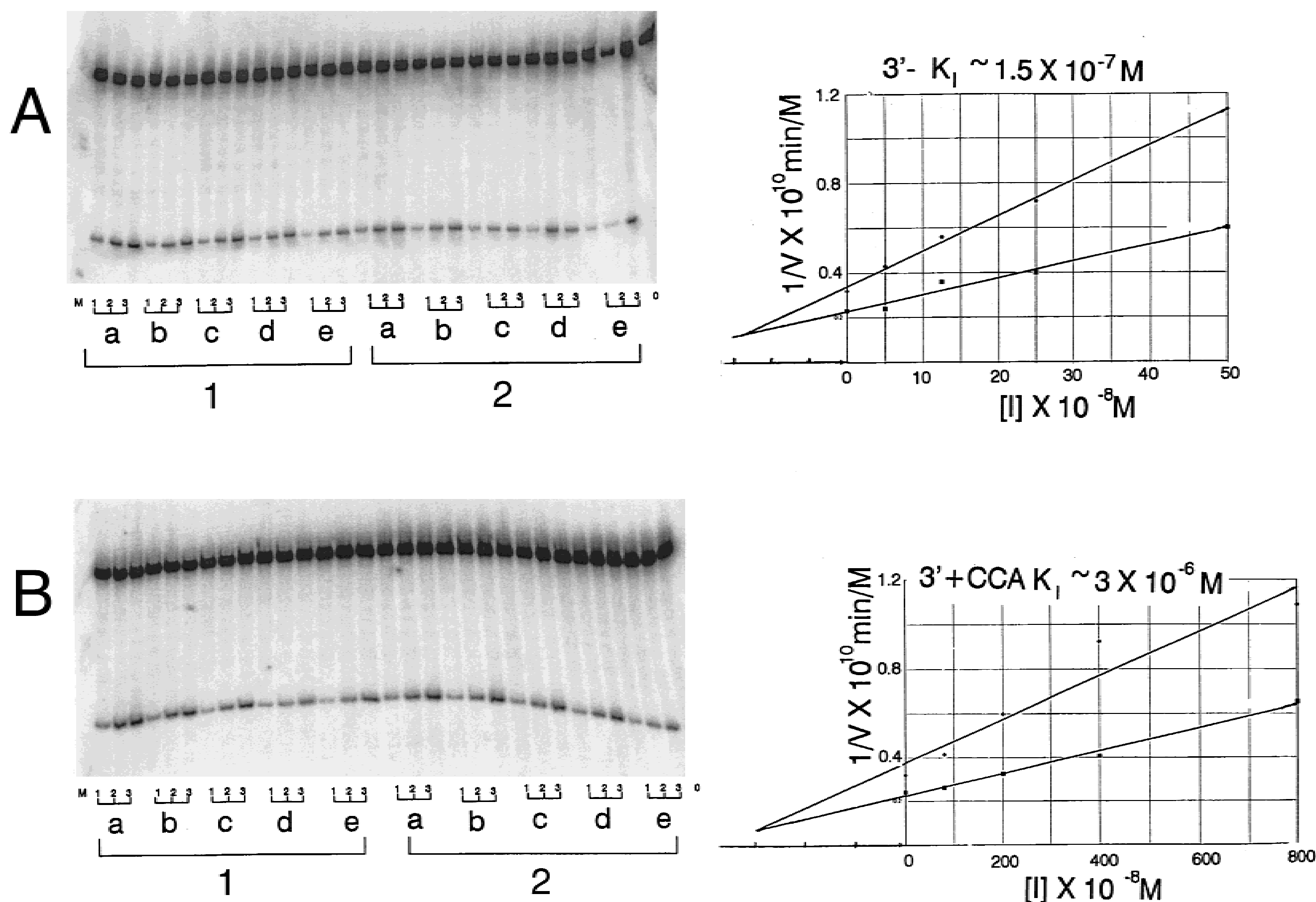


FIGURE 7. K_I determination by Dixon analysis using *Drosophila* 3′-tRNase for tRNA ending at the discriminator base (−) and with CCA added (+CCA). Reactions were performed at 28 °C and sampled after 5, 10, and 15 min. Substrate concentration was 4×10^{-8} M or 10×10^{-8} M. In **A**, the 3′-tRNase reaction product (Fig. 1C) was used as inhibitor at 0 (a), 5 (b), 12.5 (c), 25 (d), or 50×10^{-8} M (e), respectively. Layout of **B** is identical except that a different inhibitor (the tNtase reaction product; Fig. 1D) was used at 0 (a), 80 (b), 200 (c), 400 (d), or 800×10^{-8} M (e). Dixon plots of $1/V$ versus [I] for the low [S] and high [S] data in **A** and **B** show the respective K_Is. K_I is the value of −[I] at which the low [S] and high [S] curves intersect; the intersection of these lines above the abscissa indicates competitive inhibition. Note the scale difference in the [I] range used between **A** and **B**.

3′-tRNase, corresponding with the lower K_M; the K_I for tRNA-3′+CCA is around forty times K_M with pig 3′-tRNase, as it is with *Drosophila*.

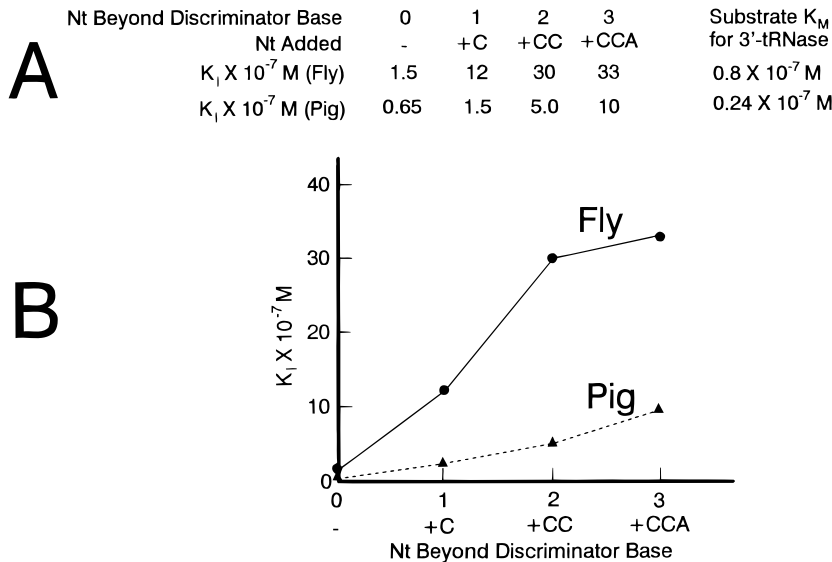
Variation in 3′ end trailer length and sequence of *Drosophila melanogaster* cytoplasmic tRNA precursors

Genbank was searched for annotated *Drosophila melanogaster* tRNA genes, and 90 tRNA genes with sequence differences between +1 and the terminator were scored. The criteria for inclusion were reliable identification of +1 and the discriminator nucleotide, based on a well paired 7 bp acceptor stem, and the presence of a terminator for RNA polymerase **III** (an uninterrupted run of four or more Ts) defining the 3′ end of the 3′ end trailer. We thus ordered the tRNAs by length of the mixed nucleotide sequence in their 3′ end trailers (Fig. 9A). Wide variation is found in the length of 3′ end trailers, between 4 and 37 nt, with a mean of 14 nt.

$tRNA^{Arg}$ and $tRNA^{His}$ (identified with * and **, respectively in Fig. 9A), which were first analyzed as substrates for 3′-tRNase (Frendewey et al., 1985), are at opposite ends of the length distribution of tRNA 3′ end trailers. Because both $tRNA^{Arg}$ with a 6-nt 3′ end trailer and $tRNA^{His}$ with a 37-nt 3′ end trailer could be efficiently 3′-end processed by 3′-tRNase, we presume that this enzyme does not discriminate greatly on the basis of 3′ end trailer length and sequence (except for the trinucleotide sequence immediately following the discriminator base; see Fig. 9B and Nashimoto, 1997).

CCA at the mature 3′ end of tRNA is not transcriptionally encoded in *Drosophila melanogaster*

The discriminator nucleotide does not itself affect the efficiency of the mammalian 3′-tRNase reaction (Nashimoto, 1997). Once the discriminator base was unambiguously located, we tabulated the distribution of


Increase in K_I with Length of Competitive Inhibitors tRNA 3'-, +C, +CC and +CCA

A

Nt Beyond Discriminator Base	0	1	2	3	Substrate K_M
Nt Added	-	+C	+CC	+CCA	for 3'-tRNase
$K_I \times 10^{-7}$ M (Fly)	1.5	12	30	33	0.8×10^{-7} M
$K_I \times 10^{-7}$ M (Pig)	0.65	1.5	5.0	10	0.24×10^{-7} M

FIGURE 8. Relation between K_I and the number of nucleotides added beyond the discriminator base with *Drosophila* and pig 3′-tRNase. The same type of analysis shown in Figure 7 was performed using fly 3′-tRNase and tRNA+C and tRNA+CC as inhibitors. In addition, all four tRNAs (−, +C, +CC, and +CCA) were tested as inhibitors of pig 3′-tRNase at 37 °C. **A**: The K_I values obtained using *Drosophila* and pig 3′-tRNase, with relation to substrate K_M. **B**: Plot of values shown in **A**.

sequence in the trinucleotide immediately following the discriminator base (Fig. 9A) and the sequence CCA at that position in mature tRNAs, as shown in Figure 9B. The sequence CCA is not encountered at this position in precursors. The sequence CC— (here we use — to represent any nucleotide other than A in the third position following the discriminator, and any nucleotide other than C in the first or second position) is found only once (on the basis of the assumption of random sequence at this position, it would be expected in 90/16 = 5.6 cases). C—A is also encountered only once. —CA is found five times. C— — occurs twice. A total of four occurrences of CC—, C—A, and C— — were thus tabulated; in a random distribution, C would be expected in the first position 22.5 times. The sequences —C— and — —A occur with a frequency of 9/90 and 18/90, respectively. The sequence — — — (not C, not C, not A) was found in 54 cases out of 90. These observations suggest that the sequence CCA, CC—, C—A, and C— — following the discriminator base are selected against in *Drosophila melanogaster* tRNA precursors.

DISCUSSION

We suggest, based on biochemical evidence (Figs. 2, 3, and 6–8), and on the distribution of sequences following the discriminator base in 90 tRNA precursors (Fig. 9), that the active site of 3′-tRNase coevolved with the tRNA precursors to establish the sequence CCA at the mature tRNA 3′ end as an antideterminant. A positive determinant is recognized in substrate by the enzyme; an antideterminant is a sequence whose presence causes an otherwise good substrate to be rejected by the enzyme, making a potential target neither a substrate nor a good inhibitor. This antagonistic relation between mature tRNA and 3′-tRNase could help prevent endless cycling of mature tRNA (Fig. 1D) through the 3′-tRNase reaction (Fig. 1B → C) and end-product inhibition of 3′-tRNase by mature tRNA.

Kinetic analysis shows that the 3′-terminal CCA of tRNA is an antideterminant for eukaryotic 3′-tRNase.

The 3′ end nucleotides −C, −CC, and −CCA added to the discriminator base almost completely prevent *Drosophila* 3′-tRNase processing (Fig. 3A). Pig 3′-tRNase activity is observed with 3′+C and +CC, but not with +CCA (Fig. 3B), and K_M and V_{Max} were determined for these combinations of enzyme with substrates (Fig. 6). Additionally, we established K_I for 3′+C, +CC, and +CCA using both *Drosophila* and pig 3′-tRNase (Fig. 7–8). These new kinetic studies add considerable depth and insight to previous understanding of mouse 3′-tRNase (Nashimoto, 1997).

The ~12× and ~37× reduction in catalytic efficiency (V_{Max}/K_M) arising from the 3′-terminal sequence −C and −CC, respectively (Fig. 6C) arises first from an increase in K_M and a decrease in V_{Max}, followed by a decrease in V_{Max} alone. When the 3′-terminal sequence −CCA is present, no product could be detected, even after 60 min of reaction at a fivefold higher concentration of enzyme than was used with the wild type

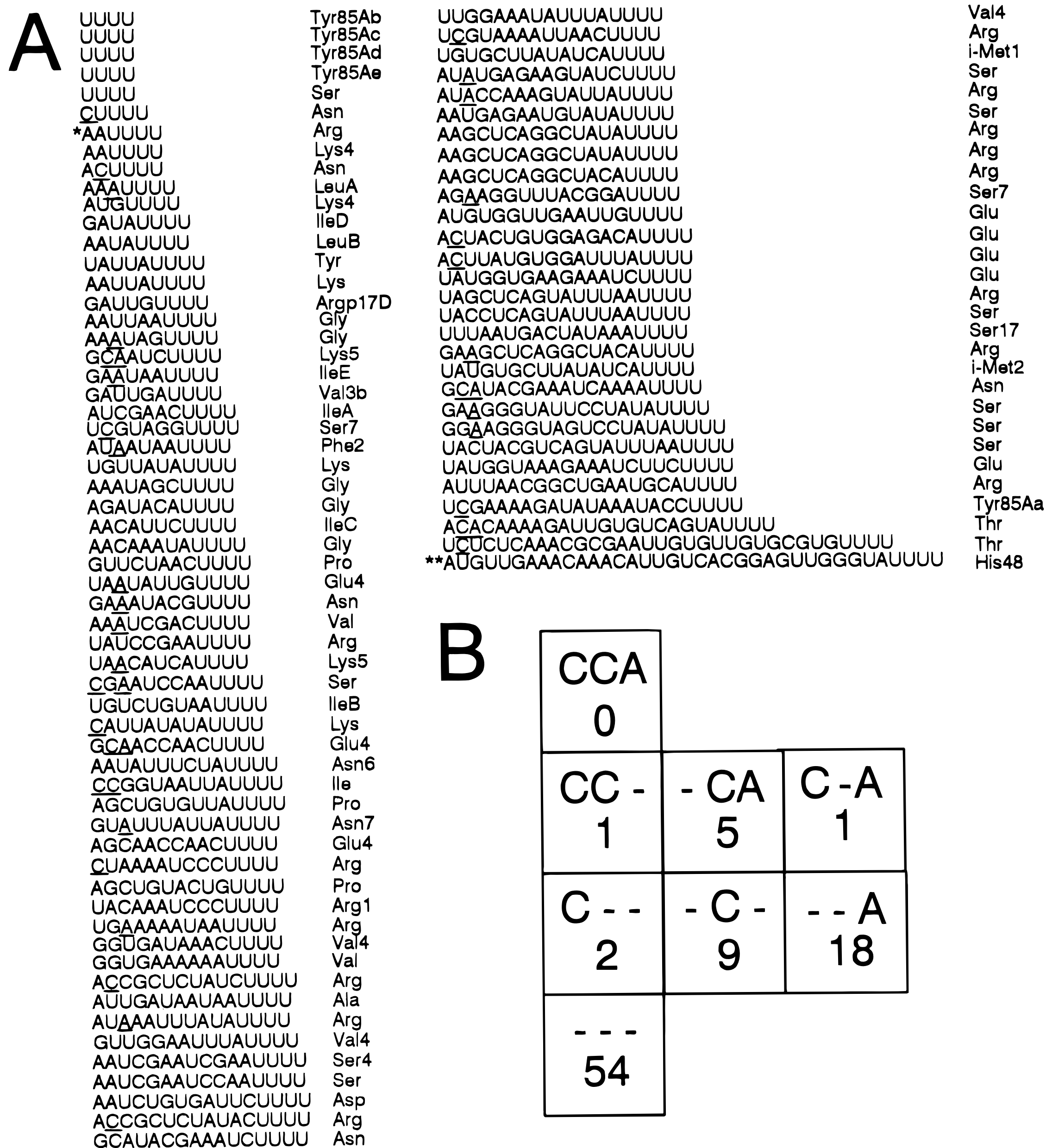


FIGURE 9. Variation in length and sequence of *Drosophila* tRNA 3′ end trailers. **A**: Ninety annotated *Drosophila melanogaster* nuclear-encoded cytoplasmic tRNA precursors were placed in order based on the length of the 3′ end trailer. C, C, and A are underlined when they occur in the first, second, and third positions following the discriminator base, respectively. * and ** signify the $tRNA^{Arg}$ and $tRNA^{His}$ first used as substrates to analyze *Drosophila* 3′-tRNase activity (Frendewey et al., 1985). **B**: Matches to CCA following the discriminator base observed in 90 natural *Drosophila melanogaster* tRNA precursors. Dashes indicate any nucleotide other than C in the first or second position and any nucleotide other than A in the third position.

(Fig. 3A, part d, lane 4; cf. part a), showing that V_{Max}/K_M must be <1/500 that of the wild-type substrate.

The inhibition of 3′-tRNase by tRNAs ending at the discriminator base, +C, +CC, and +CCA is competitive (Fig. 7–8). K_I increases with each added nucleotide to a value of $\sim 40\times$ K_M for wild-type substrate. The K_I curves (Fig. 8B) are generally consistent with the K_M and V_{Max}/K_M data (Fig. 6C).

3′ end sequence of *Drosophila melanogaster* tRNA precursors suggests that C following the discriminator base is the principle element of a 3′-tRNase antideterminant

In prokaryotes, the CCA at the 3′ end of tRNA is usually transcriptionally encoded and the 3′ end trailer of

the precursor tRNA is removed by 3′-exonuclease. tNtase can repair most damaged 3′ ends of tRNAs. In eukaryotes, on the other hand, CCA is not transcriptionally encoded, the 3′ end trailer can be removed by an endonuclease (3′-tRNase) that cleaves immediately after the discriminator base, and tNtase (the CCA-adding enzyme) serves an essential function. The analysis of the 3′ end trailer sequence in 90 *Drosophila melanogaster* tRNAs (Fig. 9) shows that the sequence CCA never occurs directly following the discriminator base, CC— and C—A each occur once, and C—— occurs twice. These frequencies are all below expectation, based on the assumption that sequence distribution is random.

We can thus extend the statement that CCA is not transcriptionally encoded to suggest that C immediately following the discriminator is avoided. We suggest that the active site of 3′-tRNase and the sequence of nucleotides in tRNA 3′ extensions immediately following the discriminator coevolved, with the first nucleotide (C) being the most critical antideterminant for 3′-tRNase. This interpretation is compatible with the kinetic results with pig 3′-tRNase (Fig. 6), in which the greatest increase in K_M occurred with the first C added and sequential reductions in V_{Max} occurred with the addition of C and CC (Fig. 6C).

tRNA localization and transport may be as important as order and kinetics of tRNA end-processing reactions

The order of tRNA end-processing reactions may vary in different systems. The reaction order presented in Fig. 1 is supported by biochemical evidence (Castaño et al., 1985; Frendewey et al., 1985). A 9-nt-long 5′ end leader prevents removal of the 3′ end trailer by mouse 3′-tRNase (Nashimoto, unpubl.), further suggesting that the RNase P reaction tends to precede the 3′-tRNase reaction (Fig. 1A → C).

End-processing kinetics needs to be considered in the larger context of tRNA localization, aminoacylation, and transport. RNase P is principally localized to the yeast nucleolus (Bertrand et al., 1998), thus 5′ end processing may be nucleolar. Splicing-defective tRNAs with CCA added accumulate in the nucleus (Hopper et al., 1980), showing that CCA addition is nuclear and can precede splicing. tRNA microinjected into the *Xenopus* oocyte must be aminoacylated in the nucleus for efficient export (Lund & Dahlberg, 1998). There is a specific mechanism for nucleocytoplasmic export of tRNAs (Simos et al., 1996; Arts et al., 1998; Kutay et al., 1998). The role of La protein, which binds to the 3′ U_3 tails of RNA polymerase III primary transcripts (Stefano, 1984), is still actively being investigated (Yoo & Wollin, 1997; Fan et al., 1998).

3′ end CCA of tRNA—a universally conserved recognition element

All mature tRNAs have CCA at their 3′ ends (nt 74–76 using the standard numbering of Sprinzl et al., 1998). To a varying extent, the enzymes and other RNAs and proteins that bind tRNA have evolved to recognize this CCA. A required Watson–Crick base pair forms between $C^{74}C^{75}$ of substrate tRNA and nt $G^{292}G^{293}$ in an internal loop of *Escherichia coli* M1 RNA, the catalytic subunit of RNase P (Kirsebom & Svaard, 1994). C^{74} of tRNA also forms a Watson–Crick base pair with conserved G^{2252} in the peptidyltransferase center of 23S rRNA (Samaha et al., 1995). Some tRNAs with an incorrect nucleotide at the 3′ end can be properly aminoacylated, but EF-Tu:GTP inefficiently forms a ternary complex with Val-$tRNA^{Val}$ when A^{76} is replaced with a pyrimidine (Liu et al., 1998). Remarkably, tNtase is a high fidelity enzyme, which reliably adds CCA to the discriminator base (Fig. 2, lane 2) without the templating based on complementary base pairing required by most nucleic acid polymerases (Yue et al., 1996).

The 3′ end CCA of tRNA is thus commonly recognized as a sequence, and possibly in some cases as a distinct secondary structure (Hou et al., 1998). CCA at the 3′ end actively discourages the binding of tRNA to 3′-tRNase (Fig. 7–8; cf. Fig. 6), serving as an antideterminant. The antibinding mechanism may be a poor fit between the active site of 3′-tRNase and 3′+CCA. A deeper understanding of why 3′-tRNase is unable to bind and cleave mature tRNA must await structural studies of the enzyme, both free and in association with various tRNAs.

MATERIALS AND METHODS

Runoff templates for transcription by T7 RNA polymerase

All derivatives of *Drosophila melanogaster* $tRNA^{His}$ (Frendewey et al., 1985) were produced by T7 RNA polymerase transcription using plasmid DNAs digested with restriction endonucleases (Levinger et al., 1995). New runoff sites were introduced using mismatched oligonucleotides and U-substituted SK^- phagemid templates (Stratagene; Kunkel, 1985). The wild type 3′-tRNase substrate with a 36-nt 3′ end trailer (Fig. 1B) was transcribed using a template *Dra*I digest. The 3′-tRNase product (tNtase substrate; Fig. 1C) and tNtase product (+CCA; Fig. 1D) were transcribed using *Nsi*I-digested runoff templates. tRNAs with one or two Cs at their 3′ ends beyond the discriminator base (3′+C and 3′+CC) were transcribed using *Pst*I runoff templates.

Transcription and gel purification of synthesized tRNAs.

Internally labeled tRNAs were transcribed for 30 min at 37 °C using 400 μM CTP, UTP, and GTP and 40 μM ATP with

10 μCi α-^{32}P-ATP per 20 μL reaction, as previously described (Levinger et al., 1998). Unlabeled tRNAs were transcribed using 8 mL reactions for 4 h at 37 °C. Initiation with pG at +1 was achieved using a fivefold excess of GMP over GTP or by priming with the dinucleotide pGpC (Levinger et al., 1998). Recombinant T7 RNA polymerase was prepared following Davanloo et al. (1984) through the S-Sepharose chromatography step, and used at a final dilution of 1:400 in the reaction mix. Both labeled and unlabeled tRNAs were purified through two denaturing 6% polyacrylamide gels to ensure a homogeneous length of product. Internally labeled tRNAs were extracted from gel slices by crush-and-soak, and unlabeled tRNAs were recovered using the Elutrap (Schleicher & Schuell). Concentrations of unlabeled tRNAs were determined from the absorbance at 260 nm using an extinction coefficient of 912,744 M^{-1} for the 108-nt 3′-tRNase substrate (Fig. 1B) and of 604,000 M^{-1} for the 75-nt tNtase product (Fig. 1D).

Source and stability of 3′-tRNase

Fly 3′-tRNase was fractionated from a *Drosophila melanogaster* KC_0 culture cell S100 (Dingermann et al., 1981) by gel filtration through Sephadex G25 and ion exchange using S-Sepharose (Pharmacia). 3′-tRNase elutes from S-Sepharose in the column flow-through (Levinger et al., 1995), which also contains substantial tNtase activity. Pig liver 3′-tRNase was purified through the first blue Sepharose chromatography step (Nashimoto, 1995). Both *Drosophila* and pig 3′-tRNase are stable for more than six months when stored in aliquots at −70 °C.

3′-tRNase reactions

3′-tRNase reactions were performed in a volume of 40 or 50 μL, and 10 μL samples were taken at timed intervals to determine reaction velocities. *Drosophila* 3′-tRNase reactions were performed at 28 °C and pig 3′-tRNase reactions were performed at either 28 °C or at 37 °C with comparable results, except that V_{Max} increased and K_M decreased at the higher temperature. The reaction buffer consisted of 30 mM K-HEPES, pH 8, 50 mM KCl, 1 mM DTT, 0.1 mM PMSF, 0.1% Tween 20, 1 U/μL RNasin (Promega), and 10% glycerol, with $MgCl_2$ at 0.3 mM or 1 mM for *Drosophila* 3′-tRNase and at 3 mM for pig 3′-tRNase. The *Drosophila* 3′-tRNase was used at a final dilution of 1:40 or 1:10 in the reaction mix; the more concentrated pig 3′-tRNase was used at 1:500 or 1:100 in the final reaction. To activate tNtase present in the *Drosophila* 3′-tRNase preparation, 1 mM CTP and ATP were added to the reaction mix.

Processing reactions were recovered and electrophoresed on 6% denaturing polyacrylamide gels (Levinger et al., 1998). Gels were exposed to the phosphor storage screen, and screens were scanned using the Molecular Dynamics Storm 840 PhosphorImager. The proportion of substrate processed by 3′-tRNase was determined using ImageQuant software.

Determination of substrate K_M and product K_I

Substrate concentration was varied in Michaelis–Menten experiments by maintaining a fixed amount of labeled substrate RNA, and varying the amount of added unlabeled substrate RNA. Product concentration was determined by multiplying the proportion of labeled product by the substrate concentration used in each reaction. Substrate K_M and V_{Max} were determined from the negative slope and ordinate intercept obtained by plotting reaction velocity versus *V*/[S] (the Eadie–Hofstee method). To determine K_I for various inhibitors, reactions were performed at two different substrate concentrations, one above and one below K_M, using five different inhibitor concentrations roughly centered on K_I (Segel, 1975). When $1/V$ is plotted against [I] for each [S], the two [S] curves intersect at a negative value of [I], which is equal to $-K_I$. When the inhibitor is competitive, the two [S] curves intersect above the abscissa, and the ordinate value at which they intersect is equal to $1/V_{Max}$. All K_M and K_I determinations were performed repeatedly.

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