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Long 5' leaders inhibit removal of a 3' trailer from a precursor tRNA by mammalian tRNA 3' processing endoribonuclease

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ABSTRACT

Mammalian tRNA 3' processing endoribonuclease (3' tRNase) can remove a 3' trailer from various pre-tRNAs without 5' leader nucleotides. To examine how 5' leader sequences affect 3' processing efficiency, we performed *in vitro* 3' processing reactions with purified pig 3' tRNase and pre-tRNA^{Arg}s containing a 13-nt 3' trailer and a 5' leader of various lengths. The 3' processing was slightly stimulated by 5' leaders containing up to 7 nt, whereas leaders of 9 nt or longer severely inhibited the reaction. Structure probing indicated that the 5' leader sequences had little effect on pre-tRNA folding. Similar results were obtained using pre-tRNA^{Val}s containing a 5' leader of various lengths. We also investigated whether 3' tRNase can remove 3' trailers that are stably base-paired with 5' leaders to form an extended acceptor stem. Even such small 5' leaders as 3 and 6 nt, when base-paired with a 3' trailer, severely hindered removal of the 3' trailer by 3' tRNase.

INTRODUCTION

Eukaryotic nuclear transfer RNAs are transcribed as precursor molecules containing 5' leader and 3' trailer sequences by RNA polymerase III (1). Some minor tRNA transcripts are interrupted by introns, which need to be excised by a tRNA splicing endonuclease (2). The tRNA 5' processing is accomplished by RNase P (3). The trailer sequence must be removed before tRNA nucleotidyltransferase adds the 3' terminal CCA sequence to the discriminator nucleotide (4). Many studies have indicated that 3' processing is achieved by an endoribonuclease (5–10), although the possibility of exonucleolytic removal of the 3' trailer has been suggested (11–13). The order of eukaryotic tRNA processing events differs depending on which eukaryotic system is analyzed for tRNA processing. In some cases, 3' processing is thought to follow removal of a 5' leader (9,14,15). On the other hand, the order is reversed in HeLa cell extracts (6).

Mammalian tRNA 3' processing endoribonuclease (3' tRNase) appears to function as a dimer of ~45 kDa protein(s) (7,8). The major determinants for substrate recognition by mammalian 3' tRNase most likely reside in the well-conserved L-shaped tRNA domain, since neither the structures nor the sequences of 3' trailers are conserved. Indeed, 3' tRNase can recognize pre-tRNA^{Arg}s containing only one 3' extra nucleotide and can remove the nucleotide very efficiently (8). Besides the L-shape domain, interactions between 3' tRNase and the 3' trailer also determine the cleavage efficiency of pre-tRNAs, which varies depending on both the 3' trailer length and the 5' end nucleotide (designated as effector nucleotide) of the 3' trailer in the order G ~ A > U > C (8). Mammalian 3' tRNase can be converted to a four-base-recognizing RNA cutter (RNase 65) by forming a relatively stable complex with a 3'-truncated tRNA of ~65 nt (7,16,17). Although nothing is known about the physiological role and substrate of RNase 65, it has been demonstrated that the 3'-truncated tRNA directs substrate specificity via four base pairings (7). The studies on RNase 65 have indicated that 3' tRNase can recognize a pre-tRNA-like complex with an additional RNA stalk extending from the middle of the acceptor stem. Furthermore, experiments using a two half-tRNA system have demonstrated that the sequence and structure of the T stem–loop domain are important, but not essential, for recognition of pre-tRNAs containing an intact D stem–loop and anticodon stem by 3' tRNase (18). Recently, we have shown that the T stem–loop and the acceptor stem plus a 3' trailer are sufficient for efficient substrate recognition and cleavage by 3' tRNase (19).

In this study, we investigated how a 5' leader sequence of pre-tRNA affects tRNA 3' processing using pig 3' tRNase. We demonstrate that 3' tRNase has difficulty in recognizing and cleaving pre-tRNAs with long 5' leaders.

MATERIALS AND METHODS

RNA synthesis

Pre-tRNAs were synthesized with T7 RNA polymerase (Promega) or SP6 RNA polymerase (Takara Shuzo) from synthetic or PCR-generated DNA templates containing a T7 or SP6 promoter. The transcription reactions were carried out in the presence or absence of [α-^{32}P]UTP (DuPont NEN) under the conditions recommended by the manufacturers (Promega and Takara Shuzo). The transcribed pre-tRNAs were gel-purified before assays.

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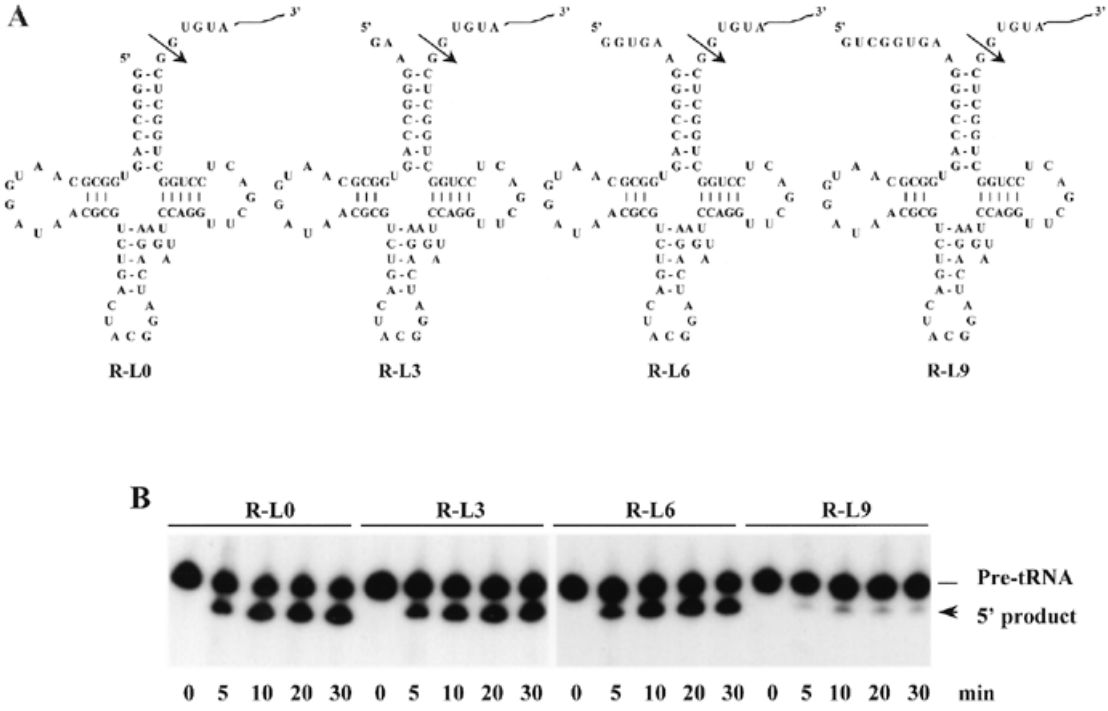

Figure 1. Effect of 5' leaders on tRNA 3' processing. (**A**) Plausible secondary structures of four pre-tRNA^{Arg}s. R-L0, R-L3, R-L6 and R-L9 contain 0-, 3-, 6- and 9-nt 5' leaders, respectively. The sequence 5'-AGCAGUUU-3' is omitted from their 3' trailers. Arrows denote the cleavage sites by 3' tRNase. (**B**) *In vitro* tRNA 3' processing assay. Uniformly ^{32}P-labeled pre-tRNA^{Arg}s, R-L0, R-L3, R-L6 and R-L9, were tested for 3' processing using pig 3' tRNase. After incubation at 37°C for the indicated times, the reaction products were analyzed on a denaturing polyacrylamide gel. Pre-tRNAs and 5' products are denoted by a bar and an arrowhead, respectively.

Preparation of 3' tRNase

We prepared 3' tRNase from pig liver basically as previously described (7). Fresh pig liver was homogenized with Buffer 1 (50 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM dithiothreitol, 10% glycerol), and centrifuged. The supernatant was heated at 55°C for 5 min and the precipitate was removed by centrifugation. The second supernatant was fractionated with ammonium sulfate (50% saturation). The precipitate was dissolved in Buffer 2 (20 mM Tris–HCl, pH 7.5, 0.2 mM EDTA, 1 mM dithiothreitol, 10% glycerol) and dialyzed against this buffer. The sample was subsequently fractionated through Q Sepharose Fast Flow (Pharmacia), Blue Sepharose (Pharmacia) and Heparin Sepharose (Pharmacia) column chromatography. In this study, instead of a Mono Q column (7), the enzyme fraction after heparin–Sepharose chromatography was layered onto a 10 ml 15–30% glycerol gradient in a solution containing 20 mM Tris–HCl, pH 7.5, 0.2 mM EDTA, and centrifuged at 38 000 r.p.m. for 48 h at 4°C. The specific activity of the most active glycerol gradient fraction was comparable to that obtained after Mono Q column chromatography (7).

The 3' processing assay

The 3' processing reactions for ^{32}P-labeled pre-tRNAs (0.1 pmol) were performed with the pig 3' tRNase fraction (10 ng) in a mixture (6 µl) containing 10 mM Tris–HCl (pH 7.5), 1.5 mM dithiothreitol and 3.2 mM spermidine at 37°C (7). After resolution of the reaction products on a 10% polyacrylamide–8 M urea gel, the gel was autoradiographed using an intensifying screen (DuPont NEN) at –80°C.

Kinetic analysis

In vitro 3' processing reactions of various pre-tRNAs by 3' tRNase were examined at various concentrations of substrate. A reaction mixture (6 µl) contained 10 mM Tris–HCl (pH 7.5), 1.5 mM dithiothreitol, 3.2 mM spermidine and 0.17–1.7 µM pre-tRNA. The reactions were incubated with the pig 3' tRNase fraction (5 ng) at 37°C for 1 min. The reaction products were resolved on a 10% polyacrylamide–8 M urea gel and quantitated with a PhosphorImager (Molecular Dynamics). Values of K_m and V_{max} were obtained from double-reciprocal plots (18).

RNA 5'-end-labeling

The 5'-triphosphates of pre-tRNAs were removed with calf intestine alkaline phosphatase (Promega). After the reaction, the RNAs were extracted with phenol, precipitated with ethanol, and redissolved in water. Pre-tRNAs were 5'-end-labeled with [γ-^{32}P]ATP (DuPont NEN) using T4 polynucleotide kinase (Life Technologies, Inc.), and purified on a denaturing gel.

Structure probing

Partial digestion of 5'-end-labeled pre-tRNAs was performed using RNases A (Sigma), T1 (Boehringer Mannheim) and V1 (Pharmacia). The reaction mixture (6 µl) contained 10 mM Tris–HCl (pH 7.5), 1.5 mM dithiothreitol, 3.2 mM spermidine, 1 µg yeast tRNA, 1.0 µM pre-tRNA, and RNase A (0.2 or 0.4 U/ml), T1 (0.1 or 0.2 U/ml) or V1 (3 or 6 U/ml). Reactions

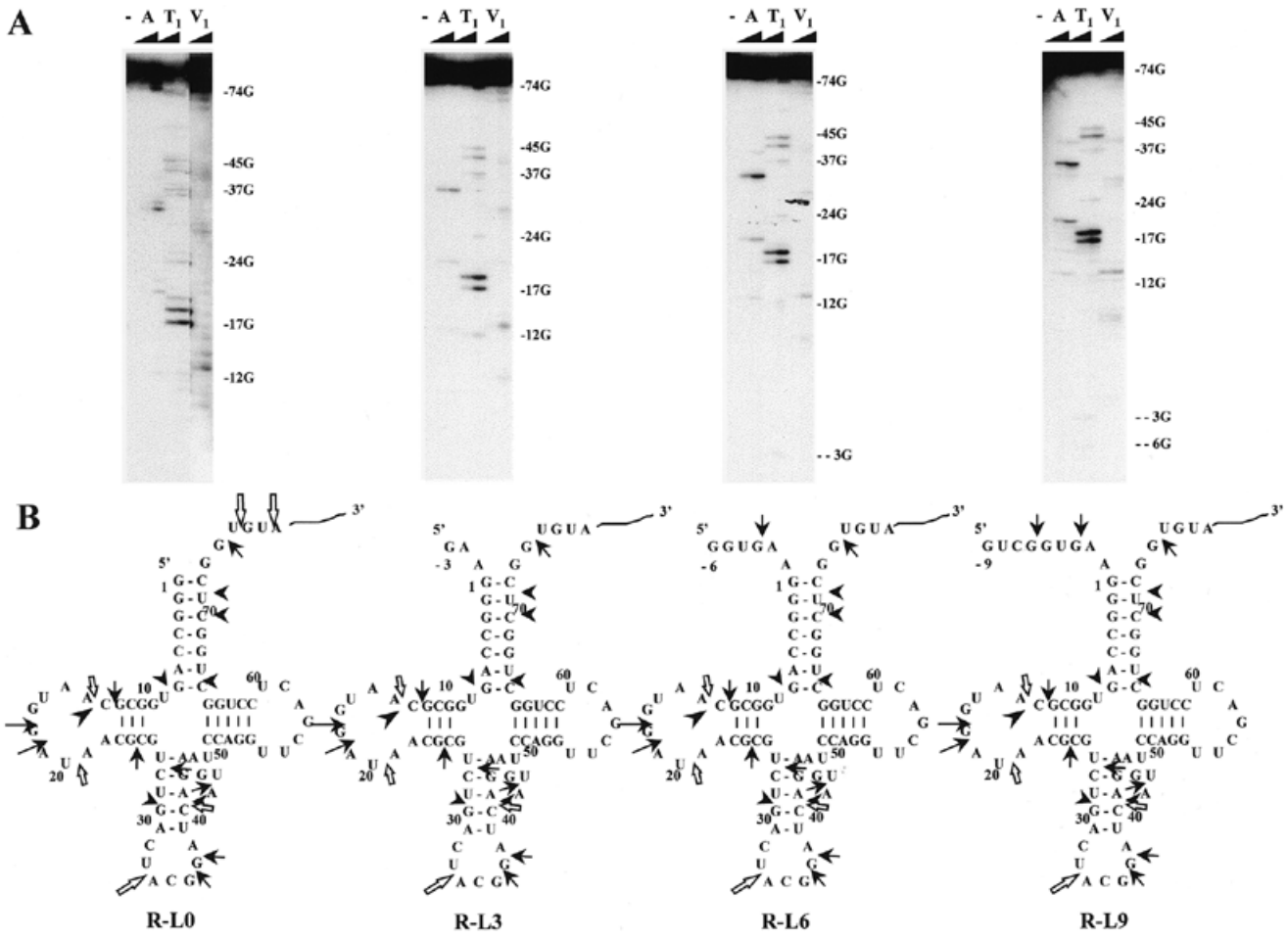

Figure 2. Structure probing of the pre-tRNA^{Arg}s R-L0, R-L3, R-L6 and R-L9. (**A**) 5'-End-labeled pre-tRNA^{Arg}s were partially digested with RNase A (0.2 and 0.4 U/ml), RNase T1 (0.1 and 0.2 U/ml) and RNase V1 (3 and 6 U/ml), and subsequently separated on a 10% polyacrylamide–8 M urea gel. (**B**) Cleavage sites of RNases A, T1 and V1 are indicated by hollow arrows, arrows and arrowheads, respectively, on the plausible secondary structures of the pre-tRNAs. Length of the symbols reflects intensity of cleavage. The sequence 5'-AGCAGUUU-3' is omitted from their 3' trailers.

were incubated at room temperature (or at 37°C in the case of RNase V1) for 10 min. After extraction with phenol/chloroform and ethanol precipitation, the samples were analyzed on a 10% polyacrylamide–8 M urea sequencing gel. After electrophoresis, the gels were dried for 45 min under vacuum at 80°C and autoradiographed using an intensifying screen at –80°C.

RESULTS

Inhibition of *in vitro* tRNA 3' processing by a 5' leader of 9 nt

We examined four human pre-tRNA^{Arg}s containing increasing 5' leader lengths for 3' processing by 3' tRNase. The pre-tRNAs R-L0, R-L3, R-L6 and R-L9 contain 0-, 3-, 6- and 9-nt 5' leaders, respectively, in addition to a common 13-nt 3' trailer (Fig. 1A). The mature tRNA domain and the 3' trailer originate from a human DNA sequence with the DDBJ/EMBL/GenBank accession no. X64282. The 5' leader sequences were selected to be uncomplementary to the 3' trailer sequence. An *in vitro* 3' processing reaction for each ^{32}P-labeled pre-tRNA was performed using purified pig 3' tRNase. After incubation for 5, 10, 20 and 30 min, the reaction products were analyzed on a denaturing polyacrylamide gel. The 3' processing of R-L3 and R-L6 was as efficient as that of R-L0, but the removal of the R-L9 3' trailer was greatly inhibited (Fig. 1B). We also carried out a kinetic analysis of 3' processing of the pre-tRNAs R-L0, R-L3 and R-L6. The K_m values decreased and the V_{max} values increased as the 5' leader sequences of the pre-tRNAs increased (Table 1). As a result, the relative 3' processing efficiency V_{max}/K_m of R-L3 and R-L6 was 1.5- and 2.0-fold higher than that of R-L0, respectively.

5' leader sequences have little effect on folding of pre-tRNAs

To rule out the possibility that the above differential 3' processing efficiency may be due to folding errors of the pre-tRNAs, we carried out structure probing assays. Each of the 5'-end-labeled pre-tRNAs R-L0, R-L3, R-L6 and R-L9 was partially digested with RNase A, T1 or V1 and analyzed on a sequencing gel. On the whole, the cleavage pattern of these pre-tRNAs was similar (Fig. 2). The D loop, the anticodon loop and the 3' trailer were susceptible to RNases A and T1, while the double-stranded RNA specific RNase V1 cleaved mainly the stem

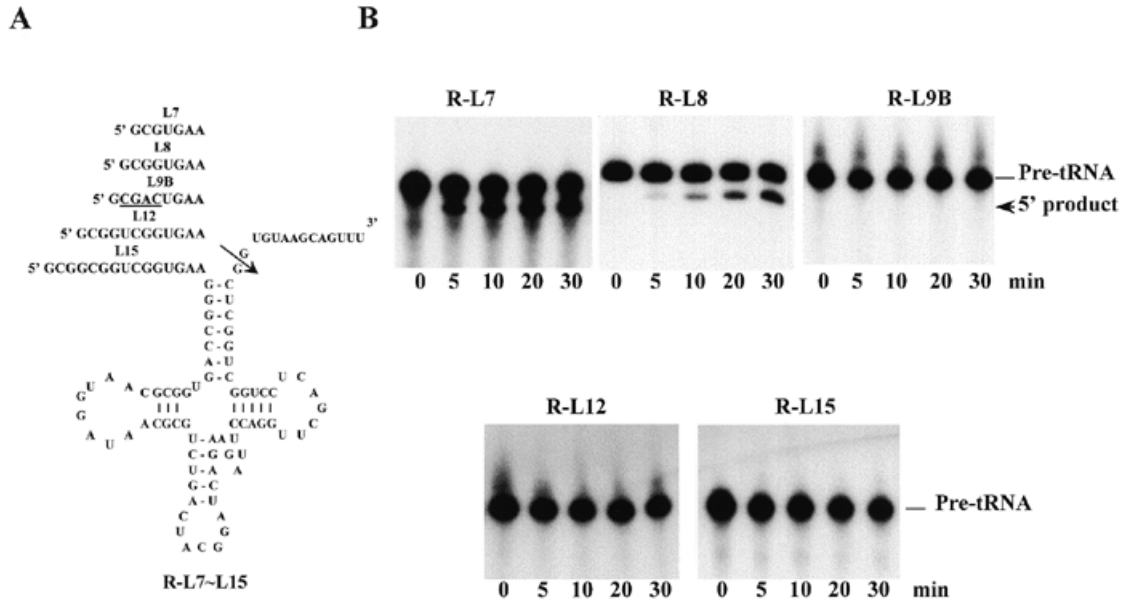

Figure 3. Inhibition of tRNA 3' processing by long 5' leaders. (**A**) Secondary structures of the pre-tRNA^{Arg}s R-L7, R-L8, R-L9B, R-L12 and R-L15. These pre-tRNAs consist of a common mature tRNA/3' trailer domain and a 5' leader of different length. The underline in the R-L9B 5' leader denotes different bases from those in the R-L9 5' leader. The 3' tRNase cleavage site is indicated by an arrow. (**B**) Time course analyses for the 3' tRNase cleavage reaction. The above ^{32}P-labeled pre-tRNA^{Arg}s were tested for 3' processing using pig 3' tRNase. After incubation at 37°C for the indicated times, the reaction products were analyzed on a denaturing polyacrylamide gel. Pre-tRNAs and 5' products are denoted by a bar and an arrowhead, respectively.

regions. RNases A and T1 also cleaved pre-tRNAs at some common sites in the D stem and the anticodon stem. The 5' leaders were digested at –6G and –3G of R-L9 and at –3G of R-L6 by RNase T1, suggesting that these 5' leaders do not interact with the other regions of pre-tRNAs through base-pairing. These results indicate that 5' leader sequences have little effect on folding of pre-tRNAs and that the difference in the 3' processing efficiency is not due to misfolding of the substrates but intrinsic to 3' tRNase itself.

Table 1. Kinetic parameters of tRNA 3' processing reaction by pig 3' tRNase

Substrate	K_m[a] (μM)	V_{max}[a] (pmol/min)	Relative V_{max}/K_m
R-L0	0.83	0.21	1.0
R-L3	0.69	0.26	1.5
R-L6	0.60	0.30	2.0

[a]The maximum velocity per nanogram of pig 3' tRNase fraction after glycerol gradient centrifugation is shown. Each measurement was from averages of three trials with a standard deviation of 5–10%.

Pre-tRNAs containing a 12-nt or longer 5' leader are not substrates for 3' tRNase

To further examine whether 7- and 8-nt 5' leaders inhibit 3' processing, we tested two pre-tRNA^{Arg}s for cleavage by 3' tRNase. The pre-tRNAs R-L7 and R-L8 contain 7- and 8-nt 5' leaders, respectively, in addition to the common 13-nt 3' trailer (Fig. 3A). The 3' trailer of R-L7 was removed by pig 3' tRNase as efficiently as that of R-L6, while the cleavage efficiency of R-L8 was intermediate between those of R-L6 and R-L9 (Figs 1B and 3B). These results suggested that 5' leaders suddenly begin to hinder the 3' processing reaction when the 5' leader length becomes 8 nt. To confirm that long 5' leaders inhibit the tRNA 3' processing, we assayed three additional pre-tRNA^{Arg}s, R-L9B, R-L12 and R-L15, for 3' tRNase cleavage. R-L9B contained a 9-nt 5' leader, in which four bases were changed from those in the R-L9 5' leader into different bases in order to analyze the effect of the 5' leader sequence on cleavage inhibition (Fig. 3A). In contrast to the slight cleavage of R-L9 (Fig. 1B), pig 3' tRNase did not cleave R-L9B at all, even after a 30 min incubation (Fig. 3B). This suggests that the sequence as well as the length of the 5' leader affect the degree of inhibition. R-L12 and R-L15, which contained 12 and 15-nt 5' leaders, respectively, were also not cleaved (Fig. 3B).

The 3' processing velocities decrease steeply as the length of the 5' leader is extended beyond 7 nt

To quantitatively compare the cleavage efficiency for the above nine pre-tRNAs, we calculated 3' processing velocities for these substrates from the amounts of the cleavage products after a 5 min reaction. The velocities (fmol/min) were plotted against the length of 5' leaders (Fig. 4). The average value of R-L9 and R-L9B was used for the 3' processing velocity for the 9-nt 5' leader. The 3' processing velocities gradually increased up to the 6-nt 5' leader, steeply decreased as the 5' leaders extended beyond 7 nt, and dropped to 0 as the length increased to 12 nt or longer (Fig. 4).

Two reports have suggested that mammalian tRNA transcription principally starts 2–7 nt upstream of mature tRNA 5' termini (6,20). In relatively few cases, pre-tRNAs containing

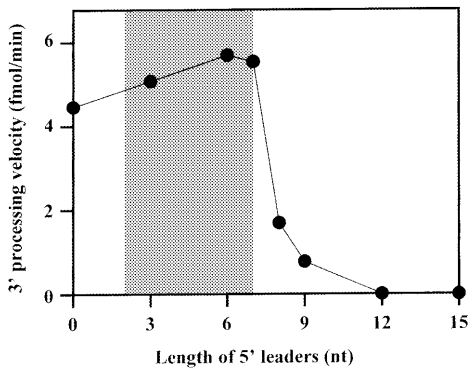

Figure 4. 3' processing velocities versus length of 5' leaders. 3' Processing velocities (fmol/min) are plotted against length of 5' leaders. The velocities were calculated from the amount of cleavage product after a 5 min reaction using the data from Figures 1B and 3B. The velocity for the 9-nt 5' leader is an average of the R-L9 and R-L9B values. Most mammalian pre-tRNAs are thought to contain 2–7-nt 5' leaders (designated by a shaded area).

8- and 9-nt 5' leaders have been detected (6). Interestingly, the distribution of 5' leader length appears to reflect the differential 3' processing efficiency by mammalian 3' tRNase (Fig. 4).

3' tRNase is unable to remove 3' trailers stably base-paired with 5' leaders

The more severe inhibition of 3' tRNase cleavage by the R-L9B 5' leader than by the L-9 5' leader may have been due to partial base-pairing between the 5' leader and the 3' trailer, as discussed below. We investigated whether 3' tRNase can remove 3' trailers stably base-paired with 5' leaders. Three pre-tRNA^{Arg}s, R-L3C, R-L6C and R-L9C, which contained 3-, 6- and 9-nt 5' leaders, respectively, were tested for 3' processing. The 5' leaders of R-L3C, R-L6C and R-L9C had complementary sequences to the discriminator and 3' trailer bases, resulting in the formation of 3, 5 and 9 bp extended acceptor stems, respectively (Fig. 5A). Time course analyses using pig 3' tRNase showed that the enzyme is unable to cleave any of these substrates even after 30 min incubations (Fig. 5B). These results indicate that even a 5' leader as small as 3 nt, when base-paired with a 3' trailer, severely hinders removal of the 3' trailer by 3' tRNase. This contrasts sharply with the observation that 3' processing is not inhibited by even such a long 5' leader as 7 nt if the 5' leader has no obvious complementarity with the 3' trailer (Fig. 3).

In vitro 3' processing of pre-tRNAs containing natural 5' leader sequences

We also tested four human pre-tRNA^{Arg}s containing increasing lengths of a natural 5' leader and a common natural 3' trailer (DDBJ/EMBL/GenBank accession no. X64282) for *in vitro* 3' processing (Fig. 6A). The pre-tRNA^{Arg}s R-L0N, R-L3N, R-L6N and R-L10N contain 0-, 3-, 6- and 10-nt 5' leaders, respectively. R-L0N, R-L3N and R-L6N were efficiently cleaved by 3' tRNase, while cleavage of R-L10N was hardly detected

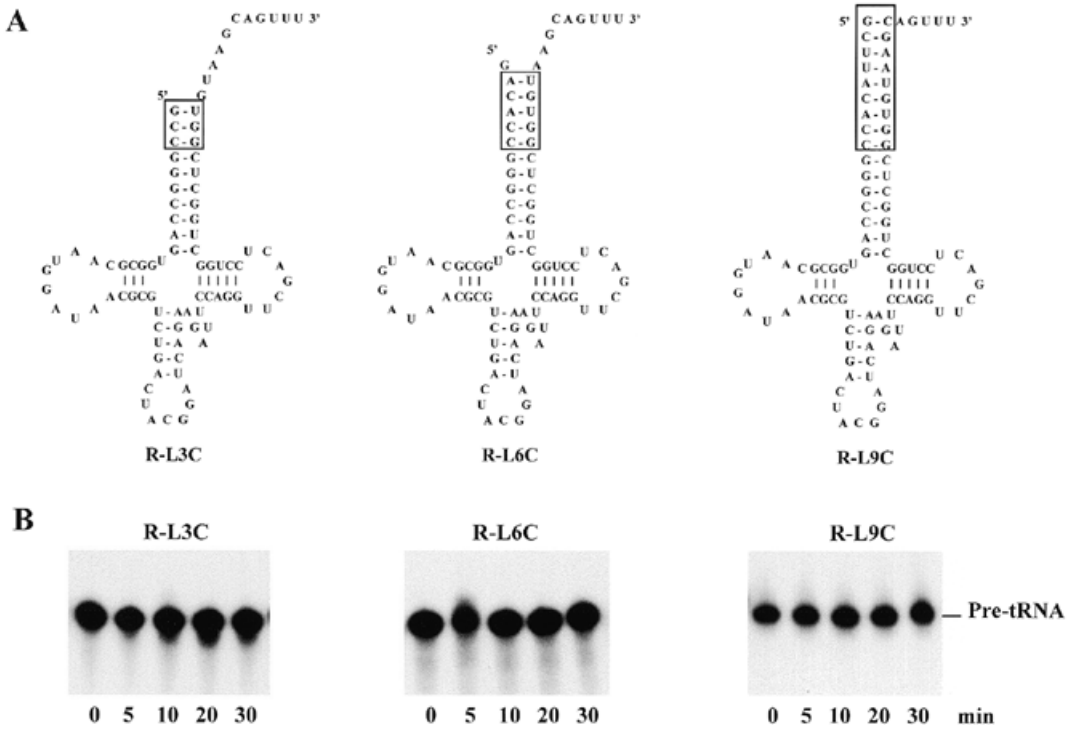

Figure 5. Effect of 5' leaders base-paired with 3' trailers on 3' processing. (**A**) Secondary structures of the pre-tRNA^{Arg}s R-L3C, R-L6C and R-L9C, which contain 3-, 6- and 9-nt 5' leaders, respectively. Regions in which the 5' leaders are base-paired with the discriminator/3' trailer sequences are surrounded by rectangles. (**B**) The 3' tRNase cleavage assays for R-L3C, R-L6C and R-L9C. The ^{32}P-labeled pre-tRNA^{Arg}s were tested for cleavage. After incubation at 37°C for the indicated times, the RNAs were analyzed on a denaturing polyacrylamide gel.

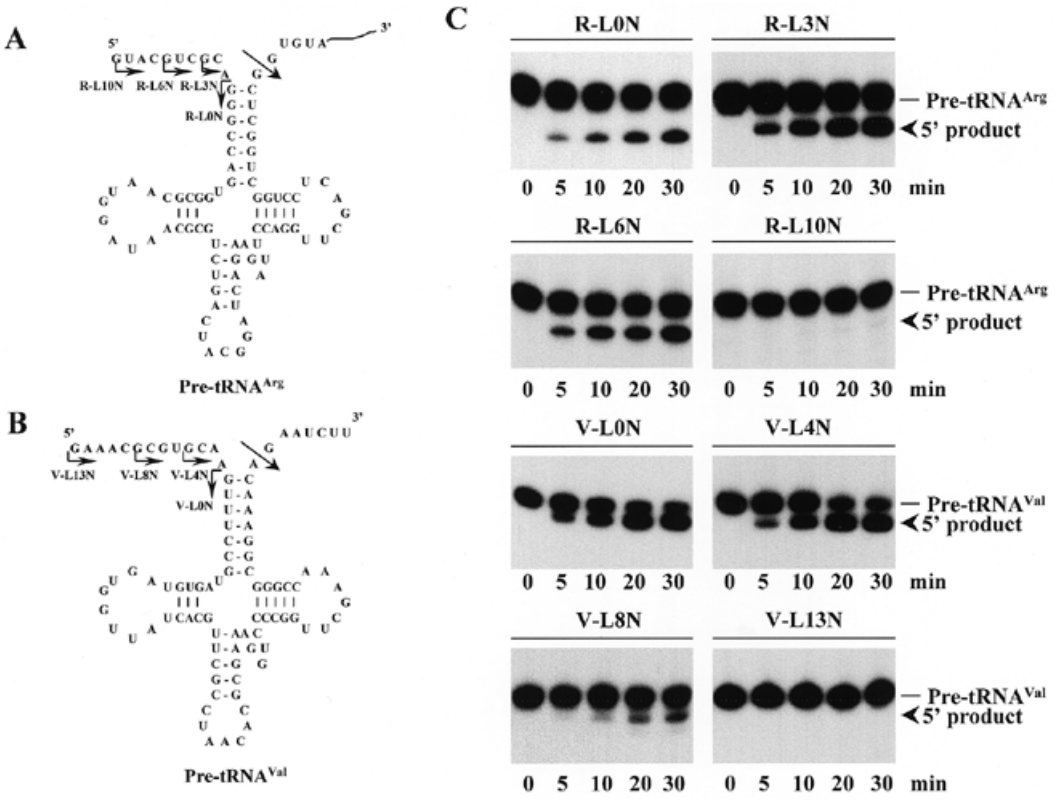

Figure 6. *In vitro* 3' processing reaction of pre-tRNAs containing natural 5' leader sequences. (**A**) Plausible secondary structures of the four pre-tRNA^{Arg}s R-L0N, R-L3N, R-L6N and R-L10N containing 0-, 3-, 6- and 10-nt 5' leaders, respectively. The sequence 5'-AGCAGGGTCGUUU-3' is omitted from their 3' trailers. A straight arrow denotes the cleavage site by 3' tRNase. (**B**) Plausible secondary structures of four pre-tRNA^{Val}s. V-L0N, V-L4N, V-L8N and V-L13N contain 0-, 4-, 8- and 13-nt 5' leaders, respectively. A straight arrow indicates the cleavage site by 3' tRNase. (**C**) Time course assays for the 3' tRNase cleavage reaction. The ^{32}P-labeled pre-tRNA^{Arg}s or pre-tRNA^{Val}s were tested for *in vitro* 3' processing. After incubation at 37°C for the indicated times, the reaction products were separated on a denaturing polyacrylamide gel. Pre-tRNAs and 5' products are indicated by bars and arrowheads, respectively.

(Fig. 6C). These results were similar to those obtained by using the pre-tRNA^{Arg}s containing artificial 5' leaders (Fig. 1).

To further examine whether long 5' leaders inhibit removal of a 3' trailer from another pre-tRNA species, we tested four human pre-tRNA^{Val}s containing increasing lengths of a natural 5' leader and a common natural 3' trailer (DDBJ/EMBL/GenBank accession no. X17514) for 3' tRNase cleavage (Fig. 6B). The pre-tRNA^{Val}s V-L0N, V-L4N, V-L8N and V-L13N have 0-, 4-, 8- and 13-nt 5' leaders, respectively. V-L4N was cleaved as efficiently as V-L0N, while cleavage of V-L8N was less efficient and cleavage of V-L13N was not detectable (Fig. 6C). These results were parallel with results from the experiments using pre-tRNA^{Arg}s containing various artificial 5' leaders (Figs 1 and 3).

DISCUSSION

Long 5' leaders may disturb substrate recognition by 3' tRNase through steric hindrance

In 3' processing reactions of pre-tRNA^{Arg}s containing increasing artificial 5' leader lengths, the K_m values decreased and the V_{max} values increased as the 5' leader sequences increased up to 6 nt (Table 1). These results indicate that 5' leader sequences up to 6 nt make the complex between pre-tRNA and 3' tRNase more stable and enhance the catalytic step and/or a dissociation rate of the product if they do not interact stably with the discriminator/3' trailer. Although the 7-nt 5' leader did not hinder 3' processing of R-L7, the 8-nt 5' leader of R-L8 was not tolerable for 3' tRNase (Fig. 3). The pre-tRNA R-L9 containing a 9-nt leader severely lost its substrate ability (Fig. 1), although its folding was correct (Fig. 2). Furthermore, R-L9B, R-L12 and R-L15, which contain 9-, 12- and 15-nt 5' leaders, respectively, were not substrates for pig 3' tRNase (Fig. 3). The natural 5' leaders of pre-tRNA^{Arg}s and pre-tRNA^{Val}s also inhibited *in vitro* 3' processing when they became 8 nt or longer (Fig. 6).

We may be able to explain the above observations by taking account of the possibility that long 5' leaders (8 nt or longer) sterically hinder recognition by 3' tRNase. The differential degree of 3' processing inhibition between by the R-L9 and R-L9B 5' leaders (Figs 1 and 3) suggested that the effect of the steric hindrance depends not only on length but also on sequence. The 9-nt 5' leader of R-L9B may inhibit 3' tRNase cleavage more severely than that of R-L9 through the interaction with the 3' trailer; the sequence 5'-GCGAC-3' in the R-L9B 5' leader may interact with the sequence 5'-GUAAGC-3' in the 3' trailer via base-pairings (indicated by underlines) (Fig. 3A). Besides simple steric hindrance by long sequences, the strong inhibitory effect of the R-L12, R-L15, R-L10N and V-L13N 5' leaders on 3' trailer removal may also arise from secondary structures

within the 5' leader and/or between the 5' leader and the 3' trailer. For example, the leader sequences 5'-GCGGUCGGU-GAA-3' (possible base-parings are shown by underlines) of R-L12 and 5'-GCGGCGGUCGGUGAA-3' of R-L15 could be folded themselves. Such interactions within the 5' leader and/or between the 5' leader and the 3' trailer become more frequent with increasing length of the 5' leader.

Stable 5' leader/3' trailer interactions are negative factors for recognition by 3' tRNase

The pre-tRNA^{Arg}s R-L3C and R-L6C were not 3'-processed at all, although their 5' leaders were only 3 and 6 nt long, respectively (Fig. 5). These results are consistent with the above notion that the 5' leader/3' trailer interaction is one of the entities that lead to the steric hindrance against substrate recognition by 3' tRNase. Recently, we have demonstrated that mammalian 3' tRNase can cleave pre-tRNA^{Arg}s containing 1 and 2 bp insertions into the acceptor stem and no 5' leaders although the cleavage becomes less efficient than the wild type depending on the number of insertions (21). The cleavage has been shown to occur after the discriminator (i.e., a nucleotide immediately 3' to the acceptor stem). We have also shown that a pre-tRNA^{Arg} that has a 3 bp insertion in the acceptor stem can hardly be cleaved by 3' tRNase (21). Taken together, our results suggest that an extension of 3 bp or more of the acceptor stem is not tolerable to mammalian 3' tRNase.

Evolutionary considerations

Like in *Neurospora* mitochondrial introns (22), tRNA-like structures may also exist in mammalian cellular RNAs other than tRNAs. Hairpin RNA structures resembling the acceptor-stem and T stem–loop domain, which are minimum substrates for mammalian 3' tRNase (19), may be found more easily in the cellular RNAs. If 3' tRNase could cleave pre-tRNAs containing long 5' leaders, those RNAs would also be cut by 3' tRNase and their cellular functions would be damaged. 3' tRNase may have needed to evolve to avoid such chaotic RNA degradation.

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