Anomalous RNA substrates for mammalian tRNA 3′ processing endoribonuclease

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Abstract Mammalian tRNA 3′ processing endoribonuclease (3′ tRNase) is an enzyme responsible for the removal of a 3′ trailer from pre-tRNA. The enzyme can also recognize and cleave any target RNA that forms a pre-tRNA-like complex with another RNA. To investigate the interaction between 3′ tRNase and substrates, we tested various anomalous pre-tRNA-like complexes for cleavage by pig 3′ tRNase. We examined how base mismatches in the acceptor stem affect 3′ tRNase cleavage of RNA complexes, and found that even one base mismatch in the acceptor stem drastically reduces the cleavage efficiency. Mammalian 3′ tRNase was able to recognize complexes between target RNAs and 5′-half tDNAs, and cleave the target RNAs, although inefficiently, whereas the enzyme had no activity to cleave phosphodiester bonds of DNA. A relatively long RNA target, the *Escherichia coli* chloramphenicol acetyltransferase (CAT) mRNA, was cleaved by 3′ tRNase in the presence of appropriate 5′-half tRNAs. We also demonstrated that an RNA complex of *lin-4* and *lin-14* from *Caenorhabditis elegans* can be recognized and cleaved by pig 3′ tRNase.

Key words: 3′ tRNase; pre-tRNA; RNase 65; Targeted RNA cleavage

1. Introduction

Mammalian nuclear tRNAs are transcribed as larger precursors containing 5′ leader and 3′ trailer sequences by RNA polymerase III [1]. The 5′-end processing of the precursor tRNA (pre-tRNA) is accomplished by RNase P [2]. Mammalian tRNA 3′ processing endoribonuclease (3′ tRNase) is an enzyme responsible for the removal of a 3′ trailer from pre-tRNA [3]. We have shown that the pre-tRNA 5′ leaders affect the 3′ processing efficiency in vitro; 9-nt or longer leaders strongly inhibit the 3′ processing reaction, and even such small 5′ leaders as three and six nucleotides, when base-paired with a 3′ trailer, severely hinder removal of the 3′ trailer by 3′ tRNase [4]. Besides the 5′ leaders, the 3′ trailers also affect the cleavage efficiency of pre-tRNAs, which varies depending on both the 3′ trailer length and the 5′-end nucleotide of the 3′ trailer in the order G ∼ A > U > C [3,5].

We have found a general rule for cleavage site selection by mammalian 3′ tRNase [6]. According to this rule, cleavage of pre-tRNAs containing a total of N base-pairs (N is less than 12) in the acceptor stem and the T stem occurs after $12-N$ and $13-N$ nt 3′ to the discriminator, while cleavage of pre-tRNAs with a total of N base-pairs (N is greater than or equal to 12) occurs after the discriminator [6].

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-tRNAs containing only one 3′ extra nucleotide and can remove the nucleotide very efficiently [3,5]. Recently we have demonstrated that minimum substrates for mammalian 3′ tRNase are minihelices or 'minihelicesΔ1' that are one base-pair smaller than minihelices and are less efficient substrates [7]. In these minimum substrates, there seems to be no essential bases [7]. The above rule for cleavage site selection slightly changes in the case of the mini-substrates [7].

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 [8–12]. 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 [12]. 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, using a two-half tRNA (designated as 5′-half tRNA and 3′-half tRNA) system, we have demonstrated that generally any 3′-half tRNAs can be specifically cleaved by mammalian 3′ tRNase under the direction of appropriate 5′-half tRNAs [13]. We have also shown that 3′ tRNase can specifically and efficiently cleave 3′-half tRNAs even in the presence of RNA heptamers that correspond to 5′ portions of the acceptor stems [14].

In this paper, we refer to 5′-half tRNA and RNA heptamer that can direct RNA cleavage by mammalian 3′ tRNase as small guide RNA (sgRNA), and 3′-half tRNA as target RNA. To further investigate the interaction between mammalian 3′ tRNase and substrates, we tested various anomalous RNA complexes for cleavage by 3′ tRNase. We confirm that the acceptor stem and the T stem/loop are important for recognition by the enzyme. We also demonstrate that 3′ tRNase can cleave a relatively large target RNA under the direction of appropriate 5′-half tRNAs.

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Abbreviations: 3′ tRNase, tRNA 3′ processing endoribonuclease

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2. Materials and methods

2.1. RNA synthesis

Target RNAs and sgRNAs were synthesized with T7 RNA polymerase (Takara Shuzo) or SP6 RNA polymerase (Takara Shuzo) from synthetic or polymerase chain reaction-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 (Amersham Japan) under the conditions recommended by the manufacturer (Takara Shuzo). The transcribed RNAs were gel-purified before assays.

2.2. The 3′ tRNase specific cleavage assay

3′ tRNase was prepared from pig liver as previously described [12]. The 3′ tRNase specific cleavage reactions for ^{32}P-labeled target RNAs (0.1 pmol) were performed with pig 3′ tRNase (0.4 U) in the presence of various sgRNAs in a mixture (10 μl) containing 10 mM Tris–HCl (pH 7.5), 1.5 mM dithiothreitol, and 3.2 mM spermidine at 37°C [14]. After resolution of the reaction products on a 10% or 5% polyacrylamide–8 M urea gel, the gel was autoradiographed using an intensifying screen (DuPont NEN) at −80°C. The cleavage products were quantitated with a Molecular Imager (Bio-Rad).

2.3. DNA 5′-end labeling

Synthetic DNAs were 5′-end labeled with [γ-^{32}P]ATP (Amersham Japan) using T4 polynucleotide kinase (Takara Shuzo) and purified on a denaturing gel.

2.4. 3′-end labeling and RNA sequencing

Unlabeled target RNAs (1 pmol) were reacted with pig 3′ tRNase (1 U) under the standard assay conditions at 37°C for 20 min, extracted with phenol/chloroform, and precipitated with ethanol. The reaction products dissolved in water were 3′-end labeled with [5′-^{32}P]pCp and T4 RNA ligase (Takara Shuzo) at 4°C for 10 h. The 5′ cleavage products were gel-purified, and their 3′-terminal sequences were determined by the chemical RNA sequencing method [15].

3. Results and discussion

3.1. The effect of base mismatches in the acceptor stem on substrate cleavage by mammalian 3′ tRNase

We examined how base mismatches in the acceptor stem

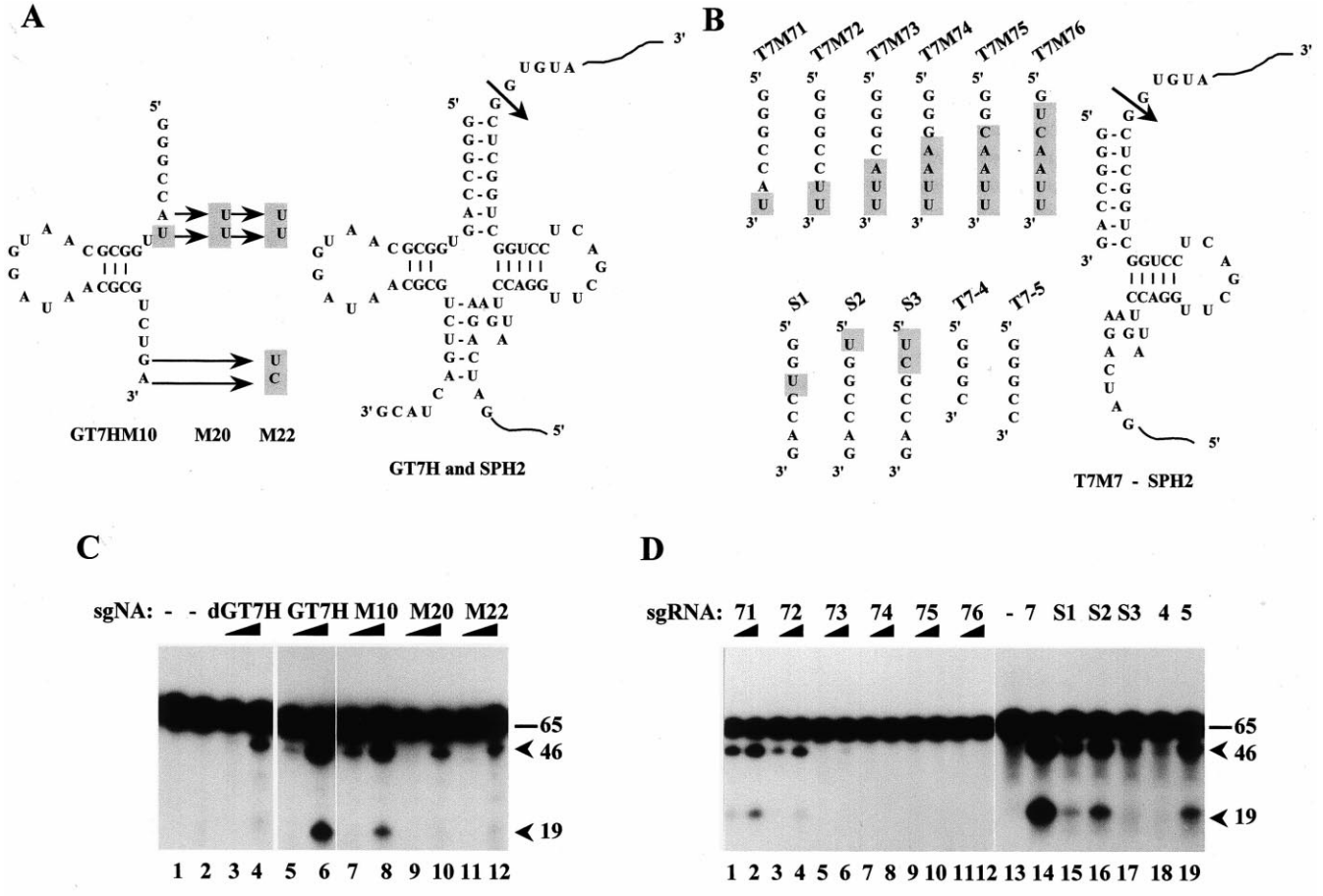

Fig. 1. Specific RNA cleavage by mammalian 3′ tRNase under the direction of various sgRNAs. A: A secondary structure of the RNA complex GT7H–SPH2 and sequences of the three sgRNAs GT7HM10, GT7HM20, and GT7HM22 derived from GT7H. The sequences 5′-GCACUAAAG-3′ and 5′-AGCAGGGUCGUUUU-3′ are omitted from the 5′ and 3′ regions, respectively, of SPH2. An arrow in the GT7H/SPH2 complex denotes the cleavage site by 3′ tRNase. In GT7HM20 and GT7HM22, the other sequences omitted in the diagram are the same as GT7HM10. The different nucleotides from GT7H are shaded. B: Sequences of the sgRNAs and a secondary structure of the T7M7/SPH2 complex. The shaded bases are different from T7M7. C: In vitro specific RNA cleavage assays. The uniformly ^{32}P-labeled target SPH2 (0.1 pmol) was tested for specific cleavage by pig 3′ tRNase in the presence of various sgRNAs (0.5 pmol in lane 5; 1 pmol in lanes 7, 9, and 11; 10 pmol in lanes 6, 8, 10, and 12) or an sgDNA (0.5 pmol in lane 3; 10 pmol in lane 4). As controls, SPH2 was incubated without the enzyme (lane 1) or with the enzyme in the absence of sgRNAs (lane 2). After incubation at 37°C for 20 min, the reaction products were analyzed on a denaturing polyacrylamide gel. The 46-nt 5′ and 19-nt 3′ cleavage products of the 65-nt SPH2 are denoted by arrowheads. D: The same as C except that RNA heptamers or shorter RNAs were used as sgRNAs. The reactions were performed in the presence of 1 pmol (lanes 1, 3, 5, 7, 9, and 11) or 10 pmol (lanes 2, 4, 6, 8, 10, 12, 14–19) of each sgRNA. As a control, SPH2 was incubated with pig 3′ tRNase in the absence of sgRNAs (lane 13).

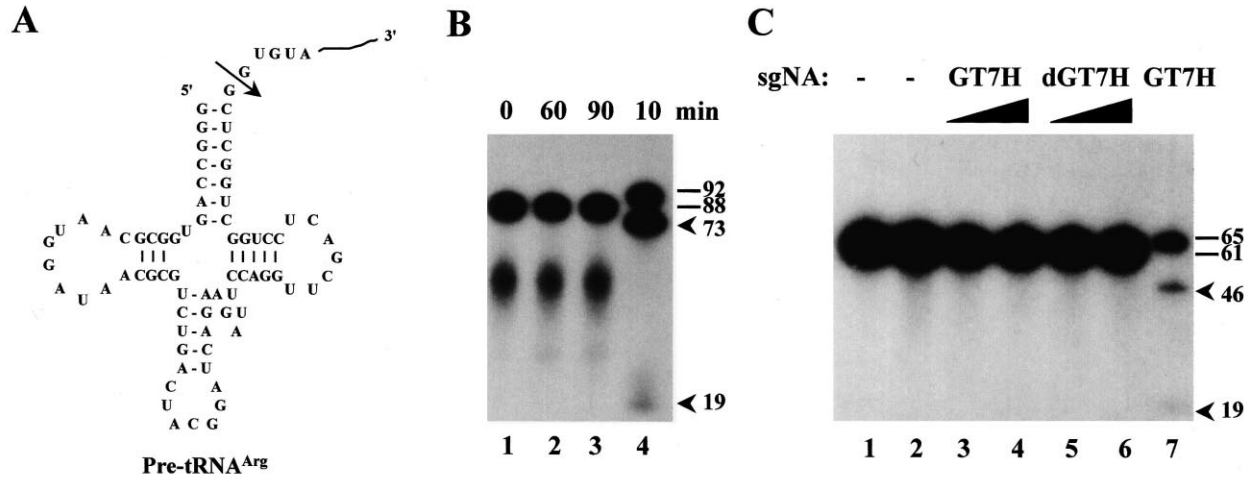

Fig. 2. Mammalian 3′ tRNase cleavage assays for DNA substrates. A: A secondary structure of pre-tRNA^{Arg}. The sequence 5′-AGCAGGGUCGUUUU-3′ is omitted from the 3′ trailer. An arrow denotes the cleavage site. B: The 5′-end-labeled 88-nt pre-tDNA^{Arg} (0.1 pmol) was tested for cleavage by pig 3′ tRNase at 37°C for the indicated times (lanes 1–3). As a control, uniformly labeled 92-nt pre-tRNA^{Arg} (0.1 pmol) was incubated at 37°C for 10 min (lane 4). Arrowheads indicate cleavage products of pre-tRNA^{Arg}. C: The 5′-end-labeled DNA target dSPH2 (0.1 pmol) was assayed for cleavage by pig 3′ tRNase at 37°C for 90 min in the absence (lane 2) or the presence of 1 pmol (lanes 3 and 5) or 50 pmol (lanes 4 and 6) of sgRNA. In lane 1, dSPH2 was incubated without the enzyme. As a control, cleavage reaction of the 65-nt SPH2 (0.1 pmol) in the presence of GT7H (0.5 pmol) was performed at 37°C for 20 min (lane 7). Arrowheads indicate cleavage products of SPH2.

and the anticodon stem affect 3′ tRNase cleavage of RNA complexes resembling pre-tRNAs. The 65-nt 3′-half tRNA SPH2 and four different 5′-half tRNAs (GT7H, GT7HM10, GT7HM20, and GT7HM22) were synthesized in vitro with T7 RNA polymerase in the presence and the absence of [α-^{32}P]UTP, respectively (Fig. 1A). The wild-type 5′-half tRNA GT7H can form a 7-bp acceptor stem and a 5-bp anticodon stem with the 3′-half tRNA SPH2, while the other three 5′-half tRNA variants contain one to four mismatched bases with regard to SPH2 (Fig. 1A). GT7HM10 and GT7HM20 have one and two mismatches in the acceptor stem, respectively, and GT7HM22 has two mismatches each in the acceptor stem and in the anticodon stem (Fig. 1A).

The labeled SPH2 (0.1 pmol) was tested for cleavage after the discriminator nucleotide G by 3′ tRNase in the presence of each unlabeled 5′-half tRNA. Assays were performed at 37°C for 20 min using pig 3′ tRNase. Percent cleavage of SPH2 directed by GT7HM10 (10 pmol) was 62.5% of that directed by the same amount of GT7H (Fig. 1C, compare lanes 6 and 8), while percent cleavage directed by GT7HM20 was much reduced to 10.7% of percent cleavage directed by GT7H (Fig. 1C, compare lanes 6 and 10). These results indicate that the increase of the number of mismatched bases in the acceptor stem gradually reduces the cleavage efficiency. GT7HM22 directed cleavage of SPH2 as efficiently as GT7HM20 (Fig. 1C, lanes 9–12), suggesting that the mismatches in the anticodon stem do not affect cleavage efficiency. This result is totally consistent with the observation that the acceptor stem and the T stem/loop plus a 3′ trailer are sufficient for substrate recognition by mammalian 3′ tRNase [7,14].

3.2. The acceptor stem is important for substrate recognition by mammalian 3′ tRNase

We further investigated the effect of base mismatches in the acceptor stem on cleavage efficiency using nine RNA heptamers. These heptamers have one to six mismatches with regard to the acceptor stem region of the 3′-half tRNA SPH2 (Fig. 1B). The ^{32}P-labeled SPH2 was tested for cleavage by pig 3′ tRNase in the presence of each unlabeled heptamer. Percent cleavage of SPH2 directed by 10 pmol of T7M71 or S2, which contains only one base mismatch, was ~40% of that directed by the same amount of T7M7 (Fig. 1D, lanes 2, 14, and 16). On the whole, the cleavage efficiency decreased as the number of mismatches increased (Fig. 1D). However, percent cleavage directed by S1, GGUCCAG (an underline denotes a mismatch), was ~50% of that directed by S2 (UGGCCAG) or T7M71 (GGGCCAU) (Fig. 1D, lanes 2, 15, and 16). This may be due to the difference in the dissociation constant among these heptamer/SPH2 complexes and/or due to inefficient recognition by 3′ tRNase of substrates containing a proximal base mismatch. Cleavage was barely detected in the presence of T7M73 (GGGCAUU), whereas no cleavage occurred in the presence of T7M74, T7M75, and T7M76 (Fig. 1D), which have less than four base matches (Fig. 1B). These results suggest that five base matches in the acceptor stem are necessary for recognition by mammalian 3′ tRNase.

We also tested two shorter sgRNAs for the ability to direct cleavage of SPH2 by 3′ tRNase. The pentamer T7-5 (GGGCC) and the tetramer T7-4 (GGGC) are complementary to the acceptor stem region of SPH2 (Fig. 1B). T7-5 directed cleavage of SPH2 efficiently (32.0%), whereas cleavage directed by T7-4 was barley detectable (Fig. 1D). This result is consistent with the above conclusion, and indicates that mammalian 3′ tRNase can cleave a target RNA with a stable T stem-like structure in the presence of a complementary pentamer, although much less efficiently than a heptamer.

Although we did not examine whether the less efficient cleavage direction by sgRNAs containing base mismatches in the acceptor stem is due to the formation of less stable RNA complexes or due to less efficient recognition of the

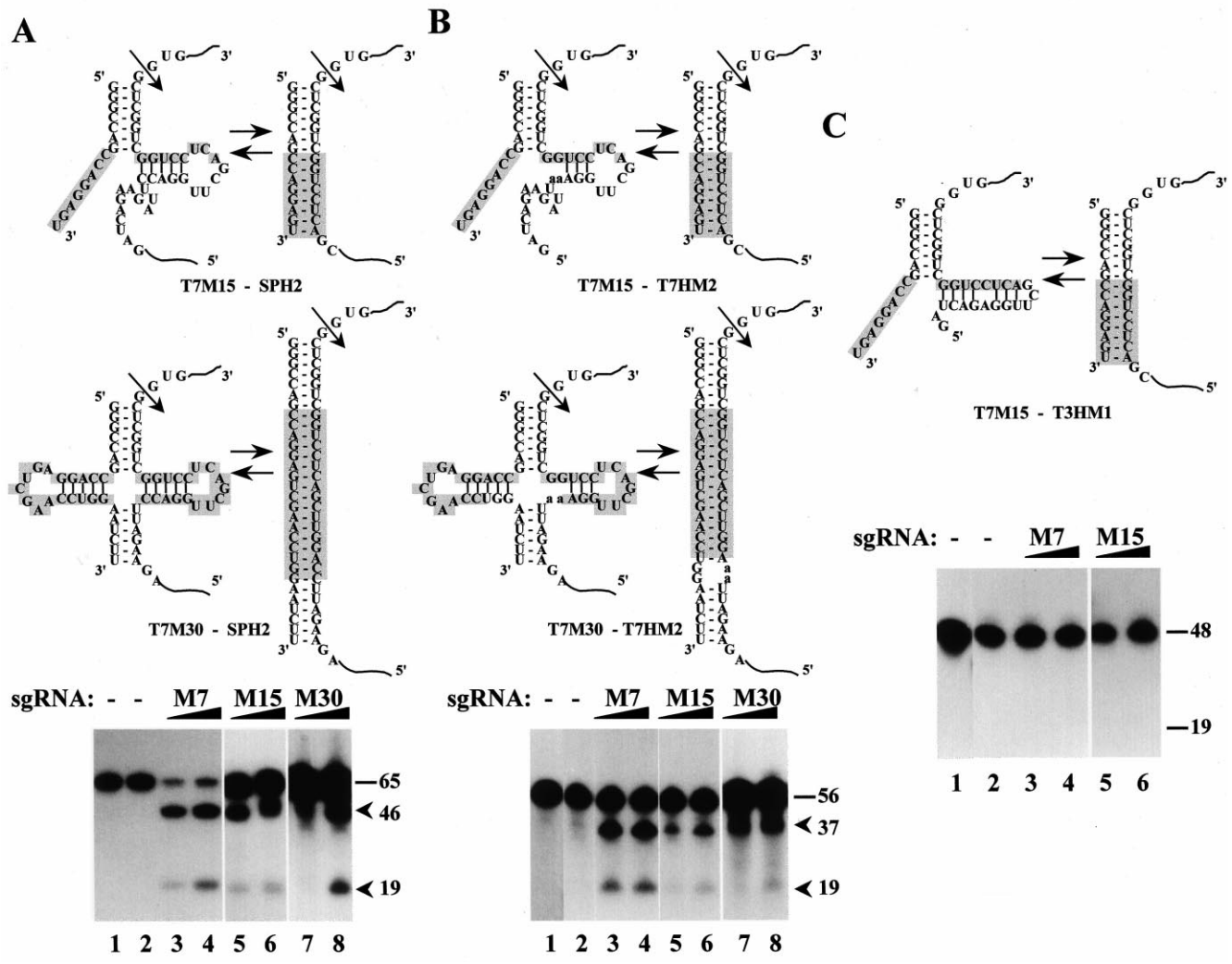

Fig. 3. Specific cleavage assays for double-stranded RNA substrates. Each ^{32}P-labeled target RNA (0.1 pmol) was incubated with pig 3′ tRNase in the presence of 1 pmol (odd lanes) or 10 pmol (even lanes) of sgRNAs at 37°C for 20 min. As controls, each target was incubated without the enzyme (lane 1) or with the enzyme in the absence of sgRNAs (lane 2). The cleavage products were analyzed on 8 M urea/10% polyacrylamide gels. Arrowheads with an nt size denote the cleavage products. Shaded sequences in a target and an sgRNA are complementary to each other. Arrows in the RNA complexes indicate the specific cleavage sites by 3′ tRNase. Diagrams of RNA complexes of T7M7 with the targets are not shown because they are the same as the complexes of T7M15 with the targets except for the 8-nt extra 3′ sequence in T7M15. A: Specific cleavage of the RNA complexes of SPH2 with the sgRNAs T7M7, T7M15, and T7M30. B: Cleavage of the RNA complexes of the target T7HM2 with the sgRNAs T7M7, T7M15, and T7M30. C: Cleavage of the target T3HM1 directed by T7M7 or T7M15.

complexes by the enzyme, the data clearly indicate that even one base mismatch in the acceptor stem drastically reduces the target cleavage efficiency by 3′ tRNase.

3.3. DNA molecules can direct RNA cleavage but cannot be cleaved by 3′ tRNase

We examined whether DNA molecules can direct cleavage of 3′-half tRNAs by mammalian 3′ tRNase. The 5′-half tDNA dGT7H that has the same sequence as GT7H (Fig. 1A) was synthesized with a DNA synthesizer. The 3′-half tRNA SPH2 was assayed for cleavage by 3′ tRNase in the presence of dGT7H under the standard conditions. dGT7H successfully directed SPH2 cleavage, although the efficiency was ~4-fold as low as the cleavage efficiency directed by GT7H (Fig. 1C, lanes 3–6).

We also examined whether mammalian 3′ tRNase can cleave DNA molecules (pre-tDNAs) that consists of pre-tRNA sequences. An 88-nt pre-tDNA^{Arg}, which is composed of the same DNA sequence as a 92-nt pre-tRNA^{Arg} except the 3′-end four uridines, was assayed for cleavage (Fig. 2A). Pig 3′ tRNase was not able to cleave the pre-tDNA^{Arg} at all even after 90 min incubation, while the enzyme cleaved the pre-tRNA^{Arg} very efficiently in 10 min (Fig. 2B). We further tested a DNA target for cleavage by 3′ tRNase in the presence of small guide nucleic acids. The 61-nt DNA target dSPH2 has the same sequence as the 3′-half tRNA SPH2 except the 3′-end U stretch (Fig. 1A). Assays were performed in the presence of GT7H or dGT7H, and no cleavage of dSPH2 was detected in each case (Fig. 2C).

Taken together, these results suggest that mammalian 3′ tRNase can recognize complexes between target RNAs and 5′-half tDNAs and cleave the target RNAs, whereas the enzyme has no activity to cleave phosphodiester bonds of DNA. This incapability may be because 3′ tRNase can hardly recognize pre-tDNAs and/or because the enzyme does not possess the chemistry.

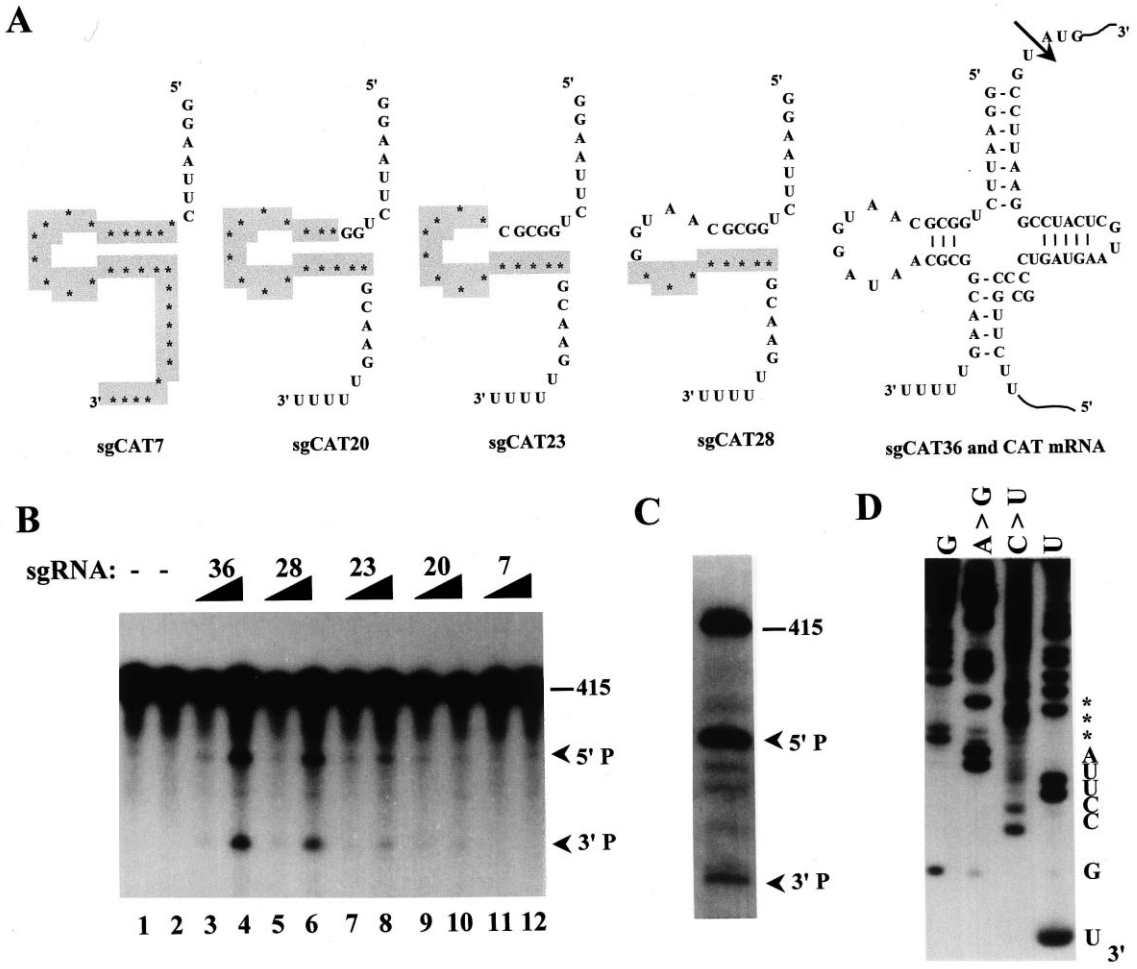

Fig. 4. Targeted cleavage of the CAT mRNA by mammalian 3′ tRNase under the direction of sgRNAs. A: A secondary structure of the RNA complex sgCAT36–CAT mRNA and the four other sgRNAs sgCAT28, sgCAT23, sgCAT20, and sgCAT7. An arrow denotes the cleavage site by 3′ tRNase. Shaded asterisks indicate deleted nucleotides from sgCAT36. B: Specific cleavage assays for a 415-nt 5′-half portion of CAT mRNA. The uniformly ^{32}P-labeled target CAT mRNA (0.1 pmol) was incubated with pig 3′ tRNase in the presence of the various sgRNAs (10 pmol). As controls, CAT mRNA was incubated without the enzyme (lane 1) or with the enzyme in the absence of sgRNAs (lane 2). After incubation at 37°C for 20 min, the reaction products were analyzed on an 8 M urea/5% polyacrylamide gel. The cleavage products are denoted by arrowheads. C: The 3′ tRNase cleavage assay for CAT mRNA in large scale. The unlabeled CAT mRNA (1 pmol) was cleaved by pig 3′ tRNase in the presence of sgCAT36 (50 pmol) at 37°C for 20 min, subsequently 3′-end labeled with [5′-^{32}P]pCp, and separated on an 8 M urea/5% polyacrylamide gel. D: Sequencing of the 5′ cleavage product of CAT mRNA. The 3′-terminal sequence was determined by the chemical RNA sequencing method.

3.4. Simple double-stranded RNAs are not substrates for mammalian 3′ tRNase

Furthermore, we investigated whether 3′-half tRNA targets can be cleaved by 3′ tRNase under the direction of sgRNAs that can form long double-stranded structures with the target RNAs. The 65-nt 3′-half tRNA SPH2 was tested for specific cleavage by pig 3′ tRNase in the presence of three different sgRNAs. The sgRNAs T7M7, T7M15, and T7M30 consist of 7-, 15-, and 30-nt sequences, respectively, which are complementary to SPH2 (Figs. 1B and 3A). More than 80% of SPH2 were cleaved in 20 min in the presence of T7M7 under the standard conditions, whereas percent cleavage of SPH2 was 30.2 and 21.5% even in the presence of 10 pmol of T7M15 and T7M30, respectively (Fig. 3A).

The RNA complexes T7M15–SPH2 and T7M30–SPH2 can take two alternative conformations: one containing an acceptor stem and a T stem loop, and the other containing a long consecutive double-stranded region (Fig. 3A). Theoretically, the latter conformer should be more dominant in the T7M30/SPH2 complex than in the T7M15/SPH2 complex. The difference in the cleavage efficiency between the T7M30/SPH2 complex and the T7M15/SPH2 complex may reflect this differential equilibrium. On the other hand, the T7M7/SPH2 complex cannot form a long double-stranded structure (Fig. 1B). Taken together, the above results suggest that mammalian 3′ tRNase cannot cleave long double-stranded RNA conformers.

Likewise, a 3′-half tRNA variant, T7HM2, was tested for cleavage under the direction of the same sgRNAs as above. T7HM2 contains a T stem composed of only three base-pairs (Fig. 3B). The RNA complexes T7M15–T7HM2 and T7M30–T7HM2 can also take two alternative conformations (Fig. 3B). T7HM2 was cleaved by 3′ tRNase in the presence of 10 pmol of T7M7 up to 36.5% in 20 min (Fig. 3B), but the cleavage was much less efficient than the cleavage of SPH2 (Fig. 3A,B). This is because of the lack of a stable T stem in

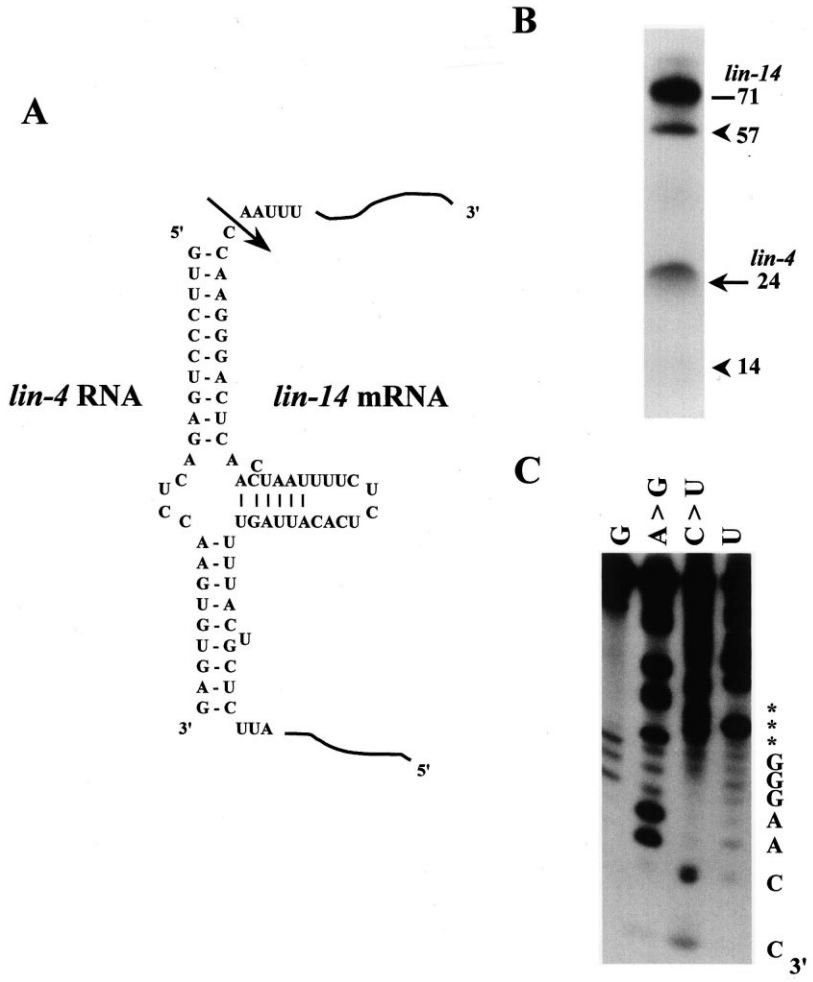

Fig. 5. In vitro cleavage reaction of the RNA complex between *lin-4* and *lin-14*. A: A possible secondary structure of the *lin-4*/*lin-14* RNA complex. The sequences 5′-GUGCGCCCA-3′ and 5′-UUUUUCUCA-3′ are omitted from the 5′ and 3′ regions, respectively, of the *lin-14* mRNA. An arrow denotes the cleavage site by 3′ tRNase. B: The specific cleavage assay. The unlabeled *lin-14* mRNA (1 pmol) was incubated with pig 3′ tRNase in the presence of the *lin-4* RNA (50 pmol) at 37°C for 20 min, subsequently 3′-end labeled with [5′-^{32}P]pCp, and analyzed on an 8 M urea/10% polyacrylamide gel. Arrowheads denote the cleavage products. C: Sequence analysis of the 5′ cleavage product of the *lin-14* mRNA. The 3′-terminal sequence was revealed by the chemical RNA sequencing method.

T7HM2, as we have demonstrated previously [14]. 3′ tRNase cleaved only 10.8% and 5.3 of T7HM2 in the presence of 10 pmol of T7M15 and T7M30, respectively (Fig. 3B), and these percent cleavage values were much smaller than those of SPH2. These results further support the conclusion that long double-stranded RNA conformers are not substrates for mammalian 3′ tRNase.

We also tested another target RNA T3HM1, which does not have an authentic T stem structure, for specific cleavage. The cleavage assay was performed in the presence of T7M7 or T7M15. In contrast to the complexes of SPH2 or T7HM2, 3′ tRNase was not able to cleave RNA complexes of T3HM1 at all (Fig. 3C). The RNA complexes T7M7–T3HM1 and T7M15–T3HM1 can form 7- and 15-bp double-stranded structures, respectively (Fig. 3C). Although an alternative conformer containing a T stem-like structure can be formed in each complex, 3′ tRNase was unable to recognize such a conformer. From these results, we confirmed that simple double-stranded RNAs cannot be recognized by mammalian 3′ tRNase.

3.5. Specific cleavage of the CAT mRNA by mammalian 3′ tRNase

To investigate whether mammalian 3′ tRNase can cleave a relatively long RNA target under the direction of 5′-half tRNAs, we used a 5′-half portion of the *Escherichia coli* chloramphenicol acetyltransferase (CAT) mRNA (GenBank/EMBL accession number X65321) as a model target. The ^{32}P-labeled 415-nt CAT mRNA, and four unlabeled 5′-half tRNAs plus one RNA heptamer that contain sequences complementary to the target were synthesized in vitro with T7 RNA polymerase (Fig. 4A). These sgRNAs were designed to cleave the CAT target after the 250th nucleotide G from the 5′-end. The expected cleavage site was located downstream of a hairpin structure with a 5-bp stem. sgCAT36 is a wild-type 5′-half tRNA, and sgCAT28, sgCAT23, and sgCAT20 have deletions in the D stem loop region (Fig. 4A). The CAT mRNA was cleaved by pig 3′ tRNase most efficiently in the presence of sgCAT36 (Fig. 4B). Percent cleavage decreased with the decrease in the length of the 5′-half tRNAs, and the RNA heptamer sgCAT7 hardly directed the cleavage of the CAT mRNA (Fig. 4B).

To determine the exact cleavage site of the CAT mRNA, the cleavage reaction for the unlabeled CAT mRNA was carried out in the presence of sgCAT36. After the 3′-end labeling with [5′-^{32}P]pCp, the cleavage products were separated on a denaturing gel (Fig. 4C). A 3′-terminal sequence of the 5′ product recovered from the gel was determined by the chemical RNA sequencing method (Fig. 4D). This sequence indicated that the cleavage occurred at one nucleotide downstream of the expected site, i.e. at a uridine immediately 3′ to the discriminator (Fig. 4A,D).

When the wild-type 5′-half tRNA was used as sgRNA, the cleavage of the CAT mRNA was very efficient, but the percent cleavage decreased with the decrease in the length of sgRNAs. In contrast to this observation, in the case of the two-half tRNA^{Arg} system, cleavage efficiency was hardly affected by the similar deletions in the D stem/loop and the anticodon stem [14]. This discrepancy may be because the hairpin structure (which has the calculated ΔG value -3.7 kcal/mol) corresponding to the T stem in the CAT mRNA is not very stable and consequently recognition of the RNA complexes by the enzyme becomes inefficient. The D stem loop and/or the anticodon stem loop may be also important determinants for recognition by mammalian 3′ tRNase of pre-tRNA-like complexes with an unstable T stem [13,14].

3.6. Implications for gene therapy

We have shown that an RNA heptamer can direct cleavage of an RNA target by mammalian 3′ tRNase as efficiently as a wild-type 5′-half tRNA if the target has a stable T stem loop-like structure [14]. Because of the necessity of stable T stem loop-like structures, roughly speaking, RNA heptamers can direct efficient RNA cleavage with a specificity of a 7 (from the acceptor stem) plus 5 (from the need for a stable T stem) = 12-nt sequence and not merely a 7-nt sequence [14]. The validity of this unexpected high specificity is further supported by the observation that the target cleavage efficiency by 3′ tRNase drastically decreases even when the cleavage is directed by RNA heptamers containing only one base mismatch (Fig. 1B,D).

As discussed previously [14], some of the antisense oligodeoxynucleotide inhibitions in cells may be due to cleavage of target RNAs by endogenous 3′ tRNase. In this case, the targets may be folded into pre-tRNA-like structures through the assistance of antisense oligodeoxynucleotides. This supposition is consistent with the observation reported in the literature that only a portion of antisense oligodeoxynucleotides works effectively in cells [16]. In this paper, we demonstrated that indeed mammalian 3′ tRNase can recognize and cleave target RNAs in the presence of long 'antisense' RNAs, although less efficiently, if the RNA complexes can form a pre-tRNA-like structure (Fig. 3). In addition, even an sgDNA was shown to be able to direct RNA cleavage by 3′ tRNase. Taken together, these results suggest that 3′ tRNase may be one of the enzymes responsible for the conventional antisense effects.

We also demonstrated that this method for targeted RNA cleavage with 3′ tRNase is valid for a relatively long mRNA such as the CAT mRNA (Fig. 4). The in vitro observations in this paper and previous studies encourage us to proceed to the application of this strategy to cellular RNA targets in living cells, which is now on the way.

3.7. An RNA complex of lin-4 and lin-14 can be recognized and cleaved by mammalian 3′ tRNase

In this paper and previous studies, it has been demonstrated that mammalian 3′ tRNase can recognize and cleave various anomalous pre-tRNA-like complexes, although cleavage efficiency differs depending on the structural abnormality. We happened to realize that RNA complexes between *lin-4* and *lin-14* of *Caenorhabditis elegans* resemble such distorted pre-tRNA structures [17,18]. To examine whether these RNA complexes are also substrates for 3′ tRNase, we synthesized a 71-nt partial *lin-14* mRNA and a 24-nt *lin-4* RNA with T7 RNA polymerase in the absence of [α-^{32}P]UTP (Fig. 5A), and tested their complex for cleavage. After an assay under the standard conditions using pig 3′ tRNase, the products were 3′-end labeled with [5′-^{32}P]pCp, and subsequently separated on a denaturing polyacrylamide gel. Pig 3′ tRNase successfully cleaved the *lin-14* mRNA and generated ∼57- and ∼14-nt products (Fig. 5B). To confirm the cleavage site, the ∼57-nt 5′ product was recovered from the gel, and its 3′-terminal sequence was determined by the chemical RNA sequencing method. The sequence indicated that the cleavage occurred after the 57th cytidine from the 5′-end as expected (Fig. 5A,C).

3.8. Other potential physiological roles of tRNA 3′ processing endoribonuclease

We have first found 3′ tRNase as a ribonucleoprotein (RNase 65) in extracts from mouse FM3A cells and various tissues of various mammals [8–11]. RNase 65 is a complex of 3′ tRNase with an ∼65-nt 3′-truncated tRNA, and functions in vitro as a four base specific RNA endoribonuclease, although its physiological roles are not known [9–12]. The sequence specificity, which is determined by the consecutive 4-nt 5′-terminal sequence of the 3′-truncated tRNA, changes depending on the tRNA species. In cells, target sites of cellular RNAs for RNase 65 may be limited due to RNA folding and a large number of attached proteins, and RNase 65 may regulate lifetimes of specific RNAs. The RNase 65 activity may be modulated by the amount of the 3′-truncated tRNAs, and may be augmented under some unusual conditions such as apoptosis. It would be worth while noting that human tyrosyl–tRNA synthetase functions as two distinct cytokines after being cleaved by protease outside the cells under apoptotic conditions [19].

Although we do not know whether a mammalian system similar to the *lin-4*/*lin-14* regulation system exists, or whether *C. elegans* 3′ tRNase is indeed involved in the *lin-4*/*lin-14* system, RNase 65 activity suggests us that there may be other roles of 3′ tRNase in cellular metabolic process. In *C. elegans*, 3′ tRNase may be also a key enzyme underlying double-stranded RNA-mediated genetic interference [20].

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