Organic & Biomolecular Chemistry

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/obc

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

Investigation of end processing and degradation of premature tRNAs and their application to stabilization of *in vitro* transcripts in wheat germ extract

Atsushi Ogawa* and Yasunori Doi

Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX
DOI: 10.1039/b000000x

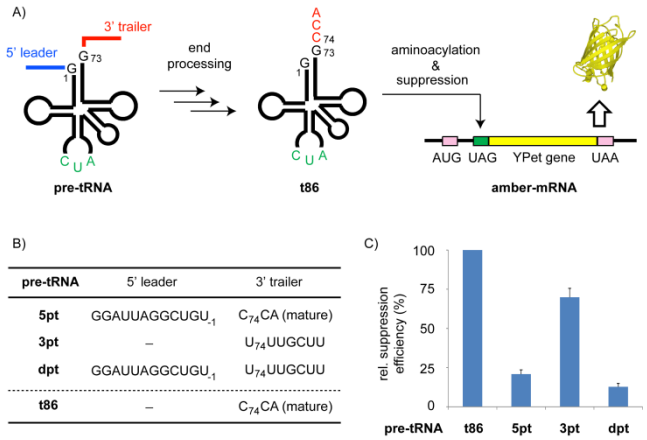
We investigated the end processing and degradation of premature tRNAs in wheat germ extract (WGE), which led to the discovery of end protectors useful for stabilizing an *in vitro* transcript against various ribonucleases and thereby enhancing its apparent activity in WGE.

Cell-free translation systems based on cell extracts from prokaryotic or eukaryotic organisms are very useful for readily preparing various types of proteins without cultivation of cells.[1] Even a protein toxic to cells can be expressed in these systems unless it adversely affects their translation systems. Thus, they are also suited for the expression of an atypical protein (or peptide) containing a non-natural amino acid, which can be obtained by using a special tRNA (*e.g.*, a suppressor tRNA) that is charged with the non-natural amino acid in some way.[2] In addition, cell-free systems are available as a platform of label-free biosensors in which an analyte induces a conformational change and/or cleavage of a functional RNA to regulate the expression of a detectable reporter protein.[3] However, despite these wide applications, many of the functions of these cell-free systems remain unclear in detail, except for the translational (and, in some cases, transcriptional) activity. It is of great importance to elucidate these unknown functions, especially the processing and degradation of exogenously added *in vitro* transcripts, to make more efficient use of cell-free systems. We herein investigated the end processing and the stability of *in vitro* transcribed premature tRNAs (pre-tRNAs) in wheat germ extract (WGE),[4] one of the most efficient eukaryotic cell-free translation systems. Based on the results, we rationally designed a considerably stable pre-tRNA, which led to the discovery of end protectors for efficiently stabilizing *in vitro* transcripts and thereby enhancing their apparent activity in WGE.

Within eukaryotic cells, tRNAs are transcribed in the nucleus with extra sequences at both the 5' and 3' ends (5' leader and 3' trailer, respectively) whose lengths are typically 6-15 nt and 5-10 nt, respectively.[5,6] These pre-tRNAs undergo end processing by nuclear enzymes to become mature.[7] The 5' leader is removed by endonuclease RNase P in the nucleolus (5' processing). The 3' trailer is eliminated by endonuclease RNase Z (and exonucleases in some cases), and then the trinucleotide CCA sequence is added to the trimmed 3' end of tRNA by a CCA-adding enzyme in the nucleoplasm (3' processing).[8] Given the fact that these enzymes are included in standard WGE,[5,9] pre-tRNAs could be terminally processed in the cell-free translation system based on WGE, even if they are *in vitro* transcripts, because base modifications generally occur after the end processing.[7] In fact, *in vitro* transcribed *Nicotiana rustica* pre-tRNA^{Tyr} has been reported to undergo the end processing in crude WGE,[9] though the experimental conditions in that report were optimal for processing, not for translation, and the CCA-adding step was intentionally suppressed for some reason.

We here chose a suppressor tRNA as the foundation of pre-tRNAs, because we can easily evaluate not only the end processing but also the function of the resulting mature tRNA with suppression efficiencies under the optimal conditions for translation in WGE (Fig. 1A). Recently, we rationally evolved an *in vitro*-transcribed amber suppressor tRNA, step by step, toward higher suppression efficiency in WGE.[10] The evolved suppressor (S2-$G_{27}C_{43}$-G_{73}, here named **t86**; Fig. S1A†) derived from *Oryza sativa* nuclear tRNA^{Ser} (Os05g0294300) is charged with Ser by endogenous wheat seryl-tRNA synthetase (SerRS) and then transfers the amino acid effectively into the ribosome at the amber codon (UAG) of mRNA in competition with eukaryotic release factor 1 (eRF1): the suppression efficiency of **t86** is as high as 60~85%. We thus prepared three types of pre-tRNAs by adding a typical length of an extra sequence to the end(s) of this highly active suppressor **t86** (Fig. 1B): 5' pre-tRNA with a 12-nt 5' leader (**5pt**); 3' pre-tRNA with a 7-nt 3' trailer (**3pt**); and 5' and 3' pre-tRNA with both extra sequences (**dpt**). The 5' leader and 3' trailer sequence were derived from the upstream and downstream sequence of the original tRNA gene, respectively.[11] Because SerRS should not be able to directly aminoacylate these pre-tRNAs, due to the structural hindrance by the 5' leader and/or the lack of the mature 3' end, they must undergo the end processing to function as a suppressor. These pre-tRNAs were incubated with **amber-mRNA**, which has the amber codon in the N-terminal region of the YPet (yellow fluorescent protein) open reading frame (Fig. 1A),[12] for 1 h in WGE (optimal for translation)[4] in order to measure their suppression efficiencies. As a result, only the 3' pre-tRNA (**3pt**) exhibited relatively high suppression efficiency (70% of that by **t86**), while the two other pre-tRNAs with the 5' leader did not work well (Fig. 1C). In view of the fact that the suppression efficiency of the mature tRNA **t86** is almost-linearly dependent on its concentration up to that of the

added pre-tRNAs (1 μM, Fig. S2†), the former result indicates that the 3' processing (including the CCA addition) took place rapidly. On the other hand, the low suppression efficiencies of the latter two mean that the 5' processing is not as effective as the 3' processing in WGE, at least under the optimal conditions for translation. Although it is considered that the 5' processing occurs prior to the 3' processing in eukaryotic cells,[7] the 3' trailer did not seem to enhance the 5' processing (**dpt**).



pre-tRNA	5' leader	3' trailer
5pt	GGAUUAGGCUGU$_{-1}$	C_{74}CA (mature)
3pt	–	U_{74}UGCUU
dpt	GGAUUAGGCUGU$_{-1}$	U_{74}UGCUU
t86	–	C_{74}CA (mature)

Fig. 1 Amber suppression assays. (A) Schematic illustration of suppression assays for evaluating the end processing of pre-tRNAs with the suppression efficiency of completely processed tRNA (**t86**). (B) The sequences of the 5' leader and the 3' trailer in pre-tRNAs. (C) The relative suppression efficiency of pre-tRNAs incubated for 1 h in WGE.

In order to more directly evaluate the end processing, we next carried out gel electrophoreses of these pre-tRNAs that were incubated for various periods of time without mRNA in WGE (Fig. 2A). As expected from the amber suppression experiments, the gel patterns revealed that **3pt** was rapidly processed, while the processing of the two other pre-tRNAs was much slower. As a result of kinetic analyses, the 5' leader removal of **5pt** ($k = 2.7 \times 10^{-2}$ min^{-1}) was 22-fold slower than the 3' trailer removal of **3pt** ($k = 6.1 \times 10^{-1}$ min^{-1}) (Fig. 2B).[13] In addition, judging from the gel pattern of **dpt**, the 5' processing was not accelerated by the 3' trailer at all, as indicated above.[14,15] Consequently, the amounts of mature tRNA produced from **5pt**, **3pt**, and **dpt** with 1-h incubation in WGE were 14%, 66%, and 11%, respectively,[16] and these values were roughly in accordance with their suppression efficiencies (21%, 70%, and 13%, respectively, Fig. 1C). It should be noted that the increase in processed pre-tRNAs was much smaller than the decrease of pre-tRNA in all cases. Given the fact that mature tRNA **t86** was highly stable in WGE (approx. 90% remained after 1-h incubation, Fig. S1†),[17] this large difference means that pre-tRNAs underwent not only processing but also degradation. We thus estimated the amount of degraded pre-tRNA at each period from the amounts of the remaining (non-decayed) and processed pre-tRNAs, and then calculated their degradation rates (Fig. 2B). The kinetic analyses elucidated that the degradation occurred in all three pre-tRNAs at a subequal rate ($k = 5\sim7 \times 10^{-2}$ min^{-1}), which was slower than the 3' trailer removal of **3pt** but faster than the 5' leader removal of **5pt**. This is why, whereas **3pt** was relatively efficiently converted to mature tRNA (via a stable intermediate, **3pt2**, Fig. S3†), the 5' pre-tRNAs (**5pt** and **dpt**) were degraded to a large extent.[18]

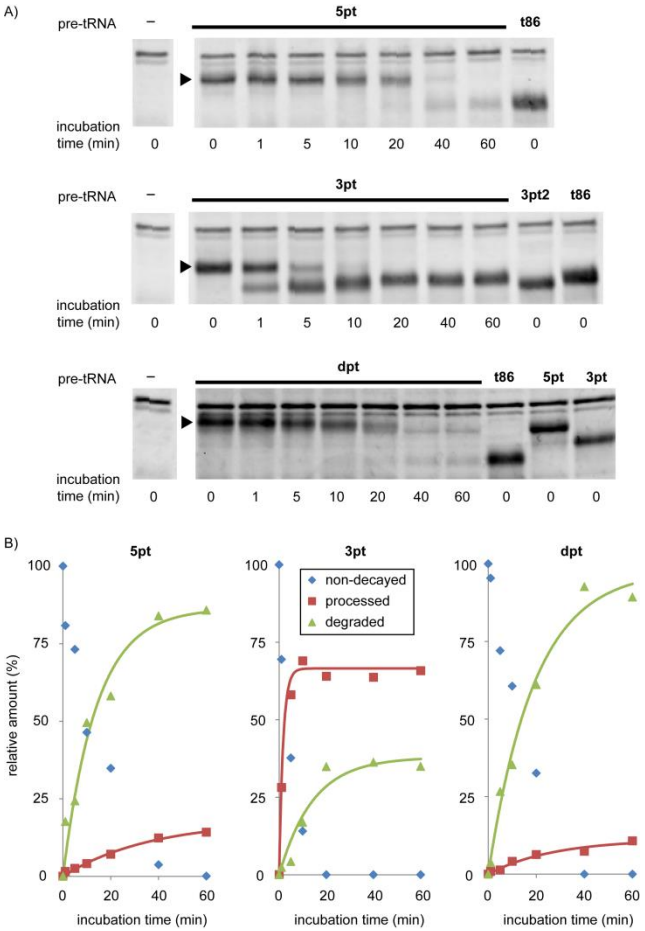


Fig. 2 Gel analyses. (A) Visualization of the end processing of pre-tRNAs on gels (top: **5pt**; middle: **3pt**; bottom: **dpt**). The arrowheads indicate initial pre-tRNAs. See the Electronic Supplementary Information† for experimental details and **3pt2**. (B) Time course of the relative amount of non-decayed, processed, and degraded pre-tRNAs (left: **5pt**; center: **3pt**; right: **dpt**), which were estimated by band intensities on the gels (A). The amount of 3' processed pre-tRNAs includes partially-processed ones before or during the CCA addition. The data of processed and degraded pre-tRNAs were fitted to a single-exponential function of the form of $y(t) = A(1 - e^{-kt})$.

It was surprising that the pre-tRNAs were so susceptible to degradation, because they are composed largely of the highly stable structure of **t86**. However, these results are in good agreement with a recent report that extensive degradation of pre-tRNAs occurs by the exosome in competition with the processing in yeast strains.[19] In the present study, therefore, it is similarly expected that the pre-tRNAs were degraded from their dangling end, without stopping at the mature end, mainly by endogenous exonucleases that are active under the optimal conditions for translation. Thus, we next attempted to inhibit exo-degradation of degradation-susceptible **5pt** by introducing a protector at its end. Specifically, we added an SL (5' GGGAGACC-ACAAC-GGUUUCCC 3') to the 5' terminus of **5pt** to prepare **5SL-5pt** (Fig. 3A), because it has been reported that 5'-3' degradation can be inhibited fairly easily by a stem-loop (SL) structure at the 5'

terminus, at least in bacteria.[20] As a result, **5SL-5pt** underwent much less degradation (20% with 1-h incubation in WGE) than **5pt** did (85%), though it was of course somewhat processed to **t86** (20%) (Figs. 3B and S4A†).[21] This clearly shows not only that a simple SL functioned well as an effective 5' end protector also in eukaryotic WGE, but that **5pt** was degraded mainly by 5'-3' exonucleases, as expected. In contrast, **5SL-dpt** prepared by adding the 5' SL to **dpt** was 40% degraded, in spite of it being moderately processed to stable **5SL-5pt** and **t86** (Fig. S4A†). This suggests that the degradation of **dpt** occurred also from the 3' end by 3'-5' exonucleases.

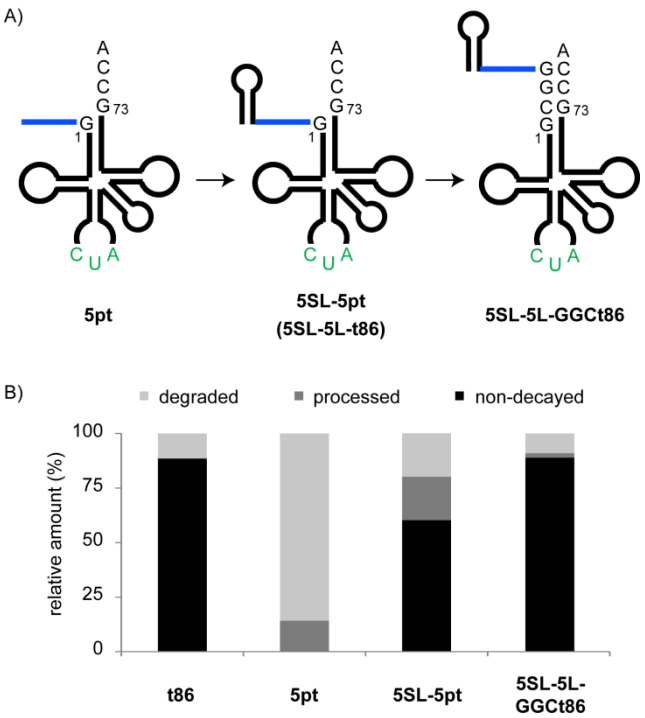


Fig. 3 Stabilization of pre-tRNAs. (A) Schematic diagrams of the stabilization. (B) Relative amounts of degraded, processed, and non-decayed pre-tRNAs after 1-h incubation in WGE, the last of which represent the stability of pre-tRNAs and were reproducible with a variation of less than 7%.

The fact that even a pre-tRNA whose framework is a rigid tRNA structure underwent rapid exo-degradation indicates that most of general *in vitro* transcripts should do so. Whereas 5'-3' degradation can be inhibited by introducing a simple SL at the end as described above, that was not applicable to 3'-5' degradation (vide infra), meaning a special protector is required for the latter. The mature tRNA **t86** is promising for this purpose, since it exhibits resistance to 3'-5' degradation (and the 3' processing). In fact, **5SL-5pt** can be considered as the 5' leader (5L) that is stabilized by the protectors at both ends (5' SL and 3' t86) (*i.e.*, it could also be designated **5SL-5L-t86**, Fig. 3A). Although it was somewhat processed to be mature in WGE, its residual (non-decayed) ratio after 1-h incubation was moderately high (60%) due to the slow 5' processing (Fig. 3B). Nonetheless, we decided to further stabilize this pre-tRNA by blocking the originally slow 5' processing, in order to search for a more efficient protector. According to a report that yeast RNase P does not work when the base at -1 forms a base pair,[22] we inserted three bases ($G_{-3}G_{-2}C_{-1}$) complementary to $G_{73}C_{74}C_{75}$ just before G_1 of **5SL-5pt** to lengthen the acceptor stem (**5SL-5L-GGCt86**, Fig. 3A), with an expectation that the duplex inhibits wheat RNase P. As expected, **5SL-5L-GGCt86** hardly underwent the 5' processing with 1-h incubation in WGE (2%) (Figs. 3B and S4A†). Moreover, the extended acceptor stem fortunately decreased the percent degradation down to as low as that of **t86** (9%). This is probably because it helped the 3' end protector (3' $G_{-3}G_{-2}C_{-1}$-t86) to be correctly folded into the tRNA structure without being hindered by the other parts (the 5' SL and 5' leader).

To demonstrate the usefulness of the identified efficient end protectors (5' SL and 3' $G_{-3}G_{-2}C_{-1}$-t86), we chose, as a model of *in vitro* transcripts, a 77-nt partially-structured eRF1-binding aptamer (**apt12**) that is widely used for nonsense suppression in eukaryotic cell-free translation systems.[2d,10,23] We prepared **apt12** and three kinds of its derivatives with end protector(s) (Fig. 4A) and then checked their stability with 1-h incubation in WGE. As a result, while protector-free **apt12** was completely degraded, one derivative with the optimized end protectors remained with almost no degradation (**5SL-apt12-GGCt86**; residual ratio: 95%) (Figs. 4B and S4B†).[24,25] In contrast, when the 3' end protector of **5SL-apt12-GGCt86** was replaced with an SL (5' CCCUUUGG-ACAAC-CCAGAGGG 3') that had the same stability as the 5' SL (**5SL-apt12-3SL**), the residual ratio decreased considerably, to a value as low as that of **5SL-apt12** (~20%), which has only the 5' protector. This result indicates that a simple SL structure is not sufficient to inhibit endogenous 3'-5' exonucleases in WGE, unlike in the case of 5'-3' exonucleases.[26] The reason why a tRNA structure is a good 3' end protector is not only that it has a complex rigid structure but also probably that it interacts with some proteins in WGE.[27]

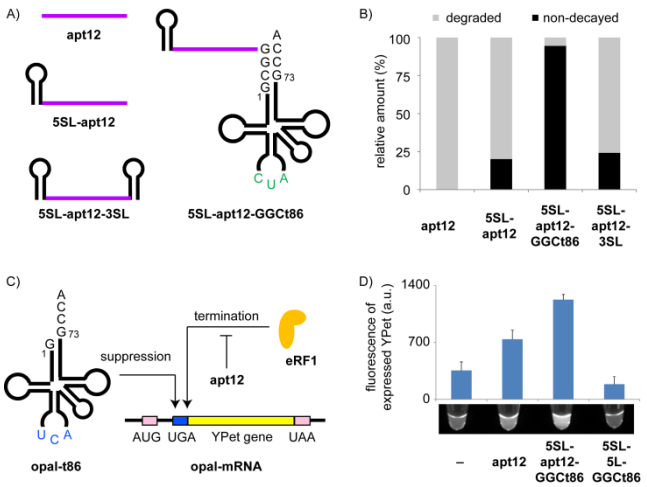


Fig. 4 Stabilization of an *in vitro* transcribed eRF1-binding aptamer, **apt12**. (A) Schematic diagrams of **apt12** and its derivatives. (B) Relative amounts of degraded and non-decayed transcripts after 1-h incubation in WGE. (C) Schematic illustration of opal suppression with **apt12**. An opal suppressor (**opal-t86**) competes with eRF1 to be incorporated into the ribosome at the opal codon (UGA). **apt12** prevents eRF1 by binding to it to facilitate the suppressor. (D) Fluorescence intensities and images of YPet translated via opal suppression in the presence of **apt12** or its derivative in WGE.

Finally, to evaluate the stabilization effect of **5SL-apt12-GGCt86** with its function, we performed opal suppression experiments (Fig. 4C).[28] Specifically, we incubated **opal-mRNA**, which was prepared by altering the amber codon in **amber-mRNA** into the opal codon (UGA), with an opal suppressor (**opal-t86**), which was prepared by altering the anticodon in **t86** into UCA, in the presence of protector-free **apt12**, **5SL-apt12-GGCt86**, or **5SL-5L-GGCt86** (the negative control), or in their absence in WGE. As shown in Fig. 4D, although protector-free **apt12** somewhat enhanced the suppression efficiency (2-fold), **5SL-apt12-GGCt86** achieved a greater enhancement (3-fold). In contrast, the negative control adversely affected the efficiency for some reason. These results clearly demonstrate that the stabilization of **apt12** with the end protectors contributes to its apparent activity: functional RNAs need to be stabilized to effectively exert their abilities, even if they function by forming a complex with a protein. Although we here used **apt12** as a model, the identified end protectors should be available for maximizing the functions of other *in vitro* transcripts, such as mRNAs, ribozymes and other aptamers. In fact, when the 3' end protector was added to mRNA, the translation efficiency was enhanced (Fig. S5†), as in the case of some plant viral mRNAs that have a tRNA-like structure at the 3' end for efficient translation.[29]

Conclusions

In summary, we used several *in vitro* transcribed pre-tRNAs derived from the highly active amber suppressor (**t86**) to investigate their end processing and degradation in WGE. As a result of the relatively easy suppression experiments, it was found that the 3' processing including the CCA addition rapidly proceeded while the 5' processing was much slower. In addition, the gel analyses revealed that pre-tRNAs underwent relatively fast degradation from the premature end(s), despite the fact that the mature tRNA is highly stable. However, we succeeded in rationally constructing a highly stable pre-tRNA (**5SL-5L-GGCt86**), which underwent almost no degradation or processing, by adding some sequences for preventing endogenous nucleases. Based on its structure, we proposed a general method for stabilizing *in vitro* transcripts in WGE: a stem-loop structure (SL) and an acceptor stem-lengthened tRNA ($G_{-3}G_{-2}C_{-1}$-t86) are added to the 5' terminus and the 3' terminus, respectively, as effective end protectors. In fact, the usefulness of this method was demonstrated by using an eRF1-binding aptamer: the addition of these end protectors to the aptamer increased not only its stability but also its apparent activity. In addition, the 3' end protector was found to enhance the translation efficiency of mRNA.

It has been one of the toughest problems for cell-free translation systems that exogenously added *in vitro* transcripts are rapidly degraded by endogenous nucleases. Out of various cell-free systems, the high-quality WGE that was used here (prepared by washing the wheat embryo as extensively as possible to exclude nucleases, proteases, and other translation inhibitors in the endosperm) has a great advantage in producing a diverse array of proteins, regardless of the original organism species, in large amounts, owing to its versatility and stability.[4] However, even in such a stable system, the *in vitro* transcripts were found to be susceptible to degradation and thus to decrease their functions.[4,30] Therefore, the present RNA-stabilizing method is expected to be widely employed to make much more efficient use of the already useful WGE.

There has recently been reported a similar method for stabilizing RNAs in *E. coli*.[31] In this method, an RNA strand is stabilized by being introduced into the anticodon loop of tRNA. However, whether the acceptor stem can be formed or not is likely to be highly dependent on the introduced RNA. In contrast, the present method could address this problem, because the two end protectors are folded independently. In addition, in comparison to other RNA-stabilizing methods such as circularization[32] and 2'-modifications,[33] the present method is very straightforward: all we have to do is to append the end protector sequences to each end when constructing DNA templates. Although the end protectors identified here are suitable for WGE, the strategy for obtaining them would be applicable to other cell free systems and even *in vivo*.

This work was supported by JSPS KAKENHI Grant Number 25708027.

Notes and references

Proteo-Science Center, Ehime University, 3 Bunkyo-cho, Matsuyama, Ehime 790-8577, Japan. Fax: +81 89 927 8450; Tel: +81 89 927 8450; E-mail: ogawa.atsushi.mf@ehime-u.ac.jp

† Electronic Supplementary Information (ESI) available: [Materials and methods, supplementary figures]. See DOI: 10.1039/b000000x/

1 (a) Y. Endo and T. Sawasaki, *Curr. Opin. Biotechnol.*, 2006, **17**, 373; (b) C. E. Hodgman and M. C. Jewett, *Metab. Eng.*, 2012, **14**, 261.

2 (a) D. Kiga, K. Sakamoto, K. Kodama, T. Kigawa, T. Matsuda, T. Yabuki, M. Shirouzu, Y. Harada, H. Nakayama, K. Takio, Y. Hasegawa, Y. Endo, I. Hirao and S. Yokoyama, *Proc. Natl. Acad. Sci. U. S. A.*, 2002, **99**, 9715; (b) H. Murakami, D. Kourouklis and H. Suga, *Chem. Biol.*, 2003, **10**, 1077; (c) S. Ohno, M. Matsui, T. Yokogawa, M. Nakamura, T. Hosoya, T. Hiramatsu, M. Suzuki, N. Hayashi and K. Nishikawa, *J. Biochem.*, 2007, **141**, 335; (d) J. Gubbens, S. J. Kim, Z. Yang, A. E. Johnson and W. R. Skach, *RNA*, 2010, **16**, 1660; (e) R. Abe, K. Shiraga, S. Ebisu, H. Takagi and T. Hohsaka, *J. Biosci. Bioeng.*, 2010, **110**, 32; (f) A. Akahoshi, Y. Suzue, M. Kitamatsu, M. Sisido and T. Ohtsuki, *Biochem. Biophys. Res. Commun.*, 2011, **414**, 625.

3 (a) A. Ogawa and M. Maeda, *ChemBioChem*, 2008, **9**, 2204; (b) A. Ogawa, *ChemBioChem*, 2009, **10**, 2465; (c) N. Muranaka, V. Sharma, Y. Nomura and Y. Yokobayashi, *Anal. Lett.*, 2009, **42**, 108; (d) A. Ogawa, *RNA*, 2011, **17**, 478; (e) A. Ogawa, *ChemBioChem*, 2011, **12**, 881; (f) A. Ogawa, *ChemBioChem*, 2013, **14**, 1539.

4 (a) K. Madin, T. Sawasaki, T. Ogasawara and Y. Endo, *Proc. Natl. Acad. Sci. U. S. A.*, 2000, ***97***, 559; (b) T. Sawasaki, T. Ogasawara, R. Morishita and Y. Endo, *Proc. Natl. Acad. Sci. U. S. A.*, 2002, **99**, 14652.

5 M. Mayer, S. Schiffer and A. Marchfelder, *Biochemistry,* 2000, ***39***, 2096.

6 Some pre-tRNAs have an additional sequence in the anticodon loop (intron).[7]

7 E. M. Phizicky and A. K. Hopper, *Genes Dev.,* 2010, **24**, 1832.

8 In addition to the end processing, some base modifications such as methylation (and the splicing of an intron if necessary) also occur by the corresponding enzymes in the nucleus or cytoplasm.[7]

9 S. Arends and A. Schön, *Eur. J. Biochem.*, 1997, **244**, 635.

10 A. Ogawa, Y. Doi and N. Matsushita, *Org. Biomol. Chem.*, 2011, **9**, 8495.

11 It is known that wheat (*Triticum aestivum*) has the same tRNA sequence (including the 5' leader and the 3' trailer sequences used here) in the mitochondrial genome (gene symbol: trnS).

12 A. W. Nguyen and P. S. Daugherty, *Nat. Biotechnol.*, 2005, **23**, 355.

13 We also roughly estimated the rate of the CCA addition ($k = 1.1 \times 10^{-1}$ min^{-1}) by using another 3' pre-tRNA with no 3' trailer (**3pt2**, Fig. S3†). Although it was 5.5-fold slower than the 3' trailer removal of **3pt**, **3pt2** was almost completely converted to mature tRNA in 1-h incubation with 18% degradation, which was less frequent than that of **3pt** (34%). This is probably because **3pt2** has no 3' trailer recognized by 3'-5' exonucleases.

14 If the 5' processing had occurred more efficiently, a larger amount of **t86** would have appeared in comparison to the case of **5pt**, because 5'-processed **dpt** (*i.e.*, **3pt**) should rapidly undergo the 3' processing.

15 The slow 5' processing can be attributed to a narrow range of optimality of wheat RNase P.[9] In contrast, wheat RNase Z has been reported to tolerate a much wider range of conditions.[5] This seems to be one reason why the 3' processing proceeds efficiently.

16 3' partially-processed tRNA may be somewhat included in the case of **dpt**.

17 Another active amber suppressor that was derived from *E. coli* $tRNA^{SerU}$ was less stable (approx. 60% remained after 1-h incubation). This indicates it is not that any tRNA body sequence can be used as the 3' end protector (vide infra).

18 The relative amounts of processed or degraded pre-tRNAs (**5pt**, **3pt**, and **dpt**) were reproducible with a variation of less than 14%.

19 R. K. Gudipati, Z. Xu, A. Lebreton, B. Séraphin, L. M. Steinmetz, A. Jacquier and D. Libri, *Mol. Cell*, 2012, **48**, 409.

20 S. A. Emory, P. Bouvet and J. G. Belasco, *Genes Dev.*, 1992, **6**, 135.

21 Due to the lower amount of degradation, the maturation efficiency was slightly higher than that of **5pt**. However, the fact that the efficiency was fairly low again demonstrates that the 5' processing was slow, though there is a possibility that the 5' SL somewhat inhibited the RNase P activity.

22 Y. Lee, D. W. Kindelberger, J. Y. Lee, S. McClennen, J. Chamberlain and D. R. Engelke, *RNA*, 1997, **3**, 175.

23 J. Carnes, L. Frolova, S. Zinnen, G. Drugeon, M. Phillippe, J. Justesen, A. L. Haenni, L. Leinwand, L. L. Kisselev and M. Yarus, *RNA*, 2000, **6**, 1468.

24 There was no processed product detected, unlike in the case of **5SL-5L-GGCt86**.

25 The fact that **5SL-apt12-GGCt86** was highly stable, despite the relatively long (77 nt) and flexible structure of **apt12**, indicates that there were almost no active nonspecific endonucleases.

26 It has recently been reported that a terminal SL structure cannot sufficiently prevent the degradation from the 3' terminus in HeLa cells; see: J. E. Wilusz, C. K. JnBaptiste, L. Y. Lu, C. D. Kuhn, L. Joshua-Tor and P. A. Sharp, *Genes Dev.*, 2012, **26**, 2392.

27 It has been indicated that there are protein(s) for protecting the 3' end of mature tRNAs in wheat extract; see: S. Schiffer, S. Rösch and A. Marchfelder, *Biol. Chem.*, 2003, **384**, 333.

28 Because **t86**, a part of the 3' end protector, is an amber suppressor, we here chose another nonsense codon, opal, to interpret the forthcoming results more simply.

29 (a) D. Matsuda and T. W. Dreher, *Virology*, 2004, **321**, 36; (b) A. Ogawa, J. Tabuchi and Y. Doi, *Bioorg. Med. Chem. Lett.*, 2014, **24**, 3724.

30 X. Shen, S. Yao, H. Fukano, S. Terada, A. Kitayama, T. Nagamune and E. Suzuki, *J. Biotechnol.*, 1999, **75**, 221.

31 L. Ponchon and F. Dardel, *Nat. Methods,* 2007, **4**, 571.

32 N. Abe, H. Abe and Y. Ito, *J. Am. Chem. Soc.*, 2007, **129**, 15108.

33 L. L. Cummins, S. R. Owens, L. M. Risen, E. A. Lesnik, S. M. Freier, D. McGee, C. J. Guinosso and P. D. Cook, *Nucleic Acids Res.*, 1995, **23**, 2019.