A Novel Endonucleolytic Mechanism to Generate the CCA 3' Termini of tRNA Molecules in *Thermotoga maritima*  

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The tRNA 3'-terminal CCA sequence is essential for aminoacylation of the tRNAs and for translation on the ribosome. The tRNAs are transcribed as larger precursor molecules containing 5' and 3' extra sequences. In the tRNAs that do not have the encoded CCA, the 3' extra sequence after the discriminator nucleotide is usually cleaved off by the tRNA 3' processing endoribonuclease (3'TRNase, or RNase Z), and the 3'-terminal CCA residues are added thereto. Here we analyzed *Thermotoga maritima* 3'TRNase for enzymatic properties using various pre-tRNAs from *T. maritima*, in which all 46 tRNA genes encode CCA with only one exception. We found that the enzyme has the unprecedented activity that cleaves CCA-containing pre-tRNAs precisely after the CCA sequence, not after the discriminator. The assays for pre-tRNA variants suggest that the CA residues at nucleotides 75 and 76 are required for the enzyme to cleave pre-tRNAs after A at nucleotide 76 and that the cleavage occurs after nucleotide 75 if the sequence is not CA. Intriguingly, the pre-tRNA*Met* that is the only tRNA 3'TRNase without the encoded CCA was cleaved after the discriminator. The kinetics data imply the existence of a CCA binding domain in *T. maritima* 3'TRNase. We also identified two amino acid residues critical for the cleavage site selection and several residues essential for the catalysis. Analysis of cleavage sites by 3'TRNases from another eubacteria *Escherichia coli* and two archaea *Thermoplasma acidophilum* and *Pyrococcus aerophilum* corroborates the importance of the two amino acid residues for the cleavage site selection.

Every single tRNA molecule ends with the sequence CCA (1). This 3'-terminal sequence is essential for aminoacylation of the tRNAs (2) and for translation on the ribosome (3) in all organisms. The tRNAs are transcribed as larger precursor molecules, which subsequently undergo various processing steps such as removal of 5' and 3' extra sequences to generate mature tRNAs (4).

Because generally eukaryotic tRNA genes do not encode the CCA sequence, the eukaryotic tRNAs are supplemented with the CCA residues by tRNA nucleotidyltransferase (4, 5). It is believed that the discriminator nucleotide that protrudes from the aminoacyl stem, to which CCA is added, is generated by removing the 3' extra sequence primarily with tRNA 3' processing endoribonuclease (3'TRNase, or RNase Z) (6–14) and possibly in some circumstances with some unidentified exoribonucleases (15, 16).

In contrast, the CCA sequences of all *Escherichia coli* tRNAs are encoded in its genome, and the six exoribonucleases RNase BN, RNase II, polynucleotide phosphorylase, RNase PH, RNase D, and RNase T are involved in the removal of 3' trailers to generate the CCA termini (17, 18). RNase II and polynucleotide phosphorylase, however, prefer unstructured RNAs such as mRNAs as substrates, so that their roles in tRNA maturation would probably be limited. In the other eubacteria and archaea, percentages of the CCA-coding tRNA genes vary with species from 0 to 100% (Table I). From the above precedents, it was assumed that prokaryotes containing 0 and 100% CCA-coding tRNA genes would primarily utilize the eukaryote-type and *E. coli*-type systems, respectively, to generate the CCA termini, and that the other prokaryotes would make use of both systems depending on the presence or absence of the encoded CCA sequence.

In the course of compilation of tRNA, 3'TRNase, and exoribonuclease genes from available prokaryote genome data, however, we became aware that the *Thermotoga maritima* genome (19) encodes orthologues to 3'TRNase, RNase II, and polynucleotide phosphorylase but no orthologues to RNase BN, RNase PH, RNase D, and RNase T, although its 46 tRNA genes encode CCA with only one exception (Table I). If *T. maritima* 3'TRNase is responsible for removal of 3' extra sequences from the pre-tRNAs containing the CCA residues, the reason why the genome preserves CCA in the tRNA gene is a mystery, because 3'TRNases so far characterized are believed to cleave pre-tRNAs immediately after the discriminator (6–14). It should be noted, however, that in some cases additional cleavages were observed in vitro 1-nt upstream, or 1- or 2-nt downstream.

To solve this enigma, we analyzed *T. maritima* 3'TRNase for enzymatic properties using various pre-tRNAs. Here we show that *T. maritima* 3'TRNase has the unprecedented activity that cleaves CCA-containing pre-tRNAs precisely after the CCA sequence, not after the discriminator. We also identify essential residues in substrate and enzyme for the cleavage site selection and the catalysis. The mechanism for 3'TRNase to select the cleavage site must have co-evolved with the gain or loss of the CCA sequence in tRNA genes.

**EXPERIMENTAL PROCEDURES**

**Construction of Expression Plasmids for 3’TRNases from** *T. maritima, Thermoplasma acidophilum,* and *Pyrococcus aerophilum*—Twelve DNA fragments were chemically synthesized to produce the double-stranded DNA encoding *T. maritima* 3'TRNase, in which the codons are optimized for translation in *E. coli*. The full-length DNA for 3’TRNase (data not shown) was created by PCR with the primer pair

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* The abbreviations used are: 3'TRNase, tRNA 3' processing endoribonuclease; nt, nucleotide(s); ATPγS, adenosine 5'-O-(3-thiotriphosphate).
The full-length 3\'-tRNA coding regions of *E. coli* (915 bp), *T. acidophilum* (921 bp), and *P. aerophilum* (861 bp) were PCR-amplified from their genomes. The primer pairs 5'-Ec/3'Ec, 5'-Ta/3'Ta, and 5'-Pa/3'Pa were used for the amplification of *E. coli*, *T. acidophilum*, and *P. aerophilum* genes, respectively. Each amplified gene was gel-purified before assays.

**Expression and Purification of Recombinant Proteins**—*E. coli* strain DH5α that harbors a pQE expression plasmid derivative (Qiagen) for prokaryotic 3′ tRNase was incubated at 37°C in a 250-ml LB medium containing 50 μg/ml ampicillin until the *A*$_{600}$ of the culture reached 0.6. At this point, the histidine-tagged protein was induced by adding 0.1 mM isopropyl-β-D-thiogalactopyranoside. After further incubation at 37°C for 1 h, the cells were harvested by centrifugation. Cell pellets were resuspended in a 10-ml lysis buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5% glycerol, 5 mM β-mercaptoethanol) containing 1 mM imidazole. The cells were sonicated and centrifuged at 9500 × g for 30 min. The cleared lysate was incubated with 0.2-ml nickel-agarose beads at 4°C for 1 h. After exhaustive washing, the retained proteins were eluted from the beads with 1 ml of the lysis buffer containing 200 mM imidazole. All of the purification steps were carried out at 4°C.

**Pre-tRNA Synthesis**—The pre-tRNAs were synthesized in *vitro* with T7 RNA polymerase (Takara Shuzo) from the synthetic pre-tDNAs containing its promoter. The transcription reactions were carried out in the presence or absence of [α-32P]UTP (Amersham Biosciences) under the conditions recommended by the manufacturer (Takara Shuzo), and the transcribed pre-tRNAs were gel-purified.

The unlabelled pre-tRNAs were subsequently labeled with fluorescein according to the manufacturer's protocol (Amersham Biosciences). Briefly, after the removal of the 5′-phosphates of the transcripts with bacterial alkaline phosphatase (Takara Shuzo), the transcripts were phosphorylated with ATP-γS using T4 polynucleotide kinase (Takara Shuzo). Then a single fluorescein moiety was appended onto the 5′-phosphonothioate site. The resulting pre-tRNAs with fluorescein were gel-purified before assays.

**In Vitro tRNA 3′ Processing Assay**—The 3′ processing reactions for 32P-labeled or fluorescein-labeled pre-tRNA were performed with 3′ DNA restriction enzymes and DNA ligase. The primer pairs used for the mutagenesis are listed in Table II. We confirmed that the insert sequences are changed correctly by DNA sequencing.

| Species                      | CA/total | Endo | BN | II | PNP | PH | D | T |
|------------------------------|----------|------|----|----|-----|----|---|---|
| *Borrelia burgdorferi*       | 0/32     |      |    |    |     |    |   |   |
| *Chlamydia pneumoniae*       | 0/32     |      |    |    |     |    |   |   |
| *Chlamydomphila trachomatis* | 1/45     |      |    |    |     |    |   |   |
| *Treponeema pallidum*        | 10/31    |      |    |    |     |    |   |   |
| *Buchnera sp.*              | 15/45    |      |    |    |     |    |   |   |
| *Myxobacterium leprae*       | 15/45    |      |    |    |     |    |   |   |
| *Staphylococcus aureus*      | 37/81    |      |    |    |     |    |   |   |
| *Bacillus subtilis*          | 63/86    |      |    |    |     |    |   |   |
| *Aquifex aeolicum*           | 32/43    |      |    |    |     |    |   |   |
| *Bacillus halodurans*        | 67/89    |      |    |    |     |    |   |   |
| *Deinococcus radiodurans*    | 48/49    |      |    |    |     |    |   |   |
| *Haemophilus influenzae*     | 55/56    |      |    |    |     |    |   |   |
| *Thermotoga maritima*        | 45/46    |      |    |    |     |    |   |   |
| *Campylobacter jejuni*       | 42/42    |      |    |    |     |    |   |   |
| *Caulobacter crescentus*     | 51/51    |      |    |    |     |    |   |   |
| *Escherichia coli*           | 88/86    |      |    |    |     |    |   |   |
| *Helicobacter pylori*        | 36/36    |      |    |    |     |    |   |   |
| *Mycoplasma genitalium*      | 35/35    |      |    |    |     |    |   |   |
| *Mycoplasma penetrans*       | 36/36    |      |    |    |     |    |   |   |
| *Pattearella maltocida*      | 51/51    |      |    |    |     |    |   |   |
| *Rickettsia prowazekii*      | 32/26    |      |    |    |     |    |   |   |
| *Pseudomonas aeruginosa*     | 62/62    |      |    |    |     |    |   |   |
| *Ureaplasma urealyticum*     | 29/29    |      |    |    |     |    |   |   |
| *Vibrio cholerae*            | 98/98    |      |    |    |     |    |   |   |
| *Xylella fastidiosa*         | 48/49    |      |    |    |     |    |   |   |
| *Archaeoglobus fulgidus*     | 0/46     |      |    |    |     |    |   |   |
| *Halobacterium sp.*          | 0/45     |      |    |    |     |    |   |   |
| *Thermoplasma vulcanium*     | 0/45     |      |    |    |     |    |   |   |
| *Sulfobolus solfatarius*     | 1/45     |      |    |    |     |    |   |   |
| *Sulfobolus toholaii*        | 1/45     |      |    |    |     |    |   |   |
| *Thermoplasma acidophilum*   | 1/45     |      |    |    |     |    |   |   |
| *Methanobacterium thermoautotrophicum* | 1/39     |      |    |    |     |    |   |   |
| *Pyrobaculum aerophilum*     | 19/46    |      |    |    |     |    |   |   |
| *Methanococcus jannaschii*   | 25/36    |      |    |    |     |    |   |   |
| *Pyrococcus abyssi*          | 44/46    |      |    |    |     |    |   |   |
| *Pyrococcus furiosus*        | 46/46    |      |    |    |     |    |   |   |
| *Pyrococcus horikoshii*      | 46/46    |      |    |    |     |    |   |   |
| *Aeropyrum pernix*           | 46/46    |      |    |    |     |    |   |   |

* The number of CCA-containing tRNA genes versus the number of the total tRNA genes.
tRNAses of various origins in a mixture (6 μl) containing 10 mM Tris-HCl (pH 8), 1.5 mM dithiothreitol, 25 mM NaCl, and 10 mM MgCl2 (or 0.2 mM MnCl2 in kinetic assays) at 60 °C. The assays for 3′ tRNases from other species than *T. maritima* were carried out at 50 °C. After resolution of the reaction products on a 10% polyacrylamide–8 M urea gel, the gel was autoradiographed using an intensifying screen at −80 °C, or analyzed with a Typhoon 9210 (Amersham Biosciences).

RNA Sequencing—Unlabeled pre-tRNA (2 pmol) was reacted with *T. maritima* 3′ tRNase (50 ng) under the standard assay conditions at 60 °C for 10 min, followed by phenol/chloroform extraction and precipitated with ethanol. The reaction products dissolved in water were 3′-end-labeled with T4 ligase (Takara Shuzo) and [5−32P]pCp (Amersham Biosciences). Uniformly 32P-labeled pre-tRNAs were synthesized by in vitro transcription of the synthetic tDNA templates. *T. maritima* 3′ tRNase cleaved pre-tRNAArg(CCG), pre-tRNAValUAG(CCA), and pre-tRNAProUAG(CCA) endonucleolytically (Fig. 1B). To determine the exact cleavage site, we performed the 3′ tRNase cleavage reactions for unlabeled pre-tRNAs and subsequently 3′-end-labeled the products with [5−32P]pCp. Each 5′ cleavage product was gel-purified, and its 3′-terminal sequence was determined by the chemical RNA sequencing method (22). The gel was autoradiographed using an intensifying screen at −80 °C.

RESULTS

*T. maritima* 3′ tRNase Cleaves Pre-tRNAs after CCA—First of all, we chemically synthesized DNA fragments to produce the double-stranded DNA encoding *T. maritima* 3′ tRNase (GenBank™ accession number NP_228673), in which the codons are optimized for translation in *E. coli* (Table II). The full-length DNA for 3′ tRNase (data not shown) was created by PCR using primer pairs (Fig. 2A) containing 5′-32P-labeled primers for T7 RNA polymerase. The reaction products were gel-purified, and 5′-32P-labeled the products with T4 polynucleotide kinase (MBI Fermentas). The 3′-end-labeled products were subsequently analyzed by 10% polyacrylamide–8 M urea gel electrophoresis. Two kinds of substrate were used to determine the 3′ tRNase cleavage sites for pre-tRNAs. The reaction products for pre-tRNAs were analyzed and compared with the reaction products for pre-tRNAArg(CCG), pre-tRNAValUAG(CCA), and pre-tRNAProUAG(CCA). The reaction products for pre-tRNAArg(CCG), pre-tRNAValUAG(CCA), and pre-tRNAProUAG(CCA) were digested with RNase A and RNase T1 (data not shown) and then analyzed by 10% polyacrylamide–8 M urea gel electrophoresis.

**Table II**

| Name               | Sequence (5′ to 3′)                                                                 |
|--------------------|--------------------------------------------------------------------------------------|
| 5′ Tm               | ACATGCGATCGATGACGTTCCATGGTACGTTTAG                                                  |
| 3′ Tm               | CCGCTGAGTTTCTAGGCTGTTCCAGATT                                                |
| 5′ Ec               | CCGCTGAGTTTAAACCTGTTAAGCCGAG                                               |
| 5′ Ta               | CCGCTGATCTGTTAATACTCATGGCTGTTACCTT                                           |
| 3′ Ta               | CCGGCTGATCTGCTTAAAGCGTTCAGGAGAATGTT                                          |
| 5′ Pa               | ATTCGTTTGCTGGCGGCGAA                                                             |
| 3′ Pa               | TGGATGCCGCAAAGAACACAGAT                                                           |
| D25A(sense)         | TGGATGCCGCAAAGAACACAGAT                                                           |
| G27A(sense)         | TGGATGCCGCAAAGAACACAGAT                                                           |
| G27A(antisense)     | TGGATGCCGCAAAGAACACAGAT                                                           |
| V30T(sense)         | TGGATGCCGCAAAGAACACAGAT                                                           |
| S31Q(sense)         | TGGATGCCGCAAAGAACACAGAT                                                           |
| T33Q(sense)         | TGGATGCCGCAAAGAACACAGAT                                                           |
| T33Q(antisense)     | TGGATGCCGCAAAGAACACAGAT                                                           |
| V38A(sense)         | TGGATGCCGCAAAGAACACAGAT                                                           |
| V38A(antisense)     | TGGATGCCGCAAAGAACACAGAT                                                           |
| H48A(sense)         | TGGATGCCGCAAAGAACACAGAT                                                           |
| H48A(antisense)     | TGGATGCCGCAAAGAACACAGAT                                                           |
| G49L(sense)         | TGGATGCCGCAAAGAACACAGAT                                                           |
| G49L(antisense)     | TGGATGCCGCAAAGAACACAGAT                                                           |
| H50A(sense)         | TGGATGCCGCAAAGAACACAGAT                                                           |
| H50A(antisense)     | TGGATGCCGCAAAGAACACAGAT                                                           |
| V51G(sense)         | TGGATGCCGCAAAGAACACAGAT                                                           |
| V51G(antisense)     | TGGATGCCGCAAAGAACACAGAT                                                           |
| D52A(sense)         | TGGATGCCGCAAAGAACACAGAT                                                           |
| D52A(antisense)     | TGGATGCCGCAAAGAACACAGAT                                                           |
| H53A(sense)         | TGGATGCCGCAAAGAACACAGAT                                                           |
| H53A(antisense)     | TGGATGCCGCAAAGAACACAGAT                                                           |
| A55L(sense)         | TGGATGCCGCAAAGAACACAGAT                                                           |
| A55L(antisense)     | TGGATGCCGCAAAGAACACAGAT                                                           |
| G49LV510(sense)     | TGGATGCCGCAAAGAACACAGAT                                                           |
| G49LV510(antisense) | TGGATGCCGCAAAGAACACAGAT                                                           |
| 43LTR44(sense)      | TGGATGCCGCAAAGAACACAGAT                                                           |
| 43LTR44(antisense)  | TGGATGCCGCAAAGAACACAGAT                                                           |

Furthermore, three variants, pre-tRNAArg(UGG), pre-tRNAArg(UUG), and pre-tRNAPro(UAG) were observed. Pre-tRNAArg(UCG) and pre-tRNAArg(UUC) were cleaved after nt 75, whereas cleavage of pre-tRNAPro(UAG) was not detected (Fig. 2B). These results suggest that the CA residues at nt 75 and 76 were required for *T. maritima* 3′ tRNase to cleave pre-tRNAs precisely after A at nt 76 and that otherwise the cleavage occurs after nt 75.

Cleavage of pre-tRNAPro(UAG) after the Discriminator—We also tested for 3′ tRNase cleavage another pre-tRNAPro(UAG) that is the only pre-tRNA without the encoded CCA.
ingly, this exceptional pre-tRNA was cleaved after the discriminator (Fig. 3). This makes sense because pre-tRNAs without the CCA termini need to be cleaved after the discriminator at nt 73, to which tRNA nucleotidyltransferase adds the CCA residues, like eukaryotic pre-tRNAs. The reason why only pre-tRNAMet(UAG) among the other pre-tRNAs tested was cleaved after the discriminator may be because this pre-tRNA contains CCA residues at nt 71-73. T. maritima 3/H11032 tRNase may be able to recognize these CCA residues as the cleavage site determinant as discussed below.

**A CCA Binding Domain in T. maritima 3’ tRNase**—The T. maritima enzyme shows its highest activity in 100 mM NaCl at pH 9 at 60 °C in the presence of 10 mM MgCl₂ or 0.5 mM MnCl₂ (data not shown). The kinetic parameters $K_m$ and $k_{cat}$ for pre-tRNA³⁰⁶⁰(CCA) and pre-tRNA³⁰⁶⁷(GUG) were determined in the presence of MgCl₂ or MnCl₂ (Table III). The $K_m$ values for pre-tRNA³⁰⁶⁰(CCA) in the presence of MgCl₂ and MnCl₂ were 0.3- and 0.4-fold, respectively, smaller than those for pre-tRNA³⁰⁶⁷(GUG). This suggests that a specific domain for CCA binding exists in the enzyme. On the other hand, the $k_{cat}$ values for pre-tRNA³⁰⁶⁰(CCA) were 0.4- and 0.6-fold, respectively, smaller than those for pre-tRNA³⁰⁶⁷(GUG). This may reflect slower release of the CCA-containing product following the chemical cleavage step. As a result, the cleavage efficiency values $k_{cat}/K_m$ for pre-tRNA³⁰⁶⁰(CCA) were 1.4-fold as large as those for pre-tRNA³⁰⁶⁷(GUG) in both conditions.

**Two Amino Acid Residues Critical for the Cleavage Site Selection**—Next we investigated which amino acid residues are responsible for making T. maritima 3’ tRNase cleave after CCA. We assumed that such residues should be in well conserved regions and be different from residues in the other enzymes that cleave after the discriminator. We selected six residues to be examined; i.e. Val-30, Ser-31, Thr-33, Gly-49, Val-51, and Ala-55 (Fig. 4A). Expression plasmids for single or double amino acid-substituted variants, Tm(V30T), Tm(S31Q), Tm(T33Q), Tm(G49L), Tm(V51G), Tm(G49L/V51G), and Tm(A55L), were constructed by site-directed mutagenesis with PCR based on pQE/Tm(WT). The 3’/H11032 tRNase variants were overexpressed in E. coli and purified as histidine-tagged proteins (Fig. 4B).

These recombinant enzymes were tested for cleavage of pre-tRNA³⁰⁶⁰(CCA) and pre-tRNA³⁰⁶⁷(GUG), which were 5’-end-labeled with fluorescein. The reaction products were analyzed on a sequencing gel with an alkaline ladder to determine the
cleavage sites. We found that the wild-type enzyme can cleave pre-tRNA\textsuperscript{Arg}(CCA) at the additional minor site between nt 75 and 76 (Fig. 5A). The ratio of the minor to major products increased with the reaction time (data not shown), suggesting that this additional product is generated by the second cut of the original product and is an \textit{in vitro} artifact.

All the above variants cleaved both pre-tRNAs, although the cleavage efficiency varied (Fig. 5, A and B). Among these variants, only Tm(S31Q) clearly changed the cleavage sites. In pre-tRNA\textsuperscript{Arg}(CCA), the major cleavage site was shifted to 2-nt downstream, and some portion of the molecules were cleaved after the discriminator. In pre-tRNA\textsuperscript{Arg}(GUG), about a half of the molecules were cleaved 1-nt downstream. With respect to Tm(T33Q), the cleavage of pre-tRNA\textsuperscript{Arg}(CCA) after nt 75 was more dominant than that after nt 76. In addition, the cleavage was also detected after nt 74. The results suggest that Ser-31 is a critical residue for selecting the cleavage site and that Thr-33 is also involved in the site selection.

These results also predict that, if 3' tRNases contain glutamines at the positions corresponding to residues 31 and 33, the enzymes would cleave pre-tRNAs after the discriminator. Indeed, ELAC1-type short 3' tRNases so far characterized have the corresponding two glutamines with the exception of the \textit{Arabidopsis thaliana} enzyme, which contains a histidine and an alanine instead of glutammines (Fig. 4A). This exceptional
case may be due to the lack of eight residues before the histidine motif.

Because three amino acids are missing in between residues 43 and 44 of T. maritima 3' tRNase compared with the other enzymes that cleave after the discriminator, we also thought that this difference could be partly responsible for the differential cleavage site selection. We tested the variant Tm(V38A), that contains a single amino acid substitution in a non-conserved region, was tested as a control. As expected, it cleaved both substrates. In contrast, cleavages by Tm(G27A) were observed at the original sites like the wild-type enzyme.

**TABLE III**

Kinetic parameters for pre-tRNA cleavage by T. maritima 3' tRNase

Various amounts (0.5–10 μM) of T. maritima pre-tRNA<sup>43A<sub>CCG</sub>(CCA) and various amounts (0.5–15 μM) of T. maritima pre-tRNA<sup>43A<sub>GUG</sub>(GUG) were assayed at 60°C for 5 min using 0.1 μM of the wild-type enzyme in the presence of 10 mM MgCl<sub>2</sub> or 0.2 mM MnCl<sub>2</sub>. The reaction was in the linear phase during the 5 min. Each set of values was from the averages of three to seven trials with a standard deviation of 7–22%.

| Substrate         | Ion   | K<sub>m</sub> | k<sub>cat</sub> | k<sub>cat</sub>/K<sub>m</sub> |
|-------------------|-------|--------------|----------------|-----------------|
| Pre-tRNA<sup>43A<sub>CCG</sub>(CCA) | Mg<sup>2+</sup> | 2.8 | 0.7 | 0.25 |
| Pre-tRNA<sup>43A<sub>GUG</sub>(GUG) | Mg<sup>2+</sup> | 10.6 | 1.8 | 0.17 |
| Pre-tRNA<sup>43A<sub>CCG</sub>(CCA) | Mn<sup>2+</sup> | 2.8 | 1.9 | 0.68 |
| Pre-tRNA<sup>43A<sub>GUG</sub>(GUG) | Mn<sup>2+</sup> | 6.5 | 3.1 | 0.48 |

**Fig. 3.** The in vitro cleavage assay for T. maritima pre-tRNA<sup>Met</sup>(UAG) and determination of its cleavage site. A, two possible alternative conformations of T. maritima pre-tRNA<sup>Met</sup>(UAG), which does not have the encoded CCA sequence, are shown with an arrow indicating the cleavage site. The discriminator at nt 73 is denoted by a semicircle. B, the upper panel shows the 5' cleavage product of the uniformly <sup>32</sup>P-labeled pre-tRNA<sup>Met</sup>(UAG) (0.1 pmol) after incubation for 10 min with 1 pmol of T. maritima 3' tRNase. A bar with the letter S and an arrowhead with the letter P denote the pre-tRNA substrate and the 5' cleavage product, respectively. The lower panel indicates the 3'-terminal sequence of the [5'–<sup>32</sup>P]<sup>3</sup>P-labeled 5'-product unveiled by chemical RNA sequencing.

**Cleavage Site Selection by Other Prokaryotic 3' tRNases**—To corroborate the above notion on the cleavage site selection, we investigated properties of 3' tRNases from another eubacteria E. coli and two archaea T. acidophilum and P. aerophilum (Fig. 4A). These enzymes were purified as histidine-tagged proteins (Fig. 6A), and assayed for in vitro tRNA 3' processing of T. maritima pre-tRNA<sup>43A<sub>CCG</sub>(CCA), pre-tRNA<sup>43A<sub>GUG</sub>(GUG), and human pre-tRNA<sup>43A<sub>GUG</sub>(GUG).

The E. coli enzyme cleaved human pre-tRNA<sup>43A<sub>GUG</sub>(GUG) primarily after the discriminator (Fig. 6D). This is consistent with the notion that the two glutamines are critical determinants for the cleavage after the discriminator (Fig. 4A). The T. maritima pre-tRNA<sup>43A<sub>CCG</sub>(CCA) and pre-tRNA<sup>43A<sub>GUG</sub>(GUG) were not substrates for this enzyme (Fig. 6, B and C). The T. acidophilum enzyme, which has two glutamates as the site selection residues, cleaved human pre-tRNA<sup>43A<sub>GUG</sub>(GUG) after the discriminator and after G at nt 74 (Fig. 6D). Although T. maritima pre-
tRNAArg(CCA) was not a substrate, T. maritima pre-tRNAArg(GUG) was cleaved after G at nt 74 (Fig. 6, B and C). 3/H11032 tRNase from P. aerophilum, which contains an arginine at 33 (in the numbering system of T. maritima 3/H11032 tRNase) instead of the second glutamine (Fig. 4A), processed only T. maritima pre-tRNAArg(CCA), and the cleavage site was after C at nt 75 (Fig. 6B). On the whole, these results agree with the notion as to the cleavage site selection.

**DISCUSSION**

**The Role of 3’ tRNase in T. maritima Cells**—To the best of our knowledge, T. maritima 3’ tRNase is the only endoribonuclease so far identified that can cleave pre-tRNAs after CCA. We showed that this enzyme can cleave three T. maritima pre-tRNAs, pre-tRNA Arg(CCA), pre-tRNA Met(CCA), and pre-tRNAPhe(CCA), after the CCA residues. This implies that the enzyme can also cleave the other 42 CCA-containing pre-tRNAs after CCA. The exceptional pre-tRNAMet(UAG) was shown to be processed only T. maritima 3’ tRNase would cleave off 3’ trailers regardless of 5’ processing by RNase P (25).

**Possible Conformational Change of Pre-tRNA Met(UAG)**—The reason why the pre-tRNA Met(UAG) containing UAG instead of CCA at nt 74–76 was cleaved after the discriminator by T. maritima 3’ tRNase (Fig. 3B) may be because the enzyme can recognize the CCA residues at nt 71–73 as the cleavage site determinant in some way. One possible mechanism is that the enzyme would recognize an alternative conformation of the pre-tRNAMet(UAG), in which the CCA residues at nt 71–73 protrude from the acceptor stem and G at nt 70 shifts to the discriminator position (Fig. 3A). The T. maritima enzyme would recognize this form of the pre-tRNAMet(UAG) containing a distorted T-stem-loop in the same fashion as normal CCA-containing pre-tRNAs and would cleave it after A at nt 73. This supposition needs to be tested by examining pre-tRNA variants containing base substitutions that affect the stability of each conformer for 3’ tRNase cleavage and structure probing.

**The Interactions of T. maritima 3’ tRNase with Pre-tRNAs**—The crystal structure of Bacillus cereus β-lactamase suggested that the histidine motif forms a part of the active site (26). Consistently, our data suggest that the histidine motif in

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**Fig. 4. Production of the recombinant T. maritima 3’ tRNase variants.** A, alignment of the histidine-motif regions of ELAC1-type short 3’ tRNases from the selected species using the computer program ClustalW (34). The positions of amino acid substitutions are denoted by arrows. The site selection residues are shaded. Identical amino acid residues are marked with asterisks. Conserved and semiconserved residues are indicated by colons and dots, respectively. The histidine motifs are boxed. B, protein profiles of the 3’ tRNase variants. The recombinant histidine-tagged enzymes were overexpressed in E. coli and purified with nickel agarose. The purified proteins (0.5–1 µg) were separated on an SDS-10% polyacrylamide gel and visualized by staining the gel with Coomassie Brilliant Blue R-250. M, size standards; W, wild type; 43–44, the variant Tm(43LTR44). The other variants are represented by the substituted amino acid positions.
**T. maritima** 3’ tRNase forms an essential part of the catalytic core and that especially the three histidines and one aspartate play a central role for the catalysis.

Pig 3’ tRNase clearly discriminates the nucleotide C from the others at nt 74 (8, 11). In addition, the very short 3’ trailers 74CC, 74CCA, and 74CCA plus one or two additional 3’nt can be distinguished by this enzyme from the other trailers, suggesting that the pig enzyme has a binding domain for the CCA residues. From the present kinetics data (Table III), a CCA binding domain also appears to exist in the **T. maritima** enzyme.

Whether 3’ tRNase cleaves pre-tRNAs after the discriminator or after the CCA residues may be determined by the relative position between the catalytic core and the CCA-binding domain. The amino acid residues at 31 and 33 (in the numbering system of **T. maritima** 3’ tRNase) appear to be critical for this positioning. When these two residues are glutamines, the active site may be located in the vicinity of the first C binding site, and the cleavage may occur after the discriminator. When the residues are other amino acids such as serine and threonine than glutamine, the catalytic site may be positioned near the A binding site, and pre-tRNAs may be cleaved after CCA. The hydroxyl groups of the serine and threonine residues may be critical for this positioning.

Human ELAC2-type long 3’ tRNase, which has a phenylalanine and a glutamine at the corresponding sites, cleaves pre-tRNAs after the discriminator (13, 27). Compared with ELAC1-type short 3’ tRNases, the human and yeast long 3’ tRNases contain 5 and 6 more residues, respectively, between the site selection residues and the histidine motif, and the yeast enzyme also has several additional residues before the site selection residues (27). This could be the reason why the selection rule for short 3’ tRNases does not seem to hold in long 3’ tRNases.

Cleavage of pre-tRNAs containing bases other than CA at nt 75 and 76 occurs after nt 75 (Fig. 2). This may be because these bases do not fit the CCA-binding domain well, and the catalytic core cannot be placed properly. Non- or inefficient cleavage of the CCG-containing pre-tRNAs (Fig. 2) may be attributed to somehow unfavorable interaction with the CCA-binding domain.

**Discrimination of Pre-tRNA Species by Prokaryotic 3’ tRNases and Their Roles in the Cells**—The four prokaryotic 3’ tRNases tested here differed in substrate specificities and cleavage sites. If we can find out a rule to discriminate pre-tRNA substrates, this would become a clue to elucidation of the physiological roles of each enzyme in the cells.

**E. coli** 3’ tRNase cleaved only human pre-tRNA<sup>Arg</sup>(GUG) after the discriminator (Fig. 6). The property that this enzyme cannot cleave **T. maritima** pre-tRNA<sup>Arg</sup>(CCA) makes sense, because removal of the CCA residues would be wasteful. The physiological roles of 3’ tRNase in **E. coli** cells are not clear, because the exonucleotides are sufficient for tRNA 3’ processing. This gene thus might have been preserved for a backup system in case of occasional mutagenesis in the CCA-coding regions. Alternatively, this enzyme may be utilized to process other RNA substrates, including T4 bacteriophage pre-tRNAs.

**Fig. 5.** Pre-tRNA cleavage assays using the recombinant **T. maritima** 3’ tRNase variants. A, the 3’ tRNase variants (1 pmol) were assayed for cleavage of **T. maritima** pre-tRNA<sup>Arg</sup>(CCA) (0.1 pmol), which was 5’-end-labeled with fluorescein. After a 15-min reaction, the products were separated on a denaturing gel. The cleavage sites were identified using alkaline ladders (L) of the fluorescein-labeled pre-tRNA<sup>Arg</sup>(CCA) and the 5’ cleavage products by the wild-type 3’ tRNase as size standards. The 5’ cleavage products are indicated by arrows with the cleavage site nucleotides. I, input RNA with no enzyme; W, wild type; 43–44, the variant Tm(43LTR44). The other variants are represented by the substituted amino acid positions. B, the same assays as above were performed with respect to the fluorescein-labeled **T. maritima** pre-tRNA<sup>Arg</sup>(GUG) (0.1 pmol).
that lack the CCA residues (28). We could not explain why
T. maritima pre-tRNAArg(GUG) was not cleaved. This is not
due to the presence of the 5’ leader, because human pre-
tRNAArg(GUG) with a 7-nt 5’ leader was processed as effi-
ciently as human pre-tRNAArg(GUG) without the leader (data
not shown).

In good contrast to the T. maritima genome, the CCA se-
quences are not encoded in all 45 tRNA genes in the T. aci-
dophilum genome with one exception (29). The cellular pre-tRNAs would probably be 3'-processed by 3' tRNase, although the cleavage site could vary from after nt 74 to after the discriminator depending on pre-tRNA species, judging from our in vitro data. Because T. maritima pre-tRNA\(^{\text{AGG}}\)(CCA) was not cleaved by T. acidophilum 3' tRNase, the exceptional CCA-containing pre-tRNA would be 3' processed by RNase PH, which is encoded in the genome as the only RNase orthologue among the six E. coli enzymes.

Although the P. aerophilum genome (30) contains 19 CCA-coding and 27 non-CCA-coding tRNA genes, P. aerophilum 3' tRNase cleaved only the CCA-containing pre-tRNA Arg coding and 27 non-CCA-coding tRNA genes, T. acidophilum cleaved by RNase PH, containing pre-tRNA would be 3' processed by RNase PH, which is encoded in the genome as the only RNase orthologue among the six exoribonucleases. With respect to the other 27 pre-tRNAs, RNase PH could trim 3' trailers up to the discriminator, or other unidentified RNases could be involved. This case contrasts sharply with the Bacillus subtilis case, where the genome has 73% CCA-coding tRNA genes, and B. subtilis 3' tRNase cleaves only CCA-less pre-tRNAs (31).

Evolution of the Mechanism to Generate the CCA 3' Termi—The above consideration implies that the mechanism for 3' tRNase to select substrates and cleavage sites must have co-evolved with the gain or loss of exoribonuclease genes and the CCA sequence encoded in tRNA genes and would currently be used properly depending on the presence or absence of CCA in pre-tRNAs. Curiously, the T. maritima CCA-adding enzyme groups with A-adding enzymes, not with CCA-adding enzymes or with CC-adding enzymes (32). The presence of the two unusual enzymes 3' tRNase and CCA-adding enzyme involved in the 3' terminal CCA generation suggests that T. maritima has evolved very uniquely.

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A Novel Endonucleolytic Mechanism to Generate the CCA 3′ Termini of tRNA Molecules in *Thermotoga maritima*

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