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Substrate tRNA Recognition Mechanism of Eubacterial tRNA (m^1A58) Methyltransferase (TrmI)*

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Background: tRNA methyltransferases specifically recognize substrate tRNAs.
Results: To clarify the tRNA recognition mechanism of TrmI, three tRNA species and 45 variants were analyzed *in vitro* and *in vivo*.
Conclusion: TrmI recognizes the aminoacyl stem, variable region, C56, purine 57, A58, and U60 in the T-loop of tRNA.
Significance: Our *in vitro* experimental results explain the regulation of *in vivo* methylation levels in tRNAs.

TrmI generates N^1-methyladenosine at position 58 (m^1A58) in tRNA. The *Thermus thermophilus* $tRNA^{Phe}$ transcript was methylated efficiently by *T. thermophilus* TrmI, whereas the yeast $tRNA^{Phe}$ transcript was poorly methylated. Fourteen chimeric tRNA transcripts derived from these two tRNAs revealed that TrmI recognized the combination of aminoacyl stem, variable region, and T-loop. This was confirmed by 10 deletion tRNA variants: TrmI methylated transcripts containing the aminoacyl stem, variable region, and T-arm. The requirement for the T-stem itself was confirmed by disrupting the T-stem. Disrupting the interaction between T- and D-arms accelerated the methylation, suggesting that this disruption is included in part of the reaction. Experiments with 17 point mutant transcripts elucidated the positive sequence determinants C56, purine 57, A58, and U60. Replacing A58 with inosine and 2-aminopurine completely abrogated methylation, demonstrating that the 6-amino group in A58 is recognized by TrmI. *T. thermophilus* $tRNA^{Thr}_{GGU}{}^{Thr}_{GGU}$ contains C60 instead of U60. The $tRNA^{Thr}_{GGU}$ transcript was poorly methylated by TrmI, and replacing C60 with U increased the methylation, consistent with the point mutation experiments. A gel shift assay revealed that $tRNA^{Thr}_{GGU}$ had a low affinity for TrmI than $tRNA^{Phe}$. Furthermore, analysis of $tRNA^{Thr}_{GGU}$ purified from the *trmI* gene disruptant strain revealed that the other modifications in tRNA accelerated the formation of m^1A58 by TrmI. Moreover, nucleoside analysis of $tRNA^{Thr}_{GGU}$ from the wild-type strain indicated that less than 50% of $tRNA^{Thr}_{GGU}$ contained m^1A58. Thus, the results from the *in vitro* experiments were confirmed by the *in vivo* methylation patterns.

To date, more than 90 modified nucleosides have been found in tRNA (1). The majority of these modified nucleosides in tRNA are methylated nucleosides whose biosynthetic pathways include one or multiple methylation steps. These methylated nucleosides in tRNA play important roles in protein synthesis (2). Furthermore, recent studies have elucidated that the modified nucleosides in tRNA are involved in higher biological phenomena such as the RNA quality control system (3, 4), regulation of the subcellular localization of RNA (5), infection (6, 7), and immune responses (8, 9).

1-Methyladenosine (m^1A)[2] is one of the methylated nucleosides that is found in tRNAs from all three domains of life. This modification has been found at positions 9, 22, 57, and 58 in tRNAs (10). Given that tRNAs from *Escherichia coli* do not contain the m^1A modification at any position, the enzymatic activities involved in generating this modification were identified using *E. coli* tRNAs as substrates in crude cell extracts from various living organisms, for example *Neurospora crassa* (11), hamster (12), and HeLa cells (13). Although enzymatic activities involved in generating the m^1A modification were detected in various living organisms, it was very difficult to purify the enzymes. Several purification trials were reported from rat liver (14, 15), plant (16), and slime mold (17). However, the genes encoding the enzymes were not identified for a long time.

At the end of the twentieth century, it was reported that the *trm6* and *trm61* genes (classical names, *gcd10* and *gcd14*) encode tRNA (m^1A58) methyltransferase in *Saccharomyces cerevisiae* (18, 19). Thus, the eukaryotic methyltransferase for m^1A58 is a heterodimeric protein. Later, genes for the eubacterial (20) and archaeal (21) enzymes were identified as *trmI*. Eubacterial TrmI modifies only A58, whereas archaeal TrmI modifies both A57 and A58 (21, 22). Consequently, to distinguish the eubacterial and archaeal enzymes, we abbreviate archaeal TrmI as aTrmI here. TrmI and aTrmI are homotetrameric enzymes. Comparison of the crystal structures of TrmI (23–25) and aTrmI (26) revealed that the tetrameric structure of TrmI is reinforced by salt bridges between the subunits, whereas that of a TrmI from *Pyrococcus abyssi* is maintained by intersubunit disulfide bonds (27). Thus, eukaryotic Trm6-Trm61, eubacterial TrmI, and aTrmI have different protein architectures even though these enzymes are responsible for

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[2] The abbreviations used are: m^1A, 1-methyladenosine; m^1A58, N^1-methyladenosine at position 58; aTrmI, archaeal TrmI; AdoMet, *S*-adenosyl-L-methionine; 2-AP, 2-aminopurine; Ψ, pseudouridine.

the same m^1A58 modification in tRNA. In addition, recently, the enzymes responsible for m^1A22 in eubacterial tRNA, m^1A9 in human mitochondrial tRNA, m^1G9 and m^1A9 in archaeal tRNA, and m^1A58 in mitochondrial tRNA were identified as TrmK (28), a subcomplex of mitochondrial RNase P (29), an archaeal Trm10 homolog (30), and Trm61B (31), respectively.

As mentioned above, the enzymatic activities involved in generating the m^1A58 modification were detected using *E. coli* tRNA in crude cell extracts or with partially purified enzymes. In these early studies, the substrate tRNA specificities of these enzymes were discussed without discriminating the sources of enzymes or the tRNA (14, 15). However, recent studies have showed that eukaryotic Trm6-Trm61, eubacterial TrmI, and aTrmI have different protein architectures. In the current study, we focused on the mechanism by which TrmI from *T. thermophilus*, an extreme thermophilic eubacterium, recognizes substrate tRNA. Given that all tRNAs reported from *T. thermophilus* commonly contain the m^1A58 modification (32–35), it was thought that eubacterial TrmI has no specificity for particular tRNAs. In this study, we show that TrmI from *T. thermophilus* does in fact prefer to act on specific tRNAs.

EXPERIMENTAL PROCEDURES

Materials—[*methyl*-^{14}C]-*S*-Adenosyl-L-methionine (AdoMet) (1.95 GBq/mmol) and [*methyl*-^{3}H]AdoMet (2.89 TBq/mmol) were purchased from ICN. Non-radioisotope-labeled AdoMet and standard modified nucleoside (m^1A) were obtained from Sigma. HiTrap Heparin HP, Superdex 75 prep grade (pg), and Q-Sepharose Fast Flow were purchased from GE Healthcare. DNA oligomers were bought from Invitrogen. All other chemical reagents were of analytical grade.

Construction of Recombinant TrmI Expression System—Briefly, the *trmI* gene from *T. thermophilus* HB8 was amplified by the polymerase chain reaction using the following primers: TrmI forward primer, 5′-GGG CAT ATG GCG TGG CCG GGA CCG CTA CTC-3′; TrmI reverse primer, 5′-GGG GGA TCC TTA GGA GGC CTT CCA TCG CCT AAG-3′. Underlined nucleotides correspond to restriction enzyme sites (NdeI and BamHI, respectively). The amplified DNA was inserted between the NdeI and BamHI sites of the pET30a plasmid vector (Novagen). Recombinant TrmI was expressed at 37 °C for 4 h by induction with isopropyl β-D-thiogalactopyranoside using *E. coli* BL21(DE3) Rosetta 2 (Novagen).

Purification of Recombinant TrmI Protein—Wet cells (10 g) were suspended in 50 ml of buffer A (50 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 100 mM KCl) and then disrupted with an Ultrasonic Disruptor Model UD-200 (Tomy, Japan). The cell debris was removed by centrifugation at 8000 × *g* at 4 °C for 20 min, and then the supernatant fraction was heated at 70 °C for 30 min. The denatured proteins were removed by centrifugation at 24,000 × *g* at 4 °C for 20 min. The supernatant fraction was loaded onto a HiTrap Heparin HP column, and the proteins were eluted with a linear gradient of 100–1000 mM KCl in buffer A. The eluted fractions of TrmI were subjected to 15% SDS-polyacrylamide gel electrophoresis (PAGE), collected, and concentrated with Centriprep YM-10 centrifugal filter devices (Millipore). The resultant sample was loaded onto a Superdex 75 prep grade column equilibrated with buffer A that contained 200 mM KCl. The eluted fractions of TrmI were combined and concentrated. Glycerol was added to a final concentration of 50%, and the protein was stored at −30 °C.

Preparation of Variant tRNA Transcripts—RNA transcripts were prepared by transcription with T7 RNA polymerase. The transcripts were purified by chromatography through a Q-Sepharose Fast Flow column and 10% PAGE (7 M urea). Transcripts 15, 16, 17, and 18 were chemically synthesized by Sigma-Aldrich. The tRNA transcript that contained inosine (I) or 2-aminopurine (2-AP) at position 58 was prepared as follows. The 5′-half that corresponded to G1–G36 of *T. thermophilus* $tRNA^{Phe}$ was synthesized with T7 RNA polymerase. The 3′-half that contained I (5′-AAU CGC AGU GUC GGC GGU UCG IUU CCG CUC CUC GGC ACC A-3′) and corresponded to A37–A76 was synthesized by Gene Design Co. Ltd. (Japan). The 3′-half that contained 2-AP (5′-AAU CGC AGU GUC GGC GGU UCG 2-APUU CCG CUC CUC GGC ACC A-3′) was synthesized by Sigma-Aldrich. The 5′-end of the 3′-half (1.0 A_{260} unit) was phosphorylated at 37 °C for 1 h with 10 units of T4 polynucleotide kinase (Takara) and 1 mM ATP. The efficiency of 5′-phosphorylation was checked by 10% PAGE (7 M urea), and the phosphorylated fragment was recovered by phenol-chloroform extraction and ethanol precipitation. The 5′- and 3′-halves (0.75 A_{260} unit each) were then annealed in 50 μl of buffer B (20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 100 mM NaCl) by cooling from 90 to 40 °C for 50 min. After annealing, 250 units of T4 RNA ligase (Takara), bovine serum albumin (final concentration, 0.01%), and $MgCl_2$ (final concentration, 10 mM) were added to the sample. The sample was incubated at 16 °C for 12 h. The ligated sample was purified by phenol-chloroform extraction and 10% PAGE (7 M urea).

Measurement of TrmI Activity—Incorporation of [^{14}C]methyl group from AdoMet into the *T. thermophilus* $tRNA^{Phe}$ transcript was used to assay TrmI activity. We used a gel assay described previously to visualize methylated RNAs (36). Briefly, 10 μl of buffer A that contained 0.1 μM TrmI, 20 μM transcript, and 37 μM [*methyl*-^{14}C]AdoMet were incubated at 55 °C for 5 min and then loaded onto a 10% polyacrylamide gel (7 M urea). To obtain the kinetic parameters of TrmI for each tRNA, the concentrations of TrmI and [*methyl*-^{3}H]AdoMet (diluted with non-radioisotope-labeled AdoMet) were fixed at 0.1 and 405 μM, respectively. The reaction was basically performed at 55 °C for 5 min, but the incubation time varied from 1 to 15 min depending on the methyl group acceptance activity of each individual transcript. The transcript concentrations in the typical assay were 0, 10, 30, 50, 100, 200, 300, and 500 nM. In the case of $tRNA^{Thr}_{GGU}$ variants, [*methyl*-^{3}H]AdoMet was used for measurements of methyl group acceptance activities in the all experiments because the methyl group acceptance activities of these variants were very low.

Gel Mobility Shift Assay—A 3% agarose gel (Agarose S, Takara) and Tris-acetate-Mg-EDTA buffer (40 mM Tris acetate (pH 8.0), 5 mM $MgCl_2$, 1 mM EDTA) was used for the electrophoresis. tRNA at a concentration of 6.8 μM and TrmI (0, 0.8, 1.3, 2.1, 3.5, 7.7, 11.5, or 19.1 μM) were mixed in 10 μl of buffer (50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 100 mM KCl, 0.1 mM sinefungin, 18% glycerol). Sinefungin (Sigma) is an analog inhibitor for AdoMet-dependent methyltransferases. The samples were incubated at 60 °C for 30 min and then cooled on ice

for 10 min. The electrophoresis was performed at 4 °C. The gel was stained with ethidium bromide.

T. thermophilus Wild-type and trmI Gene Disruptant Strains—The culture source of *T. thermophilus* HB8 was a kind gift from Dr. Tairo Oshima (Tokyo University of Pharmacy and Life Sciences). The cells were grown in rich medium (0.8% polypeptone, 0.4% yeast extract, 0.2% NaCl (pH 7.5 adjusted with NaOH)). The medium was supplemented with 0.35 mM $CaCl_2$ and 0.17 mM $MgCl_2$ after autoclaving. To make plates, gellan gum (Wako Pure Chemicals) was added to the medium (final concentration, 1.5%). The construction of the *trmI* gene disruptant strain was reported previously (37): the gene was disrupted by replacement with the highly thermostable kanamycin nucleotidyltransferase gene (38, 39).

Purification of Native $tRNA^{Phe}$ and $tRNA^{Thr}_{GGU}$ by Solid-phase DNA Probe—Native $tRNA^{Phe}$ was purified in accordance with our previous report (35, 39, 40). To purify $tRNA^{Thr}_{GGU}$, a 3′-biotinylated DNA oligomer (5′-ACC AAG GGT GTG CTC TAC CTG CT-biotin-3′) was used as a hybridization probe. The sequence of the probe is complementary to A36–A14 of *T. thermophilus* $tRNA^{Thr}_{GGU}$. The eluted tRNAs were purified further by 10% PAGE (7 M urea).

Nucleoside Analysis—Modified nucleosides were analyzed by HPLC (Hitachi L-2000 system) equipped with a reverse-phase C_{18} column (NUCLEOSIL 100 C_{18}; 25 cm × 4.6 mm, 7 μm; GL Sciences, Inc.) as described previously (35).

RESULTS

Purification of T. thermophilus TrmI—The expression and purification of His-tagged TrmI from *T. thermophilus* HB27 were reported previously by Droogmans *et al.* (20). The amino acid sequences of the TrmI proteins from *T. thermophilus* strains HB8 and HB27 are identical. In the current study, we expressed TrmI protein from *T. thermophilus* HB8 without a His tag. Given that the solubility of TrmI protein was increased in the presence of KCl as reported (20, 23), we devised purification procedures that used relatively high concentrations of KCl (greater than 100 mM). We found that the combined use of heparin and gel filtration column chromatography was effective in maintaining high KCl concentrations during the purification as described under "Experimental Procedures." As shown in Fig. 1, we successfully purified TrmI protein without a His tag.

T. thermophilus TrmI Prefers to Methylate T. thermophilus $tRNA^{Phe}$ as Compared with S. cerevisiae $tRNA^{Phe}$—The $tRNA^{Phe}$ transcript from *S. cerevisiae* has been used as a model substrate for tRNA modification enzymes (36, 42–44) because its structure is well established (45–47). However, the purified TrmI from *T. thermophilus* methylated *S. cerevisiae* $tRNA^{Phe}$ very slowly (Fig. 2 and Table 1). When the methyl group acceptance activities of the $tRNA^{Phe}$ transcripts from *T. thermophilus* and *S. cerevisiae* (Fig. 2*A*) were compared, the initial velocity for the methyl group acceptance of *S. cerevisiae* $tRNA^{Phe}$ transcript was below 5% of that for the *T. thermophilus* $tRNA^{Phe}$ transcript (Fig. 2*B*). The kinetic parameters K_m and V_{max} for the *T. thermophilus* $tRNA^{Phe}$ transcript were determined to be 130 nM and 360 mmol mg^{-1} h^{-1}, respectively (Table 1). These values are comparable with those obtained for other tRNA methyltransferases with tRNA transcripts (36, 44). Thus, the methylation of *T. thermophilus* $tRNA^{Phe}$ transcript by TrmI is not a special case. In contrast, we could not determine the kinetic parameters for the *S. cerevisiae* $tRNA^{Phe}$ transcript due to its low methyl group acceptance activity (Table 1). To clarify which features of the tRNA are responsible for this difference, we prepared 14 chimeric tRNA transcripts (Fig. 2*C*) and measured their methyl group acceptance activities (Fig. 2*D* and Table 1). In Fig. 2, the transcript numbers correspond to the *lane numbers*. When the T-arm of the *S. cerevisiae* $tRNA^{Phe}$ transcript was replaced with that of the *T. thermophilus* $tRNA^{Phe}$ transcript, the methyl group acceptance activity did not increase (Fig. 2*D*, *lane 1*). In contrast, when both the T-arm and aminoacyl stem of the *S. cerevisiae* $tRNA^{Phe}$ transcript were replaced with those of the *T. thermophilus* $tRNA^{Phe}$ transcript, the methyl acceptance activity increased clearly (Fig. 2*D*, *lane 2*). However, replacement of the aminoacyl stem alone did not have any effect on the methyl group acceptance activity (Fig. 2*D*, *lane 7*). These results show that the T-arm and aminoacyl stem in combination affect the methyl group acceptance activity. The replacement of both the aminoacyl stem and the T-loop in the *S. cerevisiae* $tRNA^{Phe}$ transcript also increased the methyl group acceptance activity (Fig. 2*D*, *lane 9*), which demonstrates that the T-loop and aminoacyl stem in combination are important for substrate tRNA recognition by TrmI. Similarly, the T-loop and variable region in combination were also important for substrate tRNA recognition by TrmI (Fig. 2*D*, *lanes 3*, *4*, *12*, *13*, and *14*). These results suggest that the sites involved in recognition by TrmI are the aminoacyl stem, T-loop, and variable region: their combined features are important for the efficient transfer of methyl groups.

Methyl Group Acceptance Activities of tRNA Deletion Variants—In general, tRNA methyltransferases recognize the local structure around the target site in the tRNA, including tertiary structural elements such as a stem-loop structure(s) (36, 48–51). Consequently, we prepared 10 deletion mutant transcripts of $tRNA^{Phe}$ from *T. thermophilus* (Fig. 3). The kinetic parameters for these transcripts are summarized in Table 2. In the previous study (49), we used two microhelices (Transcripts 15 and 16).

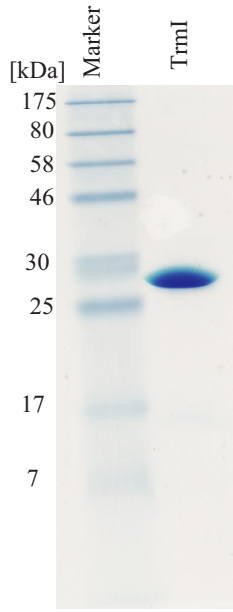

FIGURE 1. **15% SDS-PAGE analysis of purified TrmI protein.** An aliquot of 8 μg of purified TrmI was analyzed by 15% SDS-PAGE. The gel was stained with Coomassie Brilliant Blue.

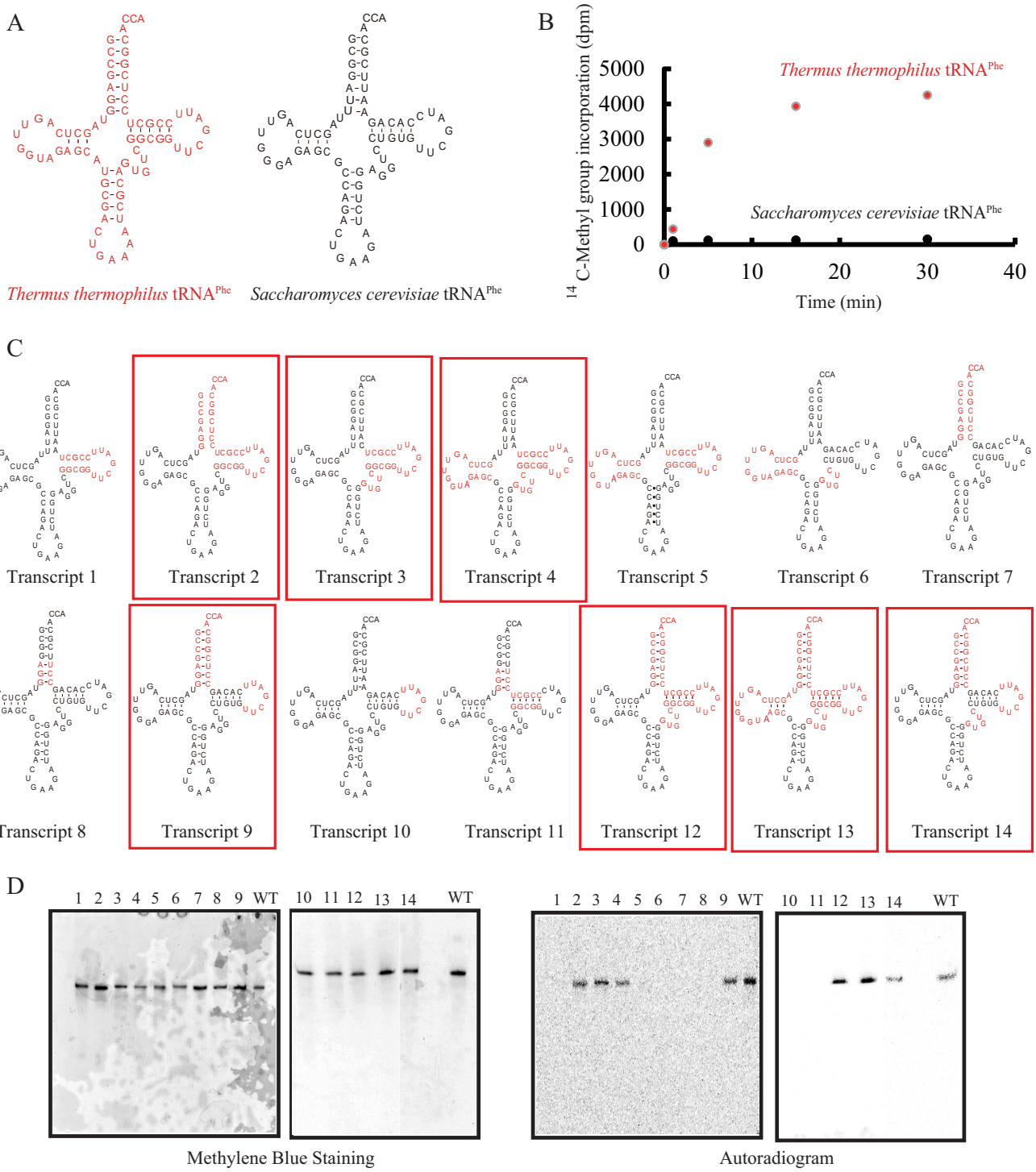

FIGURE 2. **Chimeric tRNA transcripts of *T. thermophilus* and *S. cerevisiae* tRNA^{Phe}.** *A*, *T. thermophilus* tRNA^{Phe} (*left*) and *S. cerevisiae* (*right*) tRNA^{Phe} are depicted as clover leaf structures. To distinguish the two tRNAs, *T. thermophilus* tRNA^{Phe} is indicated in *red*. *B*, the methyl group acceptance activities of *T. thermophilus* and *S. cerevisiae* tRNA^{Phe} were compared. *S. cerevisiae* tRNA^{Phe} was methylated very slowly by TrmI: the initial velocity of methyl group acceptance of *S. cerevisiae* tRNA^{Phe} was ~4% of that of *T. thermophilus* tRNA^{Phe}. *C*, 14 chimeric tRNA transcripts are depicted as clover leaf structures. The sequences derived from *T. thermophilus* tRNA^{Phe} are colored in *red*. The chimeric transcripts whose methyl group acceptance activities were more than 50% of that of *T. thermophilus* tRNA^{Phe} are enclosed with *red boxes*. *D*, after methylation by TrmI and [*methyl*-^{14}C]AdoMet, the chimeric tRNA transcripts (0.02 A_{260} unit each) were analyzed by 10% PAGE (7 M urea) and then visualized by methylene blue staining (*left panels*). The *lane numbers* correspond to the transcript numbers shown in *C*. *WT* indicates the *T. thermophilus* wild-type tRNA^{Phe} transcript. To analyze the methyl group acceptance activities of the tRNA transcripts, the same gels were subjected to autoradiography (*right panels*). The kinetic parameters are summarized in Table 1.

Transcript 15 mimics the T-arm structure of *E. coli* tRNA^{Phe} and was previously used for the crystallization of *E. coli* TrmA and RNA complex (52). Transcript 16 has an artificial sequence to reinforce the stem structure. In the previous study (49), it was confirmed that both Transcripts 15 and 16 were well methylated by *T. thermophilus* TrmFO (tRNA (m^5U54) methyltransferase). In the current study, we tested whether Transcripts 15 and 16 were methylated by TrmI. 5.1 μM transcript, 0.1 μM

TABLE 1

Kinetic parameters for chimera tRNA transcripts

The relative V_{max}/K_m for the *T. thermophilus* wild-type $tRNA^{Phe}$ transcript was expressed as 100%. ND means that the kinetic parameters could not be calculated correctly.

Transcript name	K_m	V_{max}	Relative V_{max}/K_m or relative initial velocity
	nM	*μmol mg*$^{-1}$ *h*$^{-1}$	*%*
T. thermophilus			
Wild-type $tRNA^{Phe}$	130 ± 20	360 ± 60	100
S. cerevisiae			
Wild-type $tRNA^{Phe}$	ND	ND	<5[a]
Transcript 1	ND	ND	<5[a]
Transcript 2	200 ± 40	450 ± 60	80
Transcript 3	150 ± 40	360 ± 60	70
Transcript 4	100 ± 20	260 ± 50	95
Transcript 5	>500	<130	10
Transcript 6	ND	ND	<5[a]
Transcript 7	ND	ND	<5[a]
Transcript 8	ND	ND	<5[a]
Transcript 9	80 ± 20	200 ± 50	90
Transcript 10	ND	ND	<5[a]
Transcript 11	ND	ND	<5[a]
Transcript 12	240 ± 50	710 ± 120	130
Transcript 13	180 ± 50	660 ± 100	135
Transcript 14	320 ± 60	450 ± 80	50

[a] The relative initial velocities were calculated from the time course experiment for 30 min. <5 means that the relative initial velocity was below 5% of that for *T. thermophilus* $tRNA^{Phe}$.

TrmI, and 37 μM [*methyl*-^{14}C]AdoMet were incubated at 55 °C for 30 min. However, Transcripts 15 and 16 were not methylated at all (Fig. 3*B*): the autoradiogram shown in Fig. 3*B* was obtained after a long exposure (3 days). We considered that some sequences in the T-arm might affect the methyl group acceptance activity. Therefore, next we tested the methyl group acceptance activity of Transcript 17, which mimics the T-arm of *T. thermophilus* $tRNA^{Phe}$. Transcript 17 was methylated by TrmI very slowly (Fig. 3*C* and Table 2). The initial velocity of methyl transfer to Transcript 17 was below 5% of that to the full-length tRNA transcript. It should be mentioned that the results in Fig. 3*C* do not represent the relative initial velocities: the reaction mixture was incubated for 30 min, and the full-length (WT) tRNA transcript was near fully modified under this condition as shown in Fig. 2*B*. The difference between Transcripts 15 and 17 is only the T-stem sequence. The m^1A58 modification is commonly found in *T. thermophilus* tRNAs (32–35, 41), and these tRNAs have different T-stem sequences. Consequently, these results suggested that TrmI recognizes the ribose phosphate backbone of T-stem. When the single-stranded extension corresponding to the 3′-side of acceptor stem was added to the T-arm (Transcript 18), the methyl group acceptance activity was significantly increased (Fig. 3*D* and Table 2). The data in Fig. 3, *D* and *E*, were obtained by incubations for 5 min. Consequently, the results approximated the relative initial velocities of methyl group acceptance of the transcripts. As shown in Fig. 3*D* and Table 2, Transcript 18 was efficiently methylated. Thus, the presence of the single-stranded extension corresponding to the 3′-side of acceptor stem is important for substrate RNA recognition by TrmI. When the variable region was added to Transcript 18 (Transcript 19), the methyl group acceptance activity was further increased (Fig. 3*D* and Table 2). Indeed, the initial velocity of methyl group acceptance of Transcript 19 was comparable with that of the full-length tRNA transcript (Fig. 3*E* and Table 2).

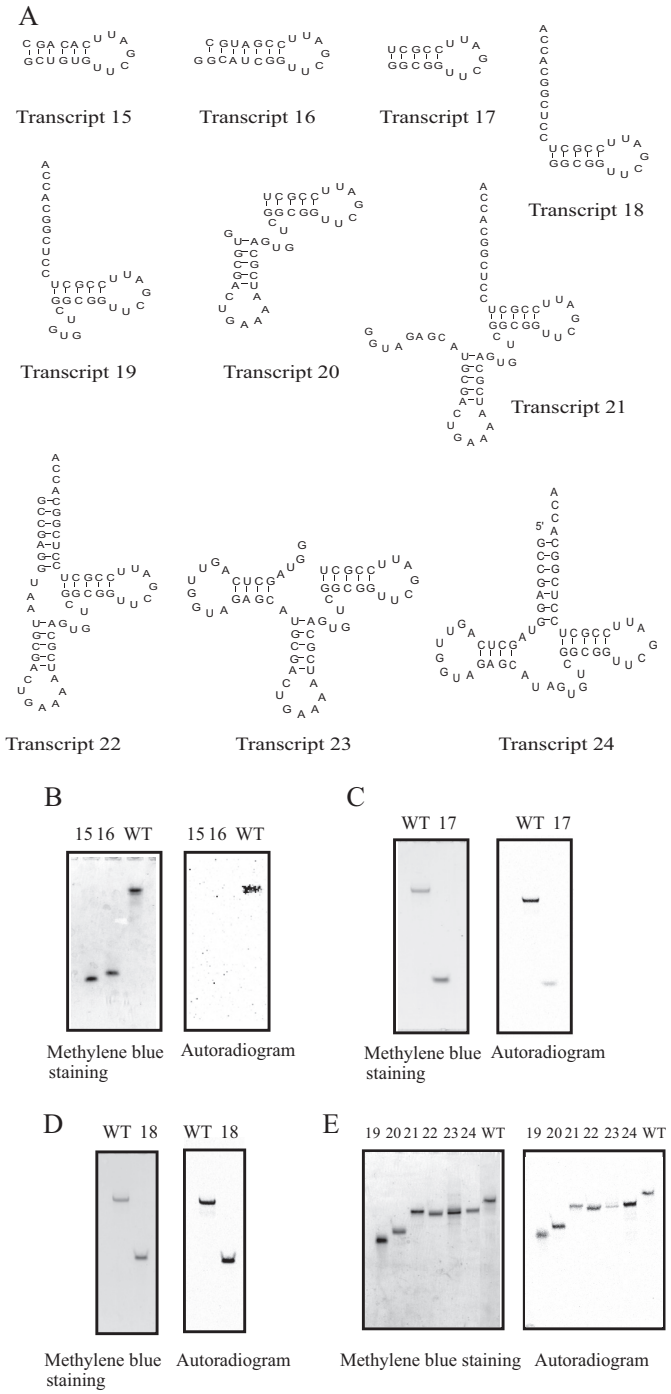

FIGURE 3. **Deletion variants of *T. thermophilus* $tRNA^{Phe}$.** *A*, sequences of 10 deletion variants are shown. *B*, the methyl group acceptance activities of Transcripts 15 and 16 were checked. *WT* indicates the full-length *T. thermophilus* $tRNA^{Phe}$ transcript. *C*, the methyl group acceptance activity of Transcript 17 was tested. Very slow methyl transfer was observed in a long incubation for 30 min (*right panel*). *D* and *E*, the methyl group acceptance activities of Transcripts 18, 19, 20, 21, 22, 23, and 24 were checked with the gel assay. As shown in the autoradiogram (*right panel*), all tested transcripts were methylated by TrmI. The kinetic parameters are summarized in Table 2.

Similarly, the transcripts that contained the T-arm structure with the aminoacyl stem or variable region were methylated by TrmI (Transcripts 20–24 in Fig. 3*D* and Table 2). However, Transcript 23, which did not have the aminoacyl stem, was methylated slowly. This result shows the importance of amino-

TABLE 2

Kinetic parameters for deletion mutant transcripts

The relative V_{max}/K_m for the *T. thermophilus* wild-type $tRNA^{Phe}$ transcript was expressed as 100%. ND means that the kinetic parameters could not be calculated correctly. Not detectable means that the methyl group incorporation was not detectable under the tested condition.

Transcript name	K_m	V_{max}	Relative V_{max}/K_m or relative initial velocity
	nM	*μmol mg⁻¹ h⁻¹*	*%*
Full-length $tRNA^{Phe}$	130 ± 20	360 ± 60	100
Transcript 15	ND	ND	Not detectable
Transcript 16	ND	ND	Not detectable
Transcript 17	ND	ND	<5[a]
Transcript 18	280 ± 40	560 ± 90	75
Transcript 19	200 ± 40	520 ± 70	95
Transcript 20	200 ± 40	730 ± 120	135
Transcript 21	220 ± 50	500 ± 60	85
Transcript 22	250 ± 50	810 ± 100	120
Transcript 23	270 ± 40	400 ± 90	25
Transcript 24	130 ± 30	680 ± 70	190

[a] The relative initial velocities were calculated from the methyl group acceptance activities at 30-min periods. <5 means that the relative initial velocity was below 5% of that for *T. thermophilus* $tRNA^{Phe}$.

acyl stem structure. Transcript 24 was methylated more rapidly than the wild-type tRNA transcript. This result suggests that the presence of the anticodon arm might disturb the methylation by TrmI. We determined the kinetic parameters for these transcripts (Table 2). The kinetic study showed the tendency that the deletion mutant transcripts have relatively large K_m and V_{max} values as compared with the wild-type tRNA transcript. This might be caused by the differences in the initial binding and structural change processes in the methyl transfer reaction. These results show that the combination of T-arm, variable region, and single-stranded extension correspond to the 3′-side of acceptor stem, which is consistent with the results from the chimeric transcripts shown in Fig. 2.

Disruption of T-stem Structure and G18-U55 and G19-C56 Tertiary Base Pairs—To determine whether the T-stem structure itself is required or not, we prepared a mutant transcript in which the T-stem was disrupted (Transcript 25 in Fig. 4*A*). This transcript was not methylated at all (Fig. 4*B*), which demonstrated that the stem structure is essential for recognition of the substrate by TrmI. The methyl group acceptance activities of the deletion mutants indicated that the tertiary interaction between the D- and T-arms was not required for recognition by TrmI (Fig. 3). To confirm this, we prepared a mutant transcript in which the G18G19 sequence was replaced with U18U19 (Transcript 26 in Fig. 4*A*). Interestingly, Transcript 26 was methylated more rapidly than the wild-type transcript (Fig. 4*C*). The kinetic parameters K_m and V_{max} for Transcript 26 were determined to be 150 nM and 480 μmol mg^{-1} h^{-1}, respectively. Thus, the difference in methyl group acceptance activities between Transcript 26 and the wild-type transcript was mainly due to a difference in V_{max} values. Given that the target site A58 forms a reverse Hoogsteen base pair with U54, A58 is embedded in the L-shaped tRNA structure. Consequently, the access of TrmI to A58 requires disruption of the tRNA structure. Disruption of the interaction between the T- and D-arms in Transcript 26 seemed to accelerate this structural change.

Introduction of Point Mutations Revealed That U55, C56, U59, and U60 Are Involved in Recognition of the Substrate tRNA by TrmI—To clarify the positive sequence determinants in the tRNA for methylation by TrmI, we prepared an additional 17

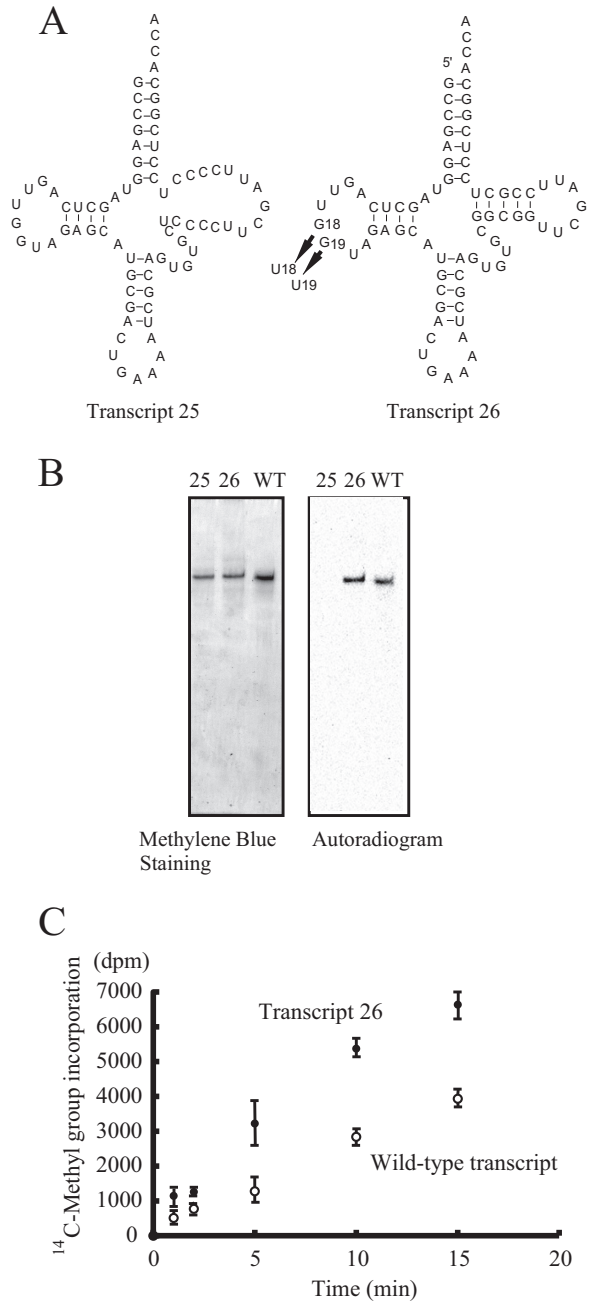

FIGURE 4. **Disruptant tRNA mutants of T-stem and interaction between D- and T-arms.** *A*, the T-stem of *T. thermophilus* $tRNA^{Phe}$ was disrupted by replacing the T-stem sequence (Transcript 25). The interaction between D- and T-arms was disrupted by replacing the G18G19 sequence with U18U19 (Transcript 26). *B*, the methyl group acceptance activities of the two disruptant tRNA mutants were analyzed with the gel assay. *WT* indicates the *T. thermophilus* wild-type $tRNA^{Phe}$ transcript. The *lane numbers* correspond to the transcript numbers. *C*, the methyl group acceptance activities of Transcript 26 and the wild-type transcript were compared. *Error bars* represent S.E.

$tRNA^{Phe}$ mutant transcripts with a single point mutation introduced in each (Transcripts 27–43 in Fig. 5). The kinetic parameters are given in Table 3. The point mutations were introduced mainly into the T-loop. Given that several tRNAs (for example $tRNA_f^{Met}$) have an A at position 46 instead of G (32), we prepared one mutant tRNA transcript in which G46 was replaced by A (Transcript 27). This transcript was methylated efficiently by TrmI (Fig. 5*B*). When U54 was replaced with A (Transcript

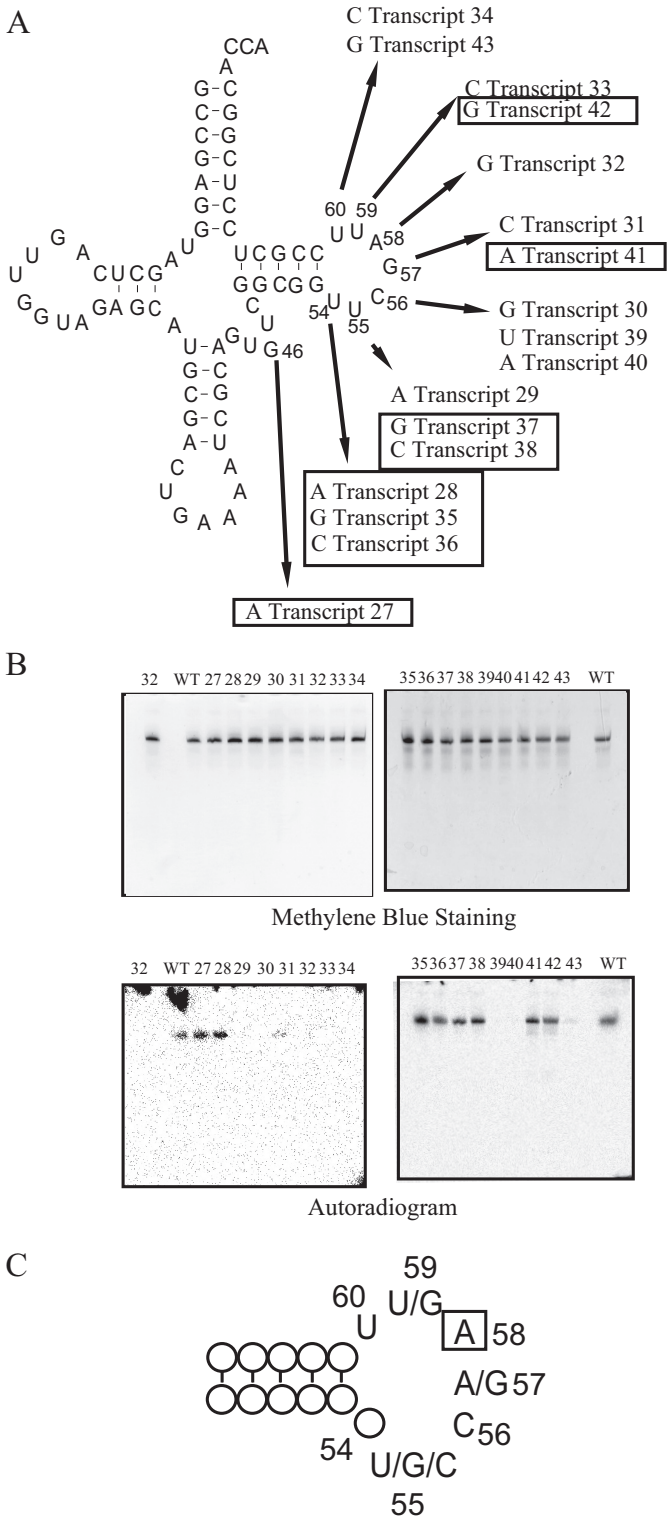

FIGURE 5. **Point mutations in the *T. thermophilus* tRNA^{Phe} transcript.** *A*, 17 point mutations were introduced individually into the *T. thermophilus* tRNA^{Phe} transcript. The *arrows* show the mutations and transcript numbers. The mutation sites and positions are indicated in the clover leaf structure. The transcripts of which the initial velocities of methyl group acceptance activity were more than 50% of that of the wild-type tRNA^{Phe} transcript are enclosed with *boxes*. The kinetic parameters are summarized in Table 3. *B*, the methyl group acceptance activities of point mutants were analyzed with the gel assay. The *upper panels* show the gel stained with methylene blue. The *lane numbers* correspond to the transcript numbers. *WT* indicates the *T. thermophilus* wild-type tRNA^{Phe} transcript. Incorporation of the [^{14}C]methyl group was monitored by autoradiography of the same gels (*lower panels*). *C*, the preferred T-loop sequence for methylation by TrmI is depicted. The methylation site A58 is enclosed with a *box*.

28), G (Transcript 35), or C (Transcript 36), the methyl group acceptance activity was not changed significantly, suggesting that the U54-A58 reverse Hoogsteen base pair is not required for the methylation by TrmI. When U55 was replaced with A (Transcript 29), the methyl group acceptance activity was decreased considerably, whereas if it was replaced with G (Transcript 37) or C (Transcript 38), the activity was not changed significantly. These results might be explained by the formation of a Watson-Crick base pair between A55 and U59 in the Transcript 29. When C56 was replaced with G (Transcript 30), U (Transcript 39), or A (Transcript 40), the methyl group acceptance activity was lost completely, which showed that C56 is a positive determinant for methylation by TrmI. The replacement of G57 with C (Transcript 31) decreased the methyl group acceptance activity considerably, whereas its replacement with A (Transcript 41) had no effect on the activity. These results show that a purine at position 57 is a positive determinant for methylation by TrmI. The replacement of U59 with C (Transcript 33) decreased the methyl group acceptance activity considerably, whereas its replacement with G (Transcript 42) had no significant effect on the activity. Although the precise mechanism of this phenomenon cannot be explained, the replacement of U59 with C might change the structure of ribose phosphate backbone of the T-loop. Furthermore, when U60 was replaced with C (Transcript 34) or G (Transcript 43), the methyl group acceptance activity was significantly decreased. These results are consistent with the low methyl group acceptance activity of the *S. cerevisiae* tRNA^{Phe} transcript, which has a C at position 60 (Fig. 2*A*). Thus, U60 is also a positive determinant for methylation by TrmI. From these results, the preferred T-loop sequence for methylation by *T. thermophilus* TrmI can be derived (Fig. 5*C*).

Replacement of A58 by I Causes the Complete Loss of Methyl Group Acceptance Activity—The replacement of A58 by G caused the complete loss of methyl group acceptance activity (Transcript 32). This result shows that TrmI correctly recognizes an adenine base at position 58. To clarify which features of the structure of adenine are recognized by TrmI, we prepared a mutant tRNA^{Phe} transcript in which A58 was replaced with I (Transcript 44) or 2-AP (Transcript 45). Hypoxanthine (the base in inosine) and 2-AP have a structure very similar to that of adenine except that it does not contain an amino group at position 6 (Fig. 6*A*). As described under "Experimental Procedures," the 3′-half of tRNA containing I or 2-AP at position 58 was synthesized chemically and ligated with the 5′-half using T4 RNA ligase (Fig. 6*B*). The ligated sample was purified further by 10% PAGE (7 M urea). As shown in Fig. 6*C*, these transcripts were not methylated at all, demonstrating that TrmI recognizes the 6-amino group in A58. During the revision of this manuscript, Hamdane *et al.* (22) reported that 2-AP in minihelices, which mimic the T-arm and aminoacyl-stem, inhibit the methylation by aTrmI. Thus, our experimental results are in good agreement with their results.

TABLE 3

Kinetic parameters for point mutated tRNA transcripts

The relative V_{max}/K_m for the *T. thermophilus* wild-type $tRNA^{Phe}$ transcript was expressed as 100%. ND means that the kinetic parameters could not be calculated correctly.

Transcript name	Mutation site	K_m	V_{max}	Relative V_{max}/K_m or relative initial velocity
		nM	$\mu mol\ mg^{-1}\ h^{-1}$	%
T. thermophilus wild-type $tRNA^{Phe}$	None	130 ± 20	360 ± 60	100
Transcript 27	46G→A	110 ± 20	340 ± 60	110
Transcript 28	54U→A	130 ± 30	440 ± 70	125
Transcript 29	55U→A	ND	ND	<5[a]
Transcript 30	56C→G	ND	ND	Not detectable
Transcript 31	57G→C	ND	ND	<10[a]
Transcript 32	58A→G	ND	ND	Not detectable
Transcript 33	59U→C	ND	ND	<5[a]
Transcript 34	60U→C	ND	ND	<5[a]
Transcript 35	54U→G	120 ± 20	480 ± 80	150
Transcript 36	54U→C	130 ± 40	250 ± 60	80
Transcript 37	55U→G	100 ± 20	380 ± 80	135
Transcript 38	55U→C	120 ± 20	390 ± 70	120
Transcript 39	56C→U	ND	ND	Not detectable
Transcript 40	56C→A	ND	ND	Not detectable
Transcript 41	57G→A	130 ± 20	480 ± 60	150
Transcript 42	59U→G	160 ± 30	340 ± 60	80
Transcript 43	60U→G	ND	ND	<5[a]

[a] The relative initial velocities were calculated from the time course experiment for 30 min. <5 and <10 mean that the relative initial velocity was below 5% and 10% of that for *T. thermophilus* $tRNA^{Phe}$, respectively.

Transfer RNA^{Thr}_{GGU} from T. thermophilus Has a C at Position 60—In general, the base at position 60 in tRNA is conserved as a pyrimidine (10). However, TrmI prefers U60 to C60 as shown in Fig. 5*C*. *T. thermophilus* has 47 tRNA species. Among them, only $tRNA^{Thr}_{GGU}$ contains C60 instead of U60 (Fig. 7*A*). Given that the anticodons of the other two $tRNA^{Thr}$ species are CGU and UGU, $tRNA^{Thr}_{GGU}$ is required to decode ACC and ACU threonine codons during protein synthesis. To check the methyl group acceptance activity of $tRNA^{Thr}_{GGU}$, we prepared the $tRNA^{Thr}_{GGU}$ transcript. As expected, the methyl group acceptance activity of the $tRNA^{Thr}_{GGU}$ transcript was considerably lower than that of the $tRNA^{Phe}$ transcript: the initial velocity of the former was only 2% of the latter (Fig. 7*B*). The replacement of C60 in the $tRNA^{Thr}_{GGU}$ transcript with U increased the initial velocity of methyl group acceptance activity by ~6-fold (Fig. 7*B*). Thus, the presence of C60 is one of the reasons for the low methyl group acceptance activity of $tRNA^{Thr}_{GGU}$. We thought that TrmI might have a lower affinity for $tRNA^{Thr}_{GGU}$ than for $tRNA^{Phe}$. To confirm this, we performed a gel mobility shift analysis. As reported by Droogmans *et al.* (20), it is much more difficult to perform gel mobility shift assays with TrmI than with other tRNA modification enzymes (53, 54) because the large TrmI tetramer (more than 100 kDa) does not migrate into a normal polyacrylamide gel under electrophoresis. Consequently, we used agarose gels for the gel mobility shift assay as reported (20) and stained them with ethidium bromide. As shown in Fig. 7*C*, *left panel*, a discrete shifted band derived from the $tRNA^{Phe}$ transcript was clearly detected. In contrast, the band derived from the $tRNA^{Thr}_{GGU}$ transcript was smeared (Fig. 7*C*, *middle panel*). These results showed that the low methyl group acceptance activity of the $tRNA^{Thr}_{GGU}$ transcript is caused by a low affinity for TrmI. The presence of C60 did not affect the pattern obtained with the gel mobility shift assay significantly because the mutant $tRNA^{Phe}$ 60U→C (Fig. 7*C*, *lower right panel*) transcript gave similar shift patterns as the wild-type tRNA transcript. Thus, the high affinity of the $tRNA^{Phe}$ transcript seems to be caused by a combination(s) of aminoacyl stem structure, sequence of the T-loop, and variable region of $tRNA^{Phe}$.

The Other Modifications in $tRNA^{Thr}_{GGU}$ Accelerate the Methylation by TrmI—Recently, we reported that the tRNA modification enzymes and modified nucleotides in *T. thermophilus* form a network, which regulates the extent of modifications in tRNA in response to changes in temperature (2, 34, 35, 51, 55). The m^1A58 modification by TrmI is required for cell viability at high temperatures (20) because it accelerates the sulfur transfer reaction that is required for m^5s^2U54 modification (37). The m^5s^2U54 modification increases the melting temperature of the tRNA by more than 4 °C (32, 33). Furthermore, the combination of the Gm18, m^5s^2U54, and m^1A58 modifications increases the melting temperature by nearly 10 °C as compared with the unmodified transcript (35). The m^1A58 modification is a relatively late modification in *T. thermophilus* tRNA. Consequently, we proposed that the other modifications in $tRNA^{Thr}_{GGU}$ might support the formation of m^1A58 by TrmI. To confirm this idea, we utilized a *T. thermophilus* HB8 *trmI* gene disruptant strain (37). Given that this HB8 gene disruptant strain did not grow at 80 °C (37) as reported with the analogous HB27 strain (20), the cells were cultured at 70 °C. We prepared the fraction of small RNAs (mainly tRNA and 5 S rRNA) from the wild-type and *trmI* gene disruptant strains and analyzed their modified nucleosides (Fig. 8*A*). The small RNA fraction from the wild-type strain contains the m^1A (Fig. 8*A*, *upper panel*). In contrast, the small RNA fraction from the *trmI* gene disruptant strain did not contain the m^1A nucleoside (Fig. 8*A*, *middle panel*). The elution point of m^1A was confirmed with the standard marker (Fig. 8*A*, *lower panel*). As shown in Fig. 8*B*, $tRNA^{Thr}_{GGU}$ was purified successfully from the *trmI* gene disruptant strain by the solid-phase DNA probe method (40, 41). The methyl transfer assay with TrmI revealed that this tRNA was methylated more rapidly than the $tRNA^{Thr}_{GGU}$ transcript (Fig. 8*C*), which demonstrated that the other modifications present in $tRNA^{Thr}_{GGU}$ accelerated the methylation by TrmI. However, the initial velocity of methyl group acceptance activity of

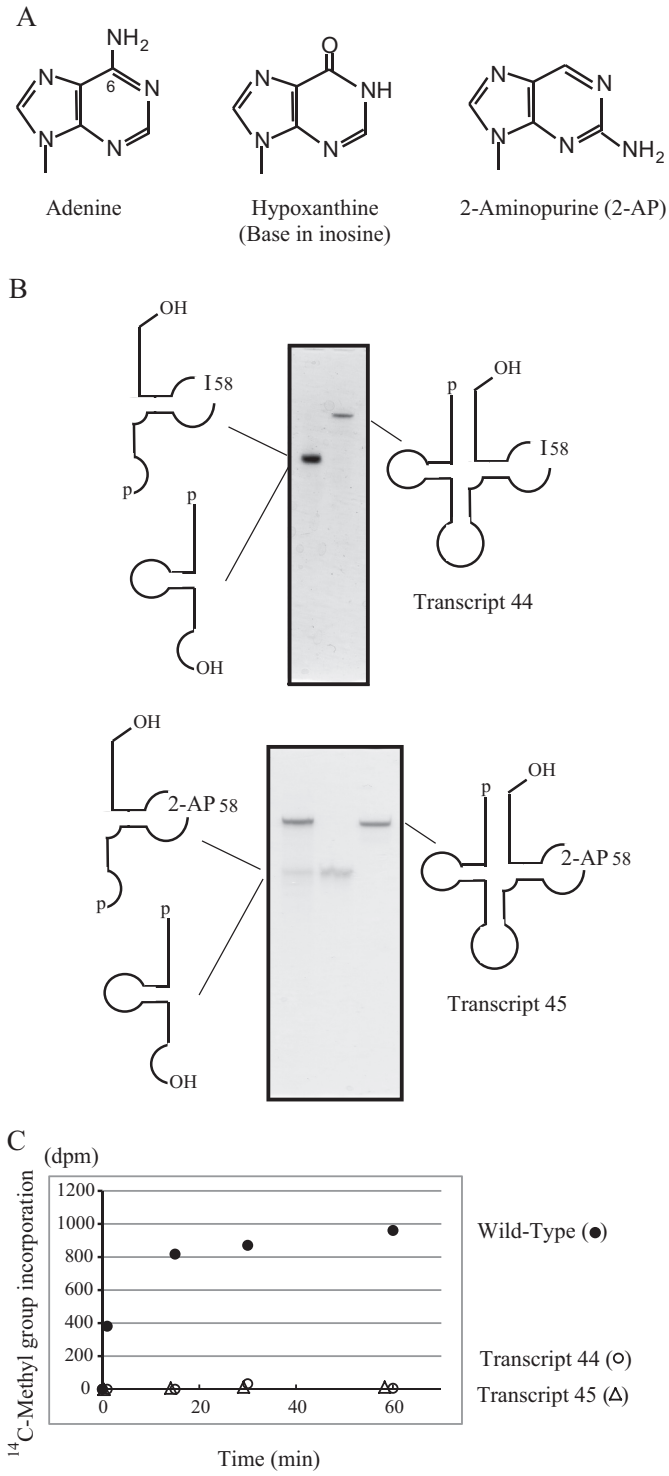

FIGURE 6. **TrmI recognizes the 6-amino group in A58.** *A*, the structures of adenine, hypoxanthine, and 2-aminopurine bases are compared. Position 6 of adenine is *numbered*. *B*, *T. thermophilus* $tRNA^{Phe}$ mutants that contained I58 and 2-AP were constructed by RNA ligation. The resultant ligated samples (Transcripts 44 and 45) were purified by 10% PAGE (7 M urea). The gel was stained with methylene blue. The bands that corresponded to 5′- and 3′-halves of $tRNA^{Phe}$ overlapped on the gel. The *left lane* of the *lower panel* shows the ligation sample before the purification. *C*, the [^{14}C]methyl group acceptance activities of Transcripts 44 (*open circles*) and 45 (*open triangles*) were tested. *Filled circles* show the methyl group acceptance activity of the wild-type $tRNA^{Phe}$.

$tRNA^{Thr}_{GGU}$ from the *trmI* gene disruptant strain was only 13% of that of the $tRNA^{Phe}$ transcript (Fig. 8*C*).

In Living Cells, $tRNA^{Thr}_{GGU}$ Is Not Modified fully by TrmI—We thought that $tRNA^{Thr}_{GGU}$ might not be modified fully in living cells because the methyl group acceptance activity of $tRNA^{Thr}_{GGU}$ purified from the *trmI* gene disruptant strain was rather low as shown in Fig. 8*C*. Consequently, we investigated the m^1A content in $tRNA^{Thr}_{GGU}$ from the wild-type strain. It should be mentioned that the content of m^1A in tRNA changes depending on the culture temperature, components of the medium, and air supply (35, 55, 56). In this experiment, the cells were cultured at 70 °C in rich medium with vigorous shaking. Under these conditions, more than 85% of $tRNA^{Phe}$ contained the m^1A58 modification (41). To estimate the m^1A content in $tRNA^{Thr}_{GGU}$, we purified $tRNA^{Phe}$ and $tRNA^{Thr}_{GGU}$ from the wild-type strain (Fig. 9*A*). The modified nucleosides in $tRNA^{Phe}$ (Fig. 9*B*) and $tRNA^{Thr}_{GGU}$ (Fig. 9*C*) were compared. The levels of m^1A were calculated from the peak areas of m^1A and pseudouridine (Ψ): $tRNA^{Phe}$ contains two Ψ residues (Ψ39 and Ψ55), whereas $tRNA^{Thr}_{GGU}$ contains one Ψ residue (Ψ55). When the level of Ψ in $tRNA^{Thr}_{GGU}$ was set as 1.00, the level of the m^1A modification in this tRNA was calculated to be 0.39. In contrast, when the level of Ψ in $tRNA^{Phe}$ was set as 2.00, the level of m^1A was calculated to be 0.90. These calculations were based on the assumption that the Ψ residues were fully modified in these tRNAs. Therefore, we tested the level of m^1A58 modification by the other method: the purified $tRNA^{Phe}$ and $tRNA^{Thr}_{GGU}$ were fully methylated by TrmI and [*methyl*-^{14}C]AdoMet. The levels of the m^1A modification in $tRNA^{Phe}$ and $tRNA^{Thr}_{GGU}$ were calculated to be 93 and 48%, respectively. These results clearly showed that $tRNA^{Thr}_{GGU}$ is not modified fully in the living cells cultured at 70 °C even though the other modifications present in the tRNA accelerate the formation of m^1A58 by TrmI. Furthermore, the level of m^5s^2U in $tRNA^{Thr}_{GGU}$ was low as compared with that in $tRNA^{Phe}$, and the level of an intermediate, m^5U, in $tRNA^{Thr}_{GGU}$ was increased (Fig. 9, *B* and *C*). This observation is consistent with the previous proposed network (2, 55). Moreover, the levels of m^7G, s^4U, and Gm in $tRNA^{Thr}_{GGU}$ were considerably low as compared with those in $tRNA^{Phe}$ (Fig. 9, *B* and *C*). These differences might be caused by the low levels of m^1A and m^5s^2U in $tRNA^{Thr}_{GGU}$.

DISCUSSION

Fig. 10 shows the crystal structures of TrmI and *S. cerevisiae* $tRNA^{Phe}$. Given that an L-shaped tRNA cannot be simply placed to the enzyme surface of the crystal structure of TrmI without a clash between the two structures (23, 27), there is no proposed docking model between TrmI and tRNA. Thus, the structure of tRNA is expected to be disrupted during methylation by TrmI. Our experiments with the chimeric transcripts and deletion mutants showed that combinations of the aminoacyl stem, variable region, and T-loop of tRNA are required for efficient methyl transfer by TrmI: these regions are colored in *gray* (Fig. 10). The earlier crystal structure study reported the presence of two grooves (grooves 1 and 2), which include many basic amino acid residues (Fig. 10, *blue* regions), on the surface of the TrmI tetramer (23). The size and orientation between grooves and the catalytic pocket are consistent with a model in

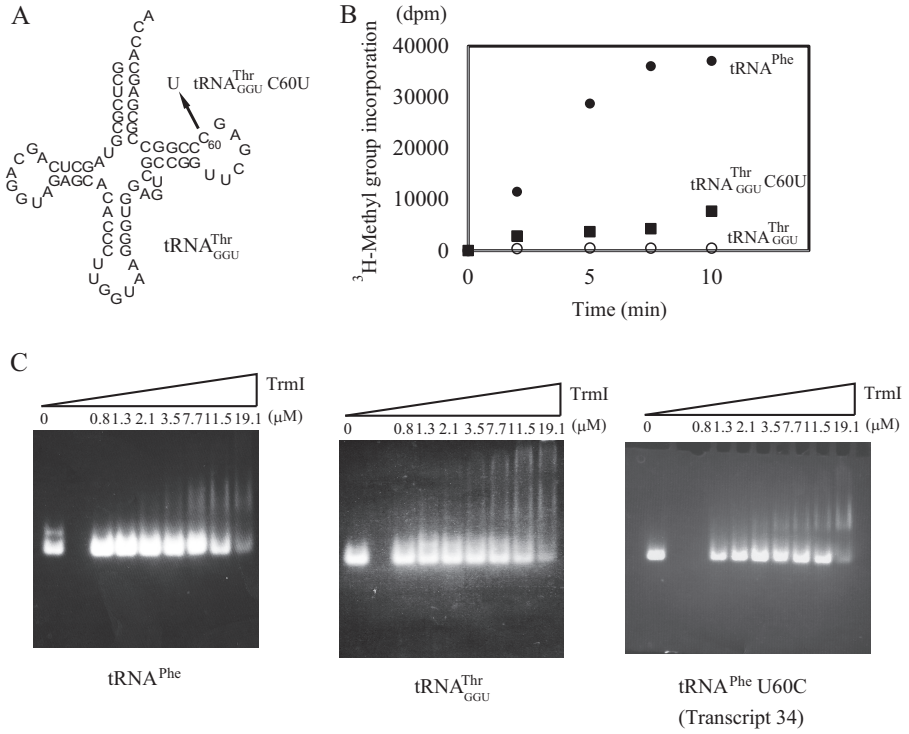

FIGURE 7. **The methyl group acceptance activity of tRNA$^{Thr}_{GGU}$ and its affinity for TrmI.** *A*, among the 47 tRNA species in *T. thermophilus*, tRNA$^{Thr}_{GGU}$ is the only one that has a C at position 60. A mutant tRNA transcript was generated (tRNA$^{Thr}_{GGU}$ 60C→U) in which C60 was replaced with U. *B*, the methyl group acceptance activities of the wild-type tRNA$^{Thr}_{GGU}$ (*open circles*) and 60C→U mutant (*filled squares*) were compared with that of tRNA^{Phe} (*filled circles*). The initial velocity of incorporation of [^{3}H]methyl group into the wild-type tRNA$^{Thr}_{GGU}$ was 2% of that of tRNA^{Phe}. The replacement of C60 with U accelerated the velocity of incorporation of [^{3}H]methyl group. *C*, the affinities of transcripts for TrmI were analyzed by the gel mobility shift assay.

which the grooves capture the aminoacyl stem in the L-shaped tRNA (23). Indeed, our current study demonstrates that the aminoacyl stem is very important for the tRNA recognition by TrmI. The stem structure itself of the aminoacyl stem is not required because Transcript 18 in Fig. 3 was methylated efficiently. When the aminoacyl stem is placed on a groove, the anticodon arm cannot be placed on another groove without a structural clash (23). The results of our experiments with the chimeric transcripts and deletion mutants are consistent with their observation because the anticodon arm was not recognized by TrmI. Instead of the anticodon arm, another groove might capture the variable region because the combination of the aminoacyl stem, variable region, and T-arm was required for efficient methyl transfer by TrmI. Under physiological conditions, one TrmI tetramer seems to capture one tRNA because grooves 1 and 2 are formed by the tetrameric structure (23). This idea was reinforced by the gel shift pattern shown in Fig. 7: the tRNA^{Phe} transcript, at least, yielded only a single shifted band. Given that the modification site A58 is located in the T-loop, the aminoacyl stem and/or variable region seems to be required for the initial binding process. The T-stem structure itself was required for methylation by TrmI. This result shows that the structure of the ribose phosphate backbone of the T-stem is required, which suggests that the distance and angle between the target site A58 and the initial binding sites (aminoacyl stem and variable region) are important for the methylation by TrmI. Given that the gel mobility shift assays with the tRNA^{Phe} variant showed that the presence of a C or U at position 60 had no obvious effect on the formation of a complex between tRNA and TrmI, the C nucleotide at position 60 might be involved in the process of structural change. The precise order in which C60 and C56 (important positive sequence determinants) are recognized is unclear. However, it is clear that the recognition of C56 occurred after the disruption of T- and D-arm interaction because C56 forms a tertiary base pair with G19. Disruption of the interaction between the T- and D-arms accelerated the methyl transfer reaction by TrmI, suggesting that the methyl transfer reaction requires the disruption of the T- and D-arm interaction. Given that the target site A58 forms a reverse Hoogsteen base pair with U54 and is embedded in the tRNA structure, introduction of A58 into the catalytic pocket requires at least the disruption of the structure of the T-arm. Disruption of the T- and D-arm interaction is probably part of this process of structural change. The U54-A58 reverse Hoogsteen base pair is not required for the recognition of A58 because point mutation of U54 did not change the methyl group acceptance activity significantly. However, the 6-amino group in A58 is recognized by TrmI because the mutant tRNA transcripts that contained I58 and 2-AP58 were not methylated at all. The earlier crystal structure study proposed a hypothetical interaction between the 6-amino group in A58 and the catalytic center (aspartate 170 in *T. thermophilus* TrmI) (23). Our result in the current study reinforces their hypothetical catalytic mechanism: the 6-amino group in A58 is absolutely required for the methyl transfer reaction. In the archaeal modification system, aTrmI methylates both A57 and A58 (21), and the resultant m^{1}A57 is further modified to m^{1}I57 by deamination (57). Unmethylated I57 has not been found

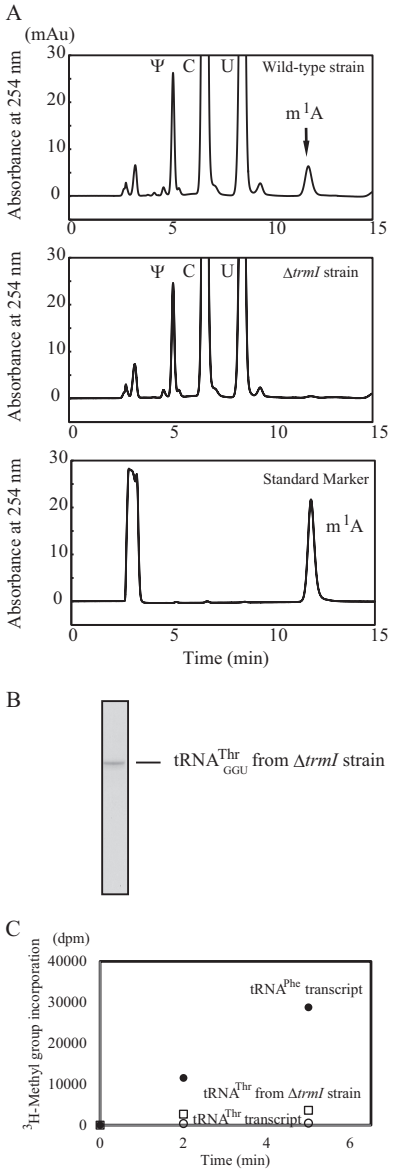

FIGURE 8. **$tRNA^{Thr}_{GGU}$ purified from the *trmI* gene disruptant strain is methylated more rapidly by TrmI than the $tRNA^{Thr}_{GGU}$ transcript.** *A*, the modified nucleosides in the fraction of small RNA from *T. thermophilus* wild-type (*upper panel*) and *trmI* gene disruptant (*middle panel*) strains were compared. The *lower panel* shows the elution point of the standard m^1A marker. *B*, transfer RNA^{Thr}_{GGU} was purified from the *trmI* gene disruptant strain by the solid-phase DNA probe method. 0.02 A_{260} unit of $tRNA^{Thr}_{GGU}$ was analyzed by 10% PAGE (7 M urea). The gel was stained with methylene blue. *C*, the methyl group acceptance activity of $tRNA^{Thr}_{GGU}$ from the *trmI* gene disruptant strain (*squares*) was compared with those of the $tRNA^{Phe}$ (*filled circles*) and $tRNA^{Thr}_{GGU}$ (*open circles*) transcripts. The presence of modified nucleotides in purified $tRNA^{Thr}_{GGU}$ accelerated the methylation by TrmI. *mAu*, milli-absorbance units.

thus far in archaeal tRNAs. Furthermore, the crystal structure of aTrmI (26) strongly suggests that TrmI and aTrmI have the same catalytic mechanism. These observations suggest that aTrmI also recognizes the 6-amino group in the target adenosine similarly to eubacterial TrmI. Indeed, during the revision of this manuscript, Hamdane *et al.* (22) reported that 2-AP inhibited the methylation of minihelices, which mimic the T-arm and aminoacyl stem, by aTrmI. Furthermore, they proposed the hypothetical mechanism that aTrmI methylates two adenosines, A57 and A58, without release of the intermediate (m^1A57-modified tRNA) (22). Thus, the reaction mechanism of aTrmI seems to be different from that of Trm1, although these enzymes consume two AdoMet molecules in the reaction: Trm1 releases the intermediate (m^2G26-modified tRNA) in the reaction (58, 59).

For a long time, it has been thought that eubacterial TrmI modifies all tRNAs at the same efficiency. The results of our current study contradict this assumption. TrmI from *T. thermophilus* at least methylated $tRNA^{Phe}$ transcript 50 times faster than the $tRNA^{Thr}_{GGU}$ transcript. Indeed, more than half of native $tRNA^{Thr}_{GGU}$ in cells was not methylated, whereas 90% of $tRNA^{Phe}$ was modified. The network among modified nucleosides and modification enzymes has mainly been investigated by examining the modification patterns of mixtures of tRNA and of $tRNA^{Phe}$ (35, 55). However, the acceleration of formation of m^5s^2U54 in the presence of m^1A58 is applicable even in the case of $tRNA^{Thr}_{GGU}$ (Fig. 9). The nucleotide at position 60 of tRNA is semiconserved as a pyrimidine. Among the 47 tRNA species from *T. thermophilus*, only $tRNA^{Thr}_{GGU}$ has a C at position 60 rather than a U. The existence of this tRNA would appear to be disadvantageous for growth at high temperatures because the m^1A58 modification is required for introduction of the m^5s^2U54 modification at high temperatures (20, 37). Why does *T. thermophilus* have this apparently disadvantageous tRNA for survival at high temperatures? There are several possibilities. The first possibility is that the precursor tRNAs have different sequences, and their structural flexibilities are also different. Therefore, each tRNA transcript is modified in the different levels for the regulation of appropriate flexibilities at suitable temperatures (for example 70 °C). The second possibility is that *T. thermophilus* lives in hot springs, and organic compounds such as amino acids in hot springs are limited. Therefore, rapid speed in protein synthesis might be not required for survival under the poor nutrient environment. Because 40–50% of $tRNA^{Thr}_{GGU}$ contains the m^1A58 modification even at 80 °C, the protein synthesis is not paused completely. The third possibility is that at high temperatures *T. thermophilus* produces heat-shock proteins (55). The population change of tRNA species might regulate the production of heat-shock proteins. The fourth possibility is that the C60 in $tRNA^{Thr}_{GGU}$ was derived from the accidental mutation, and it does not have any positive function for survival. Although there are these possibilities, the biological function of C60 in $tRNA^{Thr}_{GGU}$ is unclear. Recently, we measured the quantity of $tRNA^{Thr}_{GGU}$ in the small RNA fraction (41): the amount of $tRNA^{Thr}_{GGU}$ was only 0.25–0.30 A_{260} unit in the 50.0 A_{260} units of small RNA fraction. Thus, $tRNA^{Thr}_{GGU}$ seems to be a minor tRNA in *T. thermophilus*.

The requirement for a U at position 60 for efficient methylation by *T. thermophilus* TrmI seems to be applicable to the other thermophilic eubacterial TrmI enzymes. For example, all 43 tRNA species encoded in the genome of *Aquifex aeolicus*, a hyperthermophilic eubacterium, have the U60 nucleotide (10). In contrast, tRNA species with a C at position 60 are often found in mesophilic eubacteria. For example, three tRNA species in *E. coli* and seven tRNA species in *Bacillus subtilis* contain C60 (10): these mesophilic eubacteria do not have the TrmI protein. Therefore, the presence of TrmI in thermophilic eubacteria might function as a selective pressure for the evolu-

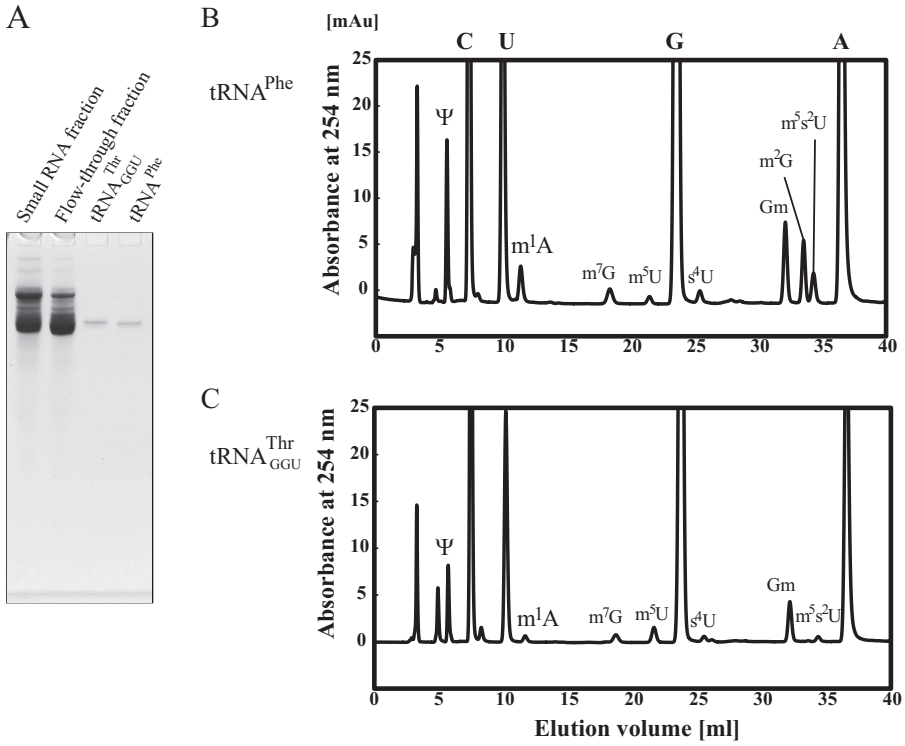

FIGURE 9. **Nucleoside analysis of tRNA^{Phe} and tRNA$^{Thr}_{GGU}$ from *T. thermophilus* wild-type strain.** To estimate the m^{1}A content in the tRNA$^{Thr}_{GGU}$ fraction, tRNA^{Phe} and tRNA$^{Thr}_{GGU}$ were purified from the *T. thermophilus* wild-type strain, and their modified nucleosides were compared. *A*, the purified tRNAs were analyzed by 10% PAGE (7 M urea). The gel was stained with toluidine blue. The modified nucleosides in tRNA^{Phe} (*B*) and tRNA$^{Thr}_{GGU}$ (*C*) were analyzed. *mAu*, milli-absorbance units.

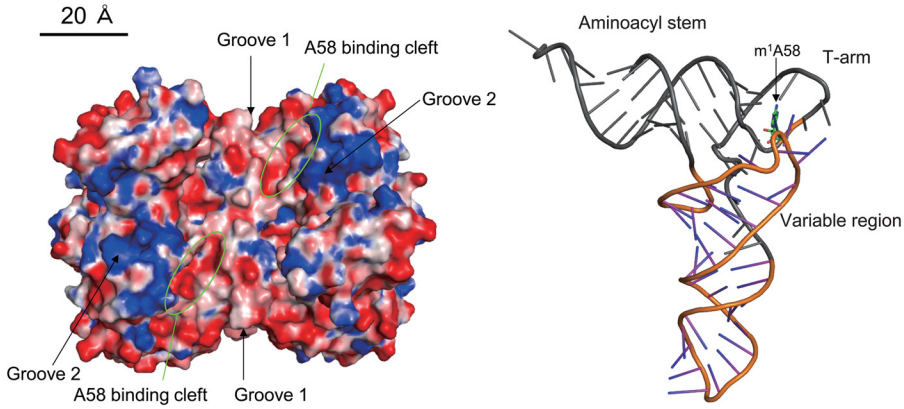

FIGURE 10. **Structures of *T. thermophilus* TrmI and tRNA.** The crystal structures of TrmI and *S. cerevisiae* tRNA^{Phe} are depicted in the same scale. The acidic and basic residues in TrmI are colored in *red* and *blue*, respectively. The A58 binding clefts are enclosed by *green circles*. The aminoacyl stem, T-arm, and variable region in tRNA are colored in *gray*: these regions are important for the recognition by TrmI. The m^{1}A58 is highlighted by a stick model (*green*).

tion of tRNA sequences. It should be mentioned that these discussions about TrmI are only applicable to eubacterial tRNAs because in eukaryotes the m^{1}A modification is generated by a different enzyme (the Trm6-Trm61 complex). In fact, 20 of 51 tRNA species from *S. cerevisiae* have a C at position 60, and these tRNAs contain the m^{1}A58 modification (10). Interestingly, the U60 nucleotide is semiconserved among archaeal tRNAs: there are very few tRNAs that have a C at this position. The semiconservation of U60 in archaeal tRNA is observed in both thermophiles and mesophiles (10). Therefore, the requirement for U60 for efficient methylation might also be applicable to aTrmI enzymes.

In thermophilic eubacteria, the m^{1}A58 modification functions as a tRNA stabilization factor and part of the tRNA modification network. However, in the case of eukaryotes, the m^{1}A58 modification functions as a part of the RNA quality control system (4). Precursor initiator tRNA^{Met} that does not contain the m^{1}A58 modification is polyadenylated by the so-called "TRAMP complex" and then degraded by Rrp6 and the nucleosome (4). Furthermore, human immunodeficiency virus utilizes the m^{1}A58 modification in human tRNA$^{Lys}_3$ as the terminator of reverse transcription (7). Therefore, studies on the formation of m^{1}A58 in eukaryotic tRNA are very important for understanding cellular biological phenomena and the control of infectious organisms. However, the enzyme architectures of eukaryotic Trm6-Trm62 and eubacterial TrmI are different. Several infectious eubacteria such as *Mycobacterium tuberculosis* contain a TrmI protein (20, 24). Therefore, eubacterial

TrmI might be an effective target for anti-infective bacterial drugs. Thus, the studies on the m^1A58 modification in eubacterial tRNA are also important.

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