Substrate Recognition of tRNA (Guanosine-2′-)methyltransferase from Thermus thermophilus HB27

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Transfer RNA (guanosine-2′-)methyltransferase (Gm-methylase, EC 2.1.1.32) from Thermus thermophilus HB27 is one of the tRNA ribose modification enzymes. The broad substrate specificity of Gm-methylase has so far been elucidated using various species of tRNAs from native sources, suggesting that the common structures in tRNAs are recognized by the enzyme. In this study, by using 28 yeast tRNA^Phe variants obtained by transcription with T7 RNA polymerase, it was revealed that the nucleotide residues G18 and G19 and the D-stem structure are essentially required for Gm-methylase recognition, and that the key sequence for the substrate is pyrimidine (Py)G17G18G19. The other conserved sequences were found not to be essential, but U8, G15, G26, G46, U54, U55, and C56 considerably affected the methylation efficiency. These residues are located within a limited space embedded in the L-shaped three-dimensional structure of tRNA. Therefore, disruption of the three-dimensional structure of the substrate tRNA is necessary for the catalytic center of Gm-methylase to be able to access the target site in the tRNA, suggesting that the interaction of Gm-methylase with tRNA consists of multiple steps. This postulation was confirmed by inhibition experiments using nonsubstrate tRNA variants which functioned as competitive inhibitors against usual substrate tRNAs.

To date, more than 80 modified nucleosides in tRNAs have been isolated and characterized (1). These nucleosides are post-transcriptionally formed at specific positions of tRNA by specific tRNA modification enzymes and are presumed to play important roles in the structure and function of tRNA (2–7).

Among tRNA modification enzymes, tRNA (guanosine-2′-)methyltransferase (Gm-methylase, EC 2.1.1.34), one of the ribose modification enzymes, specifically catalyzes the transfer of a methyl group from S-adenosyl-L-methionine to the 2′-OH of the ribose ring of guanosine at position 18 (G18) in the D-loop (8, 14). G18 is one of the hyperconserved residues located in the so-called three-dimensional core of tRNA (9, 10) and is responsible for the formation of the L-shaped three-dimensional structure by D-loop/T-loop interaction through the tertiary base pair G18–G55 and G19–C56 (11, 12). Although 2′-O-methylguanosine at position 18 (Gm18) is distributed widely in tRNAs of prokaryotes, eukaryotes, archaea, and plant mitochondria (13), purification of the enzyme has been reported solely from Thermus thermophilus (7). Recently, the Escherichia coli spoU gene has been reported to be essential for Gm18 modification, suggesting that spoU encodes E. coli Gm-methylase (14). With respect to the physiological role of the methylation of the ribose of the G18 residue, it is known that the resistance of tRNA against RNases is increased by this modification, thus probably prolonging the half-life of the tRNA (15). In higher plants, a relationship between Gm18 methylation and the transport of RNA^14u into mitochondria has also been reported (16).

The mechanisms of interactions between tRNAs and modification enzymes are of interest not only physiologically but also biochemically as typical examples of RNA-protein interaction. However, there are only a few reports on tRNA recognition by modification enzymes. The following purified enzymes have been studied: tRNA-(m1G37)-methyltransferase (17), tRNA-guanine transglycosylase for producing Q34 (18, 19), and tRNA-(m5U54)-methyltransferase (20, 21) from E. coli; tRNA-(m5C48)-methyltransferase (22) from HeLa cell line; and Gm18-methylase (23–25) and tRNA-(m1A58)-methyltransferase (26) from T. thermophilus. A crude extract, tRNA-(ψ35)-synthase from a higher plant (27), has also been investigated. In addition, several tRNA modification enzymes from Xenopus laevis (28–33) and yeast (34) have been reported using in vivo assay systems. Two technical difficulties have hindered investigations using purified enzymes. First, most tRNA modification enzymes are labile and only very scanty amounts are able to be purified. Second, special RNAs are usually required as substrates because the enzymes are highly specific for a particular nucleoside(s), sequence(s) and/or three-dimensional structure, and such tRNAs are not easy to prepare. Fortunately, the modification enzymes from T. thermophilus, one of which we used in this work, are relatively stable compared with those from other species. To overcome the second problem, we employed a T7 RNA polymerase system. A synthetic gene of yeast tRNA^Phe was chosen as the template DNA of T7 RNA

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1 The abbreviations use are: Gm-methylase, tRNA (guanosine-2′-)methyltransferase; Pu, purine; Py, pyrimidine.
polymerase, because yeast tRNA\textsuperscript{Phe} is one of the best substrate tRNAs for Gm-methylase, and its three-dimensional structure is well established (11, 12). In this report, the essential regions in the tRNA for recognition by Gm-methylase and its recognition mechanism are discussed.

**EXPERIMENTAL PROCEDURES**

**Materials**—The methyl-\textsuperscript{14}C-labeled S-adenosyl-L-methionine (55–60 C/mol) was purchased from Amersham Pharmacia Biotech. DNA oligomers were synthesized by an Applied Biosystems model 351 DNA synthesizer. DNA modifying enzymes and human placenta RNase inhibitors were obtained from Takara (Ohtsu, Japan). A T7 RNA polymerase expression system (E. coli BL21/pPAR1219) was kindly provided by Dr. F. W. Studier (Brookhaven National Laboratory) (35). T7 RNA polymerase was purified by the method of Grodberg and Dunn (36). Other chemical reagents were of analytical grade.

**Transfer RNAs from Native Sources**—Purified E. coli tRNA\textsuperscript{Met}, tRNA\textsuperscript{Asp}, and tRNA\textsuperscript{Tyr} were kindly provided by Dr. N. Hayashi (Tokyo Institute of Technology); Halobacterium volcanii tRNA\textsuperscript{Met} was a gift of Dr. Y. Kuchino (National Cancer Research); Bacillus subtilis tRNA\textsuperscript{Gly} was supplied by Dr. K. Murao (Jichi Medical School). Yeast tRNA\textsuperscript{Phe} was purchased from Boehringer Mannheim.

**Preparation of Yeast tRNA\textsuperscript{Met}**—Wild-type Transcript and Its Variants—A synthetic wild-type yeast tRNA\textsuperscript{Met} gene with a T7 promoter were constructed between the EcoRI and BamHI sites in the multicloning linker of pUC18, and the insert was then subcloned into the SalI and EcoRI sites of pUC118 for site-directed mutagenesis. In the resultant plasmid, the transcriptional initiation site was designed to be G at the 5′ terminal, the first position of yeast tRNA\textsuperscript{Met}. Yeast tRNA\textsuperscript{Met} was purified by 10% polyacrylamide (7 M urea) gel electrophoresis.

**Measurement of Melting Temperature**—The melting profiles of yeast tRNA\textsuperscript{Phe} and the wild-type transcript were measured by monitoring the change in the absorbance at 260 nm at a heating rate of 0.5 °C/min with a Gilford Response II spectrophotometer using 0.32 A\textsubscript{260} unit RNA in 400 μl of buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM MgCl\textsubscript{2}, and 100 mM NaCl. The melting temperatures were determined by the first derivative of the melting curve.

**RESULTS AND DISCUSSION**

**Transfer RNAs from Native Sources**—In tRNA of the extreme thermophile T. thermophilus, Gm18 is one of the generally existing modified nucleosides, and is produced by Gm-methylase (8, 38), which catalyzes the transfer of methyl groups to various kinds of tRNAs in vitro as well as in vivo. The tRNAs that have so far been identified as substrates of Gm-methylase in an in vitro methylation reaction are listed in Table I. The only tRNA in the table that cannot be methylated is E. coli tRNA\textsuperscript{Tyr}, because it already contains the Gm18 residue in the E. coli cells. This indicates that Gm-methylase does not catalyze the exchange reaction of the methyl group, which is in line with the reaction mechanisms of E. coli tRNA\textsuperscript{Met} (m\textsuperscript{15}U4)-methyltransferase (39) and T. thermophilus tRNA\textsuperscript{Met} (m\textsuperscript{15}A58)-methyltransferase (26). Since not only Class I but also Class II tRNAs (E. coli tRNA\textsuperscript{Ser} and B. subtilis tRNA\textsuperscript{Glu}) are methylated by Gm-methylase, the structural diversities derived from the sizes of the D-arm and the variable arm do not affect recognition by the enzyme. Moreover, the tRNA from an archaea, H. volcanii tRNA\textsuperscript{Met}, is a good substrate for Gm-methylase, suggesting that the recognition sites of Gm-methylase are common for tRNAs from three kingdoms, eukarya, prokarya, and archaea.
Variants Substituted in Conserved Sequence and D-loop—
The nucleotide residues conserved and semiconserved in the
tRNAs of the three kingdoms are shown in Fig. 1A. To clarify
the recognition sites of Gm-methylase, 28 variants of yeast
tRNA\textsuperscript{Phe} transcribed by T7 RNA polymerase were employed.
Nucleotide substitutions were mainly introduced into the con-
served residues in the three-dimensional core region in the
tRNA (Fig. 1B), because these residues interact directly or
indirectly with the D-arm, which includes the methylation
target site, the 2'-OH group of the G18 ribose. Previous results
from foot printing and experiments using half fragments indi-
cated that the essential region for the recognition by Gm-
methylase was limited within the sequence G10–G26 in E. coli
tRNA\textsuperscript{Phe}. The mutations were individually introduced into the residues
as indicated by arrows. The substituted nucleotides are indicated by the
arrowheads.

| Variant | Substitution |
|---------|--------------|
| G10A    | G to A       |
| G11A    | G to A       |
| G12A    | G to A       |
| G13A    | G to A       |
| G14A    | G to A       |
| G15A    | G to A       |
| G16A    | G to A       |
| G17A    | G to A       |
| G18A    | G to A       |
| G19A    | G to A       |
| G20A    | G to A       |

The methyl group acceptance activity of the wild-type tran-
script was about 20% of that of the full-length tRNA (24,
25). In the case of the thermophile Gm-methylase, it was dif-
ficult to discern the so-called minimalist substrate by using a
totally unmodified short fragment, since the initial velocities
for such fragments could not be measured under the standard
conditions; at least, the methylation of the chemically synthe-
sized 18-mer corresponding to positions 9–26 was not able to be
detected at either 37 or 50 °C in 24-h incubation under the
standard conditions (data not shown). Thus, it is assumed that
modified nucleosides such as m\textsuperscript{2}G10, D16, D17, m\textsuperscript{3}G26, and
Cm32 present in the native 5'-half fragment strongly affect the
methylation efficiency, probably through stabilizing the D-loop
stem structure or making the enzyme recognition toward the
substrate easier.

In the point-substituted full-length variants, the only resi-
dues essential for Gm-methylase recognition among those con-
served or semiconserved were determined to be G18 and G19
(Fig. 2A and Table II). Substitution of the nonconserved residue
U17 by purine (Pu) (U17A and U17G) resulted in a drastic
decrease in the \( V_{\text{max}}/K_m \) value. In contrast, no effect was ob-
served when U17 was substituted by C. Analysis of the kinetic
parameters indicated that the \( V_{\text{max}} \) values for U17A and U17G
were very small (Fig. 2 and Table II), suggesting that the
substitution of U17 by Pu changes the environment of the
catalytic center in the Gm-methylase-tRNA complex. Thus, the
most appropriate minimal sequence for Gm-methylase was deduced
to be Py17G18G19 (Py = pyrimidine). This is sup-
ported by the result for E. coli tRNA\textsuperscript{Phe} possessing an
A17G16G19 sequence (Table I), which was the worst substrate
for Gm-methylase among the native tRNAs tested.

In E. coli tRNAs, the G18 residue in almost all class II tRNAs
is modified to Gm18, an exception being tRNA\textsuperscript{Ser}\textsuperscript{Suu},
which has an unmodified G18 (13). Moreover, no prokaryote tRNA possessing
a Pu17Gm18 sequence has been reported (13). Thus, the
optimum sequence deduced for the thermophile Gm-methylase,
Py17G18G19, is likely to be applicable to most prokaryotic
Gm-methylases. It is also clear that positions 17–19 were dis-
tinctly recognized by the enzyme, because the G18C variant
with a C18G19G20 sequence was not methylated at all (Table
II). Judging from the results with the D-stem variants (dis-
cussed in the next section; see Table III), it is likely that the
recognition of these positions depends on the steric distance
and the angle from the phosphate-ribose backbone of the D-
stem structure. On the other hand, it has been reported that
the 2'-O-methylation of G34 (the anticodon first letter) in X
laevis is not affected by the nucleotide sequence around posi-
tion 34 (30). The recognition mechanism of Gm34-methylase
in eukaryotes is thus apparently different from that of Gm18-
methylase in prokaryotes, although the same modified nucleo-
side is produced.

The tertiary base pairs connecting the D- and T-loops were
disrupted in the variants U55A and C56G. As shown in Table II, the $K_m$ values for both variants were much larger than that of wild-type transcript. This is in good agreement with our previous experimental results using a half fragment; the 5'-half fragment of yeast tRNA Phe showed methyl group acceptance activity of about 1/5 in terms of the initial velocity, but the $K_m$ value was much larger, as compared with the values for the full-length tRNA Phe (24, 25). These data suggest that the formation of the tertiary base pair G18-U55 and G19-C56 enhances the methylation efficiency, although these base pairs or the existence of the U55 and C56 residues by themselves are not essential for methylation.

We also demonstrated in our previous study that chemical modification of the sU8 residue in E. coli tRNA$^{Met}$ decreases the efficiency of methylation by Gm-methylase (24). The variants U8A, U8C, and U8G, in which the U8 residue is substituted by A, C, and G, respectively, lack the original tertiary base pair U8-A14, although an alternative reverse Hoogsteen pairing C8-A14 may be recovered in the U8C variant. As shown in Table II, the methylation efficiency of these variants was reduced due to increased $K_m$ values, which is similar to what happened with the chemically modified E. coli tRNA$^{Met}$. Con-

### Table II

**Effect of point mutations in tRNA$^{Phe}$ transcripts on their methyl group acceptance activity**

| Variant | Conservation of substitution site | Disrupted or altered tertiary base pair | $K_m$ (nM) | $V_{max}$ (nmol/mg/h) | Relative $V_{max}/K_m$ % |
|---------|----------------------------------|----------------------------------------|------------|-----------------------|-------------------------|
| Wild-type |  |  |  |  |  |
| U8A | Conserved | U8-A14 | 80 | 85 | 100 |
| U8G | Conserved | U8-A14 | 240 | 73 | 29 |
| U8C | Conserved | U8-A14 | 170 | 67 | 37 |
| A9/U | Semi-conserved as Pu | A9-A22 | 80 | 88 | 104 |
| D-loop region |  |  |  |  |  |
| A14C | Conserved | U8-A14 | 110 | 67 | 58 |
| G15U | Semi-conserved | G15-C48 | 90 | 34 | 36 |
| U16A | No | None | 80 | 82 | 97 |
| U17A | No | None | 60 | 3 | 5 |
| U17G | No | None | 60 | 1 | 2 |
| U17C | No | None | 70 | 85 | 115 |
| G18A | Conserved | G18-U55 → A18-U55 | ND | ND | ND |
| G18C | Conserved | G18-U55 | ND | ND | ND |
| G19A | Conserved | G19-C56 | ND | ND | ND |
| G20A | No | None | 100 | 58 | 55 |
| A21C | Conserved | None | 90 | 59 | 72 |
| G26A | Conserved as Pu | G25-A44 → A26-A44 | 70 | 81 | 109 |
| G26U | Conserved as Pu | G25-A44 → U26-A44 | 130 | 48 | 35 |
| G45C | No | None | 210 | 70 | 31 |
| G46C | Semi-conserved | G22-G46 | 320 | 64 | 19 |
| C48A | Conserved | G15-C48 | 110 | 75 | 64 |
| U54A | Semi-conserved | U54-A58 | 160 | 84 | 50 |
| U55A | Conserved | G18-U55 | 440 | 78 | 17 |
| C56G | Conserved | G19-C56 | 840 | 67 | 8 |
| A58G | Conserved | U54-A58 | 110 | 92 | 79 |

**Fig. 2.** Methylation profiles (A) and Lineweaver-Burke plots (B) for the wild-type tRNA$^{Phe}$ transcript and its variants, U17A, U17G, and G18A. A, initial velocities of methyl group incorporation were measured with various concentrations of substrate tRNA transcripts: ●, wild-type transcript; ○, U17A variant; ■, U17G variant; □, G18A variant. B, Lineweaver-Burke plots for wild-type transcript (●; left and right) and for U17G (■) and U17A (□) variants.
sidering the result with the variant A14C, in which the same U8-A14 base pair was disrupted, it appears that disruption of the U8-A14 base pair slightly affected the methylation efficiency, but the substitution of U8 gave rise to a pronounced decrease in affinity with Gm-methylase. These findings suggest that the decrease in methylation efficiency following chemical modification observed in the previous study (24) arose from steric hindrance due to the s^1Us adduct resulting from the chemical modification.

Other tertiary base pairs, G15-C48, U54-A58, G26-A44, and G21-G46, were also disrupted individually by nucleotide substitutions (G15U, C48A, U54A, A58G, G26U, G26A, and G46C; see Fig. 1B and Table II). Although these tertiary base pairs are not essential, the variants G15U, G26U, G46C, and U54A clearly showed reduced methylation efficiency. In G15U this was due to a decrease in \( V_{\text{max}} \) in G46C and U54A to an increase in \( K_m \), and in G26U to both a decrease in \( V_{\text{max}} \) and an increase in \( K_m \).

The tertiary Levitt base pair G15-C48 did not affect the methylation efficiency, since the variant C48A in which this tertiary base pair was disrupted, showed no marked reduction in methylation efficiency. Therefore, the G15 residue itself and/or the D-loop structure influenced by G15 probably affected the environment around the catalytic center of Gm-methylase.

The mechanism in the case of the variant G46C is less clear. In the native tRNA, G46 is usually modified to m7G46 and the tertiary base pair G22-m7G46 is formed; the variable loop region is located nearby the D-arm in the three-dimensional structure of the tRNA. Since a relatively large \( K_m \) value was also observed when the neighboring nonconserved G45 residue was substituted by C, it is considered that the G45-G46 region in the variable loop may be located near the enzyme surface, thus affecting the affinity of the enzyme.

The G26 residue is conserved as purine and in yeast tRNA_Phe its modified to m2G26 resulting in formation of the tertiary base pair m2G26-A44 (11, 12). When G26 was substituted by A, no appreciable effect was observed. In contrast, substitution of G26 by U reduced the methylation efficiency by 70%. In the variant G26A, the tertiary base pair G26-A44 was disrupted and an alternative base pair A26-A44 was probably formed. Similarly, in the variant G26U, the tertiary base pair G26-A44 was disrupted and an alternative Watson-Crick base pair U26-A44 was formed. This Watson-Crick base pair newly formed in the variant G26U probably affected not only the connection between the D-stem and the anticodon-stem, but also the local structure of the variable loop. Since, as described above, the G45-G46 region in the variable loop has an influence on the methylation efficiency, the substitution of the G26 residue by U may affect the methylation efficiency through the newly formed U26-A44 base pair.

The variant U54A showed decreased methylation efficiency (to approximately half of that of the wild type in initial velocity) through an increase of the \( K_m \) value. Since the U54 residue is usually modified to m7U54 (13) and affects the stability of the L-shaped tRNA structure (40), the effect of substituting U54 by A might arise from structural destabilization. The decrease in the methylation efficiency of U54A was smaller than that of the variant U55A and C56G (Table II).

**The D-stem Variants**—The methylation activity of the D-stem variants (Fig. 3) was also tested, because our previous footprinting data demonstrated that Gm-methylase was bound to the D-arm region in the enzyme-yeast tRNA_Phe complex (25). The results are shown in Table III. Since there is no conserved sequence in the D-stem region of all tRNAs (Fig. 1A), the D-stem variants II and III were designed so as to form a new, artificial stem structure as shown in Fig. 3. In the D-stem variant II, the base pair Py11-Pu24 conserved in all elongator tRNAs was substituted by an alternative base pair A11-U24, and the tertiary base pair A9-A23 was disrupted; in the D-stem variant III, the semiconserved base pairs (Pu10-Pu25 is conserved in almost all tRNAs and Py11-Pu24 is conserved in elongator tRNAs) were substituted by alternative base pairs, and the tertiary base pairs (G22-G46 and A9-A23) were disrupted.

As shown in Table III, the D-stem variant I lacking the D-stem structure was not methylated at all, but the D-stem variants II and III were methylated at about 50% of the methylation of the wild-type transcript. These results clearly show that the structure of the D-stem is one of the essential factors for tRNA recognition by Gm-methylase. Further, it is suggested that the phosphate-ribose backbone of the D-stem structure was recognized by the enzyme, because the nucleotide sequence of the D-stem seemed to have nothing to do with the methylation activity. Moreover, the effect of the disruption of the tertiary base pairs (G22-G46 and A9-A23) on the methyla-
tion efficiency was smaller than that of the substitution of the G46 residue by C (see the variant G46C in Table II).

Multistep Recognition Mechanism Inferred from Inhibition Experiments—The regions essential for recognition by Gm-methylase so far elucidated are depicted on the L-shaped tRNA structure shown in Fig. 4, in which the most significant residues are circled. These features are in agreement with our previous foot printing data (25). However, since the residues involved in substrate recognition by Gm-methylase appear to be embedded inside the L-shaped tRNA molecule, disruption of the tertiary structure of the tRNA may be necessary for Gm-methylase to gain access to these residues. This is supported by the previous foot printing data, which showed that the sensitivity of the aminoacyl stem and T-loop toward RNases is enhanced in the enzyme-tRNA complex (25). This assumption suggests the existence of at least two steps in the interaction of substrate tRNA with Gm-methylase; the first step would involve association of the tRNA with Gm-methylase, while in the second step the structural change of the tRNA, involving interaction between the enzyme and the essential regions of the tRNA, would take place. Such a kind of multistep reaction mechanism has recently been proposed for some cases of DNA-protein interaction (41).

To clarify the above postulation, inhibition experiments were carried out using two variants different in type but both lacking methyl group acceptance activity (Fig. 5A). In the first variant, G18A, the tertiary base pairs connecting the T-loop with the D-loop were not likely to be disrupted because an alternative A18-U55 and the conserved G19-C56 base pairs were probably formed. The other variant, named ΔD-arm, lacked the entire D-arm structure. Surprisingly, as shown in Fig. 5B, both variants inhibited methyl group incorporation of the wild-type transcript. The Kᵢ of G18A indicates that this variant possessed comparable affinity to that of the variant C56G. This finding is in line with an earlier report on tRNA methylation enzymes such as m5C(48)- and m7G(46)-methylases in rat liver (42). The other variant used in the inhibition experiments, ΔD-arm, also showed unexpected inhibition toward the methylation reaction despite the deletion of all the regions in the tRNA essential for methylation. These results clearly show that Gm-methylase has the potential to form a complex with all tRNAs in the first binding step, irrespective of whether they are substrates for Gm-methylase or not. The L-shaped tRNA structure is probably desirable but not essen-

![Fig. 4. Essential regions for recognition by Gm-methylase in the L-shaped tRNA structure.](image)

![Fig. 5. A, nucleotide sequences of tRNA variants used for inhibition experiments. Left, variant G18A, in which the substituted residue is indicated by an arrow; right, variant ΔD-arm in which the whole D-arm structure is deleted. B, Dixon plots derived from the inhibition experiments. Left, inhibition pattern by variant G18A; right, inhibition pattern for variant ΔD-arm. S1 and S2 are the concentrations of the wild-type transcript in each experiment.](image)
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