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Review

7-Methylguanosine modifications in tRNA

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Abstract: More than 90 different modified nucleosides have been identified in tRNA. Among the tRNA modifications, the 7-methylguanosine (m^7G) modification is found widely in eubacteria, eukaryotes, and a few archaea. In most cases, the m^7G modification occurs at position 46 in the variable region and is a product of tRNA (m^7G46) methyltransferase. The m^7G46 modification forms a tertiary base pair with C13-G22, and stabilizes the tRNA structure. Recently, we have proposed a reaction mechanism for eubacterial tRNA m^7G methyltransferase (TrmB) based on the results of biochemical studies and previous biochemical, bioinformatic, and structural studies by others. However, an experimentally determined mechanism of methyl-transfer remains to be ascertained. The physiological functions of m^7G46 in tRNA have started to be determined over the past decade. To be able to better respond to diseases and infections in which the m^7G modification is considered to be involved, it is still necessary to further understand the catalytic mechanism of AdoMet and/or the tRNA bound form of m^7G methyltransferases. In this review, information of tRNA m^7G modifications and tRNA m^7G methyltransferases are summarized and the differences in reaction mechanism between tRNA m^7G methyltransferase and rRNA or mRNA m^7G methylation enzyme are discussed.

Keywords: RNA modification, tRNA methyltransferase, tRNA modification, methylase

1. Introduction

Transfer RNA, which is one of the classical non-coding RNAs, functions as an adaptor molecule supplying amino acids to ribosomes according to the codon of the mRNA. It is important that tRNA forms a precise L-shape structure for its full function [1] and this requires tRNA modification. More than 100 different modified nucleosides have been reported to date and found throughout the different families of RNA molecules [2,3]. tRNA in particular is the most heavily modified [3,4]. The modified nucleosides include thiolation, deamination, isomerization conversion of uridine to pseudouridine, or the combination of several modifications. Of the modification, methylation is the most abundant. This modification encompasses 1-methyladenosine (m^1A), 5-methyluridine (m^5U), 5-methylcytidine (m^5C), 1-, 2-, or 7- position methylation of G (m^1G, m^2G, m^7G), 2′-*O*-methylation of ribonucleoside (Nm), and others [3,5,6]. The most widely prevalent tRNA methylation is S-adenosyl-L-methionine (AdoMet)-dependent methylation by AdoMet dependent methyltransferases. 7-methylguanosine (m^7G) is one of the most conserved modified nucleosides and is common in eubacteria, eukaryotes [4], and a few archaea [7]. Even in psychrophiles which have low levels of modified nucleoside content, m^7G has been found in addition to dihydrouridine (D), pseudouridine (ψ), and m^5U [8]. Additionally, m^7G is present in intron containing pre-tRNA together with $m^{2}{}_{2}G$, ψ, and m^1A [9]. For this reason, it is thought that m^7G is generated immediately after the transcription. m^7G is most frequently located at position 46 in the tRNA variable region, and forms a tertiary base pair with C13-G22 in the three-dimensional core of tRNA (The nucleotide positions in tRNA are numbered, according to Sprinzl *et al*. [10])[11–13]. m^7G has no (net) charge under physiological conditions, but is positively charged in position 46 in tRNA via hydrogen bonding to bases G22 and

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C13 [14–16] (Figure 1). Thus, 7-methylation of m^{7}G produces a site-specific electrostatic charge within the tRNA structure [16].

There are some examples where m^{7}G is found in positions other than at position 46 (Figure 2, Table 1). Chloroplast tRNA^{Leu}(UAG) from *Chlamydomonas reinhardtii* has m^{7}G at position 36 in the tRNA anticodon [17]. In animal mitochondria in which there is deviation from the universal genetic code, m^{7}G is present at position 34 in the anticodon of mitochondorial (mt) tRNA^{Ser}(GCU) of starfish, *Asterina amurensis*, and squid, *Loligo bleekeri* [18,19]. Furthermore, the D-arms of the mt tRNA^{Ser}(m^{7}GCU) in starfish and squid have an unusual secondary structure, and the mt tRNA^{Ser}(m^{7}GCU) recognizes not only the serine codons AGU and AGC, but also the unusual serine codons AGA and AGG. Thus, m^{7}G can make base-pairing with the four basic bases of A, U, G, and C [20]. With respect to m^{7}G in archaeal tRNA, in 1991, Edmonds *et al.* reported that tRNA mixtures from some archaea contain m^{7}G nucleoside, however, the position(s) in tRNA has remained unidentified [7]. More than twenty years later, we demonstrated the presence of the m^{7}G modification in a thermo-acidophilic archaeon, *Thermooplasma acidophilum* at the novel, irregular position 49 in class II tRNA^{Leu} [21]. However, the gene encoding the methyltransferase for m^{7}G49 in tRNA has not thus far been identified.

Table1. *N*7-methylguanosine methyltransferase in tRNA.

domains of life	organisms	positions of m^{7}G	enzyme names	higher order structure	references
eubacteria	*E. coli*	46	TrmB	monomer	[55][60]
	A. aeolicus	46	TrmB	monomer	[53][61][62]
	T. thermophilus	46	TrmB	?	[93]
	B. subtilis	46	TrmB	homodimer	[54]
	S. pneumoniae	46	TrmB	homodimer	PDB: 1YZH
archaea	*T. acidophilum*	49 (tRNA^{Leu}(UAG))	?	?	[7][21]
	T. neutrophilus	?	?	?	[7]
eukaryote	*S. cerevisiae*	46	Trm8/Trm82	heterodimer	[50][56][70]
	S. cerevisiae	5' termini of pre-tRNA	Ceg1p	?	[25]
	C. lagenarium	46	Aph1	?	[96]
	human	46	METTL1/WDR4	?	[50][51]
	C. reinhardtii	37 (chloroplast tRNA^{Leu}(UAG))	?	?	[17]
	A. amurensis	34 (mt tRNASer(GCU))	?	?	[18][20]
	L. bleekeri	34 (mt tRNASer(GCU))	?	?	[19][20]

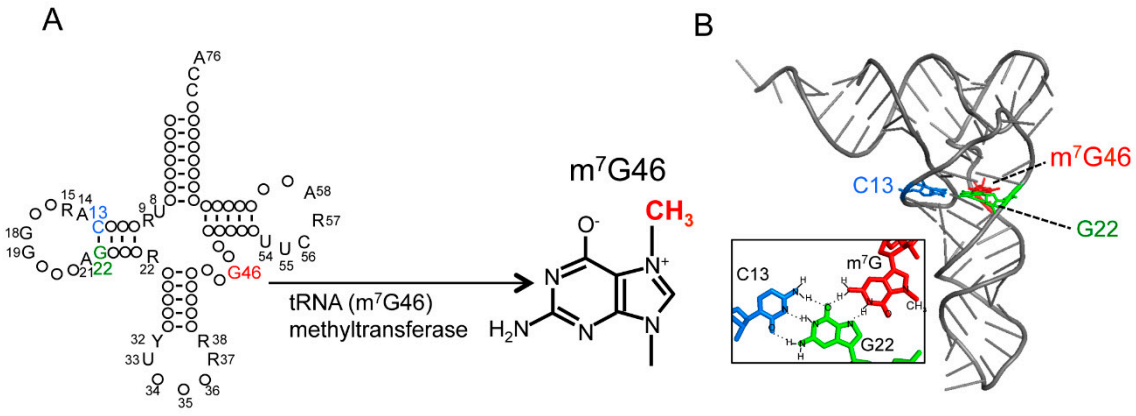

Figure 1. tRNA m^{7}G46 methyltransferase methylates the 7-atom of guanine at position 46 in tRNA and forms m^{7}G46. (A) The secondary structure of tRNA is presented in cloverleaf form. Conserved nucleotides are depicted as follows: adenosine, A; guanosine, G; cytidine, C; uridine, U; purine, R; pyrimidine, Y. tRNA (m^{7}G46) methyltransferase transfers a methyl-group to the 7-atom of guanine at position 46 in tRNA and forms 7-methylguanine. (B) The L-shaped structure of tRNA is presented. The m7G46 forms a tertiary base pair with the C13-G22 base pair in the L-shaped tRNA structure.

The 7-methylguanosine modifications occur not only in tRNA but also in other RNA species such as mRNA, ribosomal RNA (rRNA), small nuclear RNA (snRNA), and small nucleolar RNA (snoRNA). The 5' terminus of eukaryotic mRNA is blocked by $m^7G^5ppp^5N$ cap structure [22,23], and gene of cap-m^7G methyltransferase (Abd1) is essential in *Saccharomyces cerevisiae* [24]. Surprisingly, recent work has detected a cap structure in tRNA. In yeast, an m^7G cap structure is found at the 5' termini of pre-tRNA bearing 5' leader sequences (Figure 2). The capped pre-tRNAs accumulate due to inhibition of 5' exonucleases activities and protect pre-tRNAs from 5'-exonucleolytic degradation during maturation [25]. 7-methylguanosine is observed in 16S rRNA of aminoglycoside-producing Actinobacteria, including *Streptomyces tenebrarius* and *Micromonospora purpurea*. The m^7G modification is at position G1405 in the 16S rRNA and has an aminoglycoside resistance activity [26–28]. Also, in *E. coli*, there is an rRNA methyltransferase RlmL encoded by *rlmL/ycbY* (subsequently renamed *rlmKL*) [29], which contains two methyltransferase domains. In Helix 74 of the 23S rRNA, the N-terminal domain of the rRNA methyltransferase forms m^7G at position 2069, and the C-terminal domain forms m^2G at position 2445 [30]. Cooperative methylation of m^7G2069 and m^2G2445 in Helix74 by RlmKL occurs during biogenesis of the 50S subunit, and RlmKL plays a key role in efficient assembly of the 50S subunit [30].

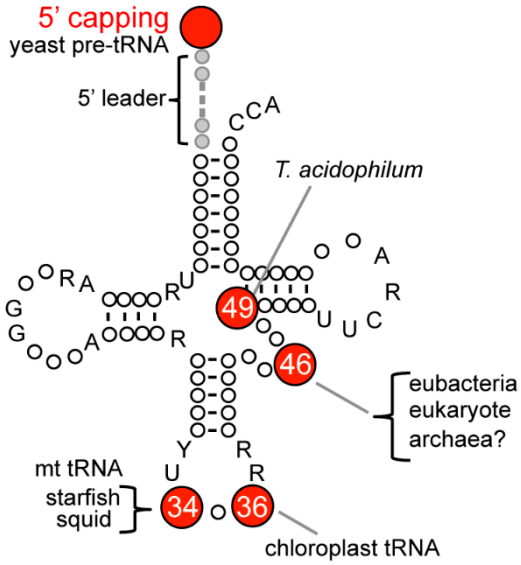

Figure 2. Positions of the m^7G modification in tRNA The numbers in circles indicate the positions of m^7G in tRNA.

Since Holley *et al.* determined the sequence of yeast $tRNA^{Ala}$ in 1965 [31], various tRNA sequences have been reported, and the presences of modified nucleosides in tRNA have been revealed. Also the technical method of m^7G detection has a history as well as tRNA sequencing. Initially, Wintermeyer and Zachau described a specific chemical method in which m^7G detection is achieved via aniline-induced cleavage of the tRNA strand by β-elimination after additional treatment under alkaline conditions or after its reduction by sodium borohydride ($NaBH_4$) in tRNA [32,33]. By combining of the aniline cleavage method and the Donis-Keller-method which uses ribonucleases [34,35], it is possible to identify the position of m^7G in tRNA. These methods are utilized for m^7G detection not only in tRNA but also in rRNA [36]. In the Donis-Keller-method, RNsase T1 is used for detection of guanosine position. Although RNase T1 specifically digests the phosphodiester bond of guanosine-3' phosphate in ribonucleic acid and ribonucleotide, the 7-methyl modification o guanosine prevents RNase T1 cleavage. Additionally, in pre-tRNA containing m^7G at the 5'-end of the acceptor stem, the m^7G modified nucleoside absolutely prevents cleavage by M1 RNA, the catalytic RNA subunit of RNase P [37]. Kuchino's post labeling method, which is a method which results in tRNA fragments upon formamide treatment and subsequent analysis of the nucleotide at the 5' end by two-dimensional thin-layer chromatography, allowed faster sequence analysis of the

modified nucleotides of tRNA [38]. In addition, antibiotics which specifically target m^6A and m^7G were prepared by immunization of rabbits with nucleoside conjugates of bovine serum albumin (m^6A-BSA, m^7G-BSA) [39]. Both the anti-m^7G and anti-m^6A antibody adsorbents became a tool for fractionation of oligonucleotides and nucleic acids. Currently, not only the m^7G modification but also a variety of other modified nucleosides can be detected more quickly and accurately by mass spectrometry or high performance liquid chromatography [40].

The enzymatic activity of tRNA (m^7G46) methyltransferase was initially confirmed in cell extracts from Escherichia coli [41] and has been purified more than 1000 fold [42]. The enzymatic activities have also been detected from Salmonella typhimurium [43,44], Thermus flavus [45], Xenopus laevis [46], human [47], and plant [9]. The m^7G46 modification is generated by tRNA (m^7G46) methyltransferase [tRNA (guanine-7-)- methyltransferase, EC 2. 1. 1. 33; TrMet (m^7G46)] [48,49]. The gene encoding tRNA (m^7G46) methyltransferase was first identified in yeast, and shown to be composed of two protein subunits Trm8 and Trm82 encoded by YDL201w and YDR165w, respectively [50]. Although Trm8 is the catalytic subunit, formation of a complex with Trm82 is required for the enzymatic activity [51]. Following this report, eubacterial genes have also been identified as trmB, whose classical name is yggh, in Escherichia coli [52], Aquifex aeolicus [53], and Bacillus subtilis [54] (Table 1).

In this review, information of tRNA m^7G modifications and tRNA m^7G methyltransferases since m^7G was discovered in tRNA are summarized, and the differences in reaction mechanism between tRNA m^7G methyltransferase and rRNA or mRNA m^7G methylation enzyme are discussed.

2. Structural analyses and catalytic mechanisms of m^7G methyltransferases

Structural studies of tRNA modification enzymes can be informative both to the specificity and catalytic mechanism of the enzymes. Furthermore, the combination of structural and biochemical analysis data allows comparison of reaction mechanisms from different species. This makes it possible to infer information about molecular evolution. The crystal structures of tRNA (m^7G46) methyltransferase from *B. subtilis* [54], *E. coli* [55], *Streptococcus pneumoniae* (PDB: 1YZH), and *S. cerevisiae* [56] have been reported (Table 1). X-ray crystallographies of TrmB and Trm8 have revealed a classic class I AdoMet-dependent methyltransferase structures. These AdoMet-dependent tRNA MTases belongs to two protein super families, which are structurally and phylogenetically unrelated, namely the Rossmann fold MTases (RFM) and SPOUT MTases (SpoU and TrmD) [57]. SPOUT MTases have a deep trefoil knot structure which forms the catalytic site and the cofactor-binding pocket [57–59]. TrmB and Trm8 belongs to the RFM family of MTases [52,56].

2.1. Eubacterial tRNA m^7G46 methyltransferases (TrmB)

Eubacterial TrmB exists as either a single subunit or a homodimer. TrmB of *B. subtilis* and *S. pneumoniae* have a dimeric structure both in solution and in the crystal form. However, dimerization does not seem to be a common feature of the enzymes, as the interface is not conserved and the TrmB enzymes of *E. coli* and *A. aeolicus* are monomeric. Analysis of TrmB activity using mutant proteins based on bioinformatic studies has revealed residues important for function [60]. The thermophilic TrmB has a longer C-terminal region compared to the methophilic TrmB [53,61] (Figure 3B). It has been reported that the C-terminal region is required for protein stability at high temperatures and contributes to the selection of the precise guanine nucleotide (i.e. G46) to be modified [61]. Alanine substitution of Arg287 in the long C-terminal region considerably reduces the methyltransfer activity. Thus, a part of the C-terminal region may make contact with tRNA. In contrast, the methophilic TrmB proteins from *E. coli* and *B. subtilis* have a long N-terminal region, and it has been shown that Arg26 in *E. coli* TrmB is involved in activity [60]. Furthermore, when Asp133 is replaced by alanine or asparagine in *A. aeolicus* TrmB, the methyltransfer activity is completely lost [61]. The aspartic acid residue is highly conserved in both TrmB and Trm8. Therefore, the Asp133 of *A. aeolicus* TrmB is considered as the catalytic center. In a docking model of a guanine base and *E. coli* TrmB, the aspartic acid is observed in the vicinity of the 1 atom of guanine [55]. Furthermore, in a docking model of guanine and the Trm8-Trm82 complex, the corresponding aspartic acid has the same positional

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relationship [56]. For this reason, it is suggested that Asp133 of *A. aeolicus* TrmB captures the G46 base of tRNA. Mutagenesis study has shown that Asp133 may contribute to AdoMet binding. Substitution of Thr132 by alanine, cysteine, or serine increases the *Km* values and/or decreases the *Vmax* values for AdoMet. Additionally, the kinetic studies have demonstrated that several amino acid residues are important for AdoMet binding, including Arg108 and Thr165 (Figure 4A). Taken together, it has been proposed a hypothetical mechanism for TrmB in which the carboxyl group of Asp133 captures the proton of N-H of the guanine base and the 7 atom of the guanine base itself attacks the methyl group in AdoMet [62] (Figure 4B). A hypothetical catalytic mechanism for TrmI which is a methyltransferase for the 1 atom of adenosine at position 58 in tRNA, has also been proposed [63]. In this mechanism, the 1 atom causes a nucleophilic attack on the methyl group of AdoMet. Since the reactivity of nitrogen atoms in the bases is generally higher than the reactivity of carbon and oxygen atoms [49], in some methyl group transfer reactions, the nitrogen atom itself seems to directly attack the methyl group of AdoMet.

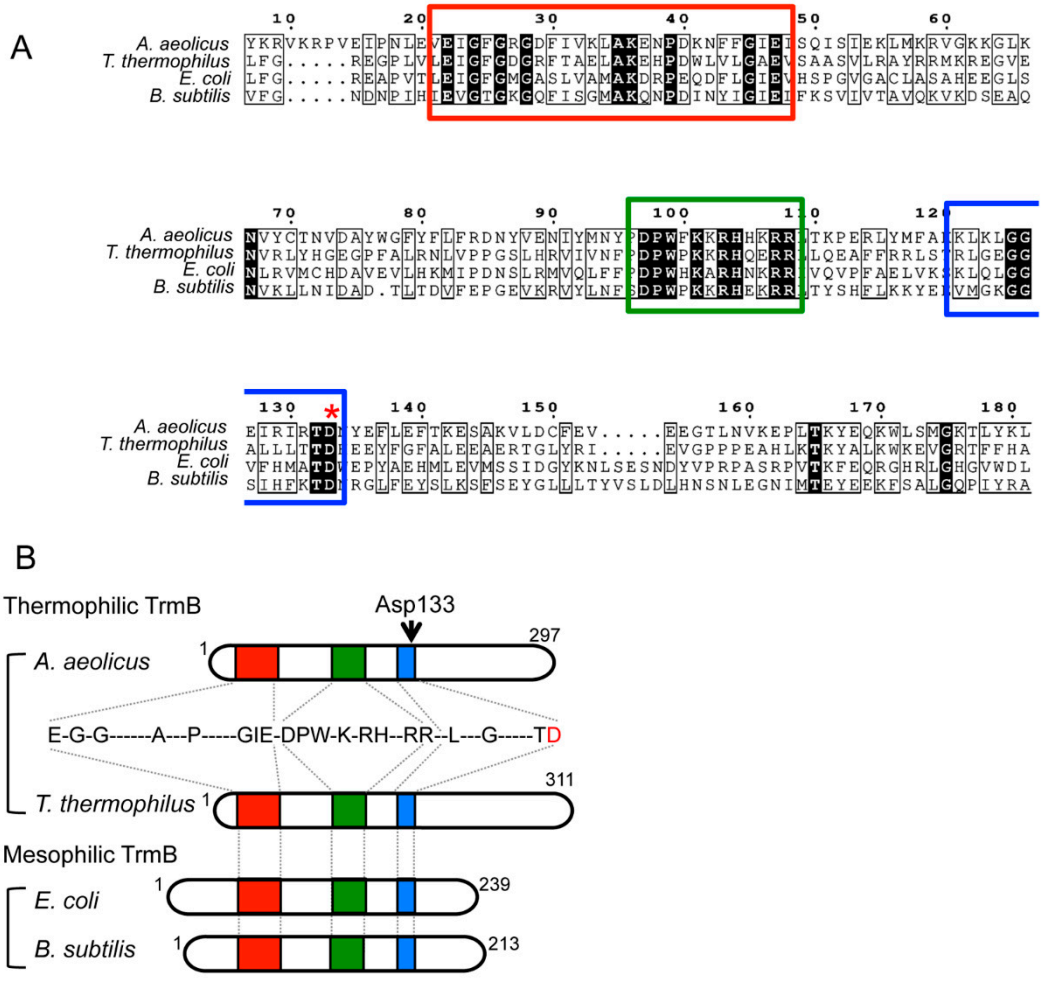

Figure 3. Comparison of thermophilic and methophilic TrmB (A) Sequence alignment of TrmB. Conserved regions are highlighted in three squares colored squares (red, green, blue). Asp133 is indicated by an asterisk. (B) Thermophilic and mesophilic TrmB proteins are illustrated schematically. The three colored regions correspond to the amino acid sequences in panel A. Thermophilic TrmB has a distinct long C-terminal region. Asp133 highlighted by an arrow.

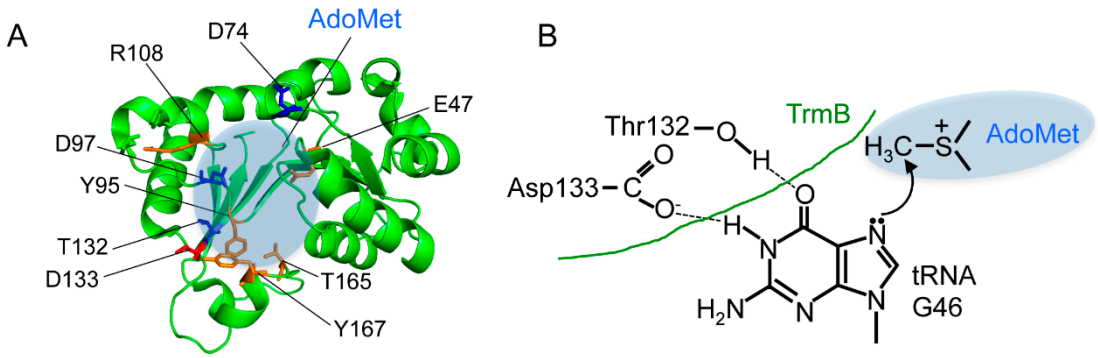

Figure 4. The amino acid residues for AdoMet binding and hypothetical reaction mechanism of TrmB (A) The amino acid residues (E47, Y95, R108, T165 and Y167) involved in AdoMet binding (orange) are indicated on the catalytic domain of *B. subtilis* TrmB structure (PDB:2FCA). The hypothetical catalytic center (grey shading), Asp133 (red) and the other important amino acid residues (D74, D97 and T132; blue) are highlighted. (B) Hypothetical reaction mechanism of eubacterial TrmB proteins is drawn.

2.2. Heterodimeric tRNA m⁷G methyltransferase of yeast (Trm8/Trm82)

Yeast Trm8/Trm82 proteins are unrelated and have no homology each other. This two proteins complex is conserved in eukaryotes. The homologous human proteins METTL/WDR4 complement Trm8/Trm82 in yeast [50]. Structural analysis demonstrated that Trm82 protein is a member of the WD fold family. Trm82 adopts a β propeller fold and contains seven blades [56]. It is possible that Trm82 might be a target of other protein partners that regulate the Trm8/Trm82 complex, since β propeller interacting proteins often interact with the top of the β propeller. In yeast, two-subunit tRNA methyltransferases have been identified. There are Trm11-Trm112 (m^2G10), Trm7-Trm732 (Cm32), Trm7-Trm734 (Nm34), Trm9-Trm112 (mnm^5U and mcm^5s^2U), and Trm6-Trm61 (m^1A58) (Modified nucleosides are in parentheses)[64–68]. Trm112, which is a small protein and conserved in all three domains of life, interacts and activates four methyltransferases, Bud23, Trm9, Trm11 and Mtq2. The targets of these methyltransferase are components of different parts of the translation machinery, namely rRNA, tRNAs, and release factors [69]. These complexes are composed of a methyltransferase catalytic subunit and a noncatalytic subunit. The noncatalytic subunits are involved in stabilizing the catalytic subunit or activating and fine tuning the activity. By using a wheat germ cell free system we have expressed Trm8 and Trm82 from their respective mRNAs. Separate expression of Trm8 and Trm82 proteins and their subsequent mixing resulted in proteins with no activity. However, active Trm8/Trm82 heterodimer was synthesized when mRNAs of both Trm8 and Trm82 were co-translated [70]. These results strongly suggest that the association of the Trm8 and Trm82 subunits is translationally controlled in living cells.

It has been suggested that the manner of tRNA binding to eukaryotic tRNA m^7G methyltransferase Trm8 is different from that in eubacterial TrmB [56]. This proposal is consistent with the results of biochemical studies. For methylation in the yeast Trm8/Trm82 complex, it is important between the D-loop and T-loop interaction while the aminoacyl-stem does not contact either Trm8 or trm82 [71]. In contrast, for eubacterial TrmB methylation, the most important site is located on the T-arm, and the tertiary base pairs in the tRNA three-dimensional core are not essential [53]. Thus, yeast Trm8/Trm82 has stricter recognition requirements for substrate tRNA than eubacterial TrmB.

2.3. m⁷G methyltransferases other than tRNA m⁷G methyltransferase

TrmB and mRNA cap-m^7G methyltransferase (Abd1) have different targets for methylation. In the catalytic center of TrmB, the amino acid residues present differ totally from those in the catalytic center of Abd1. Thus, the reaction mechanism of TrmB is expected to differ from that of Abd1 [60,72]. The m^7G methyltransferase of vaccinia virus mRNA capping enzyme is also a heterodimeric protein

composed of the vD1 subunit and the vD12 subunit. The vD1 subunit constitutes an autonomous functional unit containing both the RNA triphosphate and RNA guanyltransferase activities [73,74]. The m^{7}G methyltransferase domain is heterodimerized with a stimulatory vD12 subunit [75,76]. An allosteric mechanism, whereby the vD12 subunit enhances the affinity of the catalytic vD1 subunit for AdoMet and the guanine acceptor, has been proposed. It has been shown that the catalytic subunits of vD1, as well as the yeast mRNA capping enzyme Abd1, are unrelated to Trm8. Furthermore, from sequence analysis, the noncatalytic subunit vD12 is not structurally related to Trm82. With respect to the relationship with rRNA m^{7}G methyltransferase, neither Trm8 nor Trm82 have significant similarity to KgmB [the kanamycin-gentamicin resistance methylase, 16S rRNA (m^{7}G1405) methyltransferase] from *Streptomyces tenebrarius*, a rRNA m^{7}G methyltransferase associated with aminoglycoside resistance [26,27], other than the methyltransferase domain shared by numerous methyltransferases [77]

3. Physiological functions

A large number of tRNA modifications have important roles in tRNA function [78]. In particular, tRNA modifications in the anticodon region play a major role in translation and growth [79]. The role of many tRNA modifications outside of the anticodon region are considered auxiliary to correct structure formation and fine tuning of the translation because it hardly appears as phenotypic defects [78,79]. Because of this, information about the role of the m^{7}G46 modification in tRNA was limited for a long time, even though the modification is widely found in eubacteria and eukaryotes. However, clarification of the function of m^{7}G46 in tRNA has begun over the past decade.

3.1. tRNA m^{7}G46 modification in yeast

TrmB gene disruption in *E. coli* demonstrated no phenotypic defects [52]. Although tRNA m^{7}G46 methylation requires both Trm8 and Trm82 in yeast, phenotypic analysis of double deletion mutant strain of *trm8* and *trm82* also has not shown detectable phenotypes [50]. In restrictive nutrient conditions, however, it has been reported that a double mutant strain lacking both *trm8* and *trm82* yeast strains are temperature-sensitive in synthetic media containing glycerol as the sole carbon source [80]. This conditional temperature sensitivity is the first example of a physiological function for m^{7}G modification for tRNA. Since this report, various double gene deletion mutants of yeast have shown phenotypes. Hypo-modified mature tRNA^{Val}(AAC) deacylates and degrades rapidly in a double deletion mutant strain of *trm8* and *trm4* (*Δtrm4Δtrm8*), (Trm4 is a methyltransferase for 5-methyl cytidine at positions 34, 40, 48, and 49 in tRNA) at 37℃, resulting in a temperature sensitive phenotype [81]. The temperature-sensitivity indicates that it relates rapid tRNA decay (RTD) pathways. Deletion of MET22, which likely regulates the 5'-3' exonuclease Rat1 and Xrn1 activity indirectly, prevents the tRNA^{Val}(AAC) degradation in *Δtrm4Δtrm8* strain. In this mutant strain, the tRNA^{Val}(AAC) restores its aminoacylation and the growth defect is rescued. Thus, the RTD is mediated by Met22 and the 5'-3' exonuclease Rat1 and Xrn1 [82]. Hypomodified tRNA, such as tRNA^{Val}(AAC) from a *Δtrm4Δtrm8* mutant, is subject to degradation by RTD. However, stability of tRNA^{Val}(AAC) is restored, upon overexpression *TEF1* and *VAS1*, which encode the elongation factor *eEF1A* and valyl-tRNA synthetase respectively, and which protect the hypomodified tRNA by direct interaction [83]. In addition, Maf1 indirectly affects maturation of tRNA precursor [84]. In yeast, Maf1 is a negative regulator of Pol III which mediates several signaling pathways [85,86]. Maf1 inhibits tRNA transcription via a mechanism dependent on phosphorylation and nuclear accumulation of Maf1, followed by physical association with Pol III in the tRNA genes. In a *Δtrm4Δtrm8* mutant, inhibition of Pol III activity reduces degradation of tRNA^{Val}(AAC). Thus, reduction of tRNA transcription prevents degradation of hypomodified tRNA [83]. Surveillance of RNA quality and clearance of aberrant tRNA is important in all organisms. As with the study of *Δtrm4Δtrm8* mutant, mature tRNA^{Ser}(CGA) and tRNA^{Ser}(UGA) are degraded at 37℃ in strains lacking Um44(2'-*O*-methyluridine) and ac^{4}C12(4-acetylcytidine) which are a result of Trm44 and TAN1 activity, respectively, resulting in a temperature-sensitive phenotype [87]. In HeLa cells, initiator tRNA [tRNA(iMet)] is degraded at high temperature with the degradation dependent upon

Xrn1/2 nucleases. However, the levels of not only m^7G46 but also other modified nucleosides in tRNA are not significantly changed. The problem of the structural stabilization of the acceptor and T-stem in tRNA(iMet) in HeLa cells has not been clarified [88] .

3.2. tRNA m^7G46 modification in thermophilic eubacteria

In comparison with these eukaryotic enzymes, there is limited information about eubacterial enzymes. However, a part of the physiological importance of the m^7G modification in tRNA has been revealed. We focused on characterization of a thermophilic *trmB* gene disruptant (*ΔtrmB*) strain of *Thermus thermophilus* HB8. *T. thermophilus* is an extreme thermophilic eubacteria and can live at wide range of temperatures, from 50 to 83℃. A combination of distinct tRNA modifications, namely Gm18, m^5s^2U54, and m^1A58, increase the tRNA melting temperature by nearly 10℃ compared with unmodified tRNA transcript [89–91]. These modifications do not affect translational fidelity under 65℃, and the rate of the modification of tRNA is very low in cells cultured at 50℃. The levels of these modified nucleosides in tRNA control translation via tRNA flexibility [92]. However, the mechanism by which modifications are controlled remained unknown until the beginning of the 21st century. When the *trmB* gene was disrupted, the introduction ratio of Gm18, m^5s^2U54, and m^1A58 was dramatically changed, and the melting temperature of the hypo-modified tRNA decreased. In particular, degradation of $tRNA^{Phe}$ and $tRNA^{Ile}$ was detected. Furthermore, protein synthesis was depressed in the *ΔtrmB* strain at 70℃, and exhibited a severe growth defect at 80℃ [93]. Above 65℃, m^7G functions as a marker of precursor tRNA and increases the reaction rate of other modification enzymes. In contrast, at low temperature (50℃), Ψ55 decreased the rate of formation of Gm, m^5s^2U54, and m^1A58 and controls structural rigidity of tRNA [94]. In addition, the protein synthesis activity of the tRNA Ψ55 synthase (*truB*) disruptant strain was lower than that of the wild-type strain and cold-shock proteins were absent in *ΔtruB* cells at low temperature [94]. Thus, the m^7G46 and Ψ55 modifications work as an accelerator and a brake, respectively [95]. Therefore, these tRNA modification networks regulate the degree of modifications in response to temperature, and the response of the network to environmental change is very rapid. This is a typical strategy of eubacteria whose genome size is limited.

3.3. Involvement of tRNA m^7G46 modification in fungal pathogenicity

The first report of the relationship between tRNA modification enzymes and fungal pathogenicity was by Takano *et al.* who showed that the tRNA m^7G46 modification is required for plant infection by the phytopathogenic fungus *Colletotrichum lagenarium*, the cause of cucumber anthracnose [96]. *aph1* (Appressorial Penetration into Host) is required for efficient tRNA m^7G46 modification in *C. lagenarium*, and experiments with *aph1* gene knockout mutants suggest that Aph1 is required for appressorium-mediated host invasion and also has important roles in resistance to several stresses including the basic defense response of the host plant. Given that in addition to m^7G46 there are other tRNA modifications which are related to infection [6], tRNA modification and tRNA modification enzymes are likely be an important factor in the relationship between host and infectious organisms.

3.4. Involvement of tRNA m^7G46 methyltransferase in diseases

Since tRNA modification regulates protein synthesis, there are several reports on the relationship between tRNA modification and genetic disease. METTL1 and WDR4 are the human homologue of Trm8 and Trm82, respectively. METTL1 has been initially identified as a substrate of protein kinase Bα, and METTL1 phosphorylated at Ser27 is inactive [51]. Also, NSUN2 (NOP2/Sun domain family, member 2) is the mammalian ortholog of yeast Trm4 and has been identified as a substrate of protein kinase (Aurora-B) in HeLa cells [97]. As mentioned above, the yeast tRNA modification enzymes, Trm8 and Trm4, are required to protect tRNA from RTD. Moreover, in a yeast *Δtrm8* strain, it has been observed that the cytotoxic effect of 5-fluorouracil (5-FU) is enhanced by heat stress [98]. 5-FU, which is a pyrimidine analogue, is a widely used chemotherapeutic agent for

treatment of solid cancerous tumours. However, increasing doses of 5-FU causes serious side effects. In order to reduce the side effects of 5-FU treatment, it is necessary to consider a new strategy. The observation of the influence of 5-FU in the yeast *Δtrm8* strain leads to the hypothesis that non-essential tRNA modifications catalyzed by NSUN2 and METTL1 might affect the efficiency of 5-FU in human cancer cells. In fact, Okamoto *et al*. have shown that a knockdown strain of both NSNU2 and METTL1 genes drastically increases sensitivity to 5-FU in HeLa cells [99]. NSNU2 and METTL1 are phosphorylated by Aurora-B and protein Akt1/kinase Bα, respectively, and the enzymatic activities of NSNU2 and METTL1 are suppressed by phosphorylation. Therefore, constitutive overexpression of both dephosphorylated tRNA methyltransferases can repress 5-FU sensitivity. Thus, NSUN2 and METTL1 are involved 5-FU sensitivity in HeLa cells.

With respect to homologues of Trm82, in recent years, various important points have come out to light. In Drosophila, the gene wh (wuho) which has WD40 repeats is a homologue of Trm82 and is essential for spermatogenesis and has a critical function in cellular differentiation for germline cells during gametogenesis [100]. In human, the WDR4 gene mutation abolishes the m^7G46 modification in tRNA and causes microcephalic primordial dwarfism characterized by facial dysmorphism, brain malformation, and severe encephalopathy with seizure. Amino acid substitution of WDR4 has been revealed as the cause mutation [101,102]

The molecular mechanism of microcephaly has not been well understood. In order to profile the m^7G tRNA methylome in mouse embryonic stem cell (mESCs), two independent methods have been developed: m^7G methylated immunoprecipitation sequencing (MeRIP-seq) and tRNA reduction and cleavage sequencing (TRAC-seq) [103]. In mESCs, the global m^7G tRNA methylome is essential for appropriate translation of cell cycle genes and genes associated with brain malformation. Further to this observation, Mettl1 or Wdr4 knockout causes defective mESC self-renewal and neural lineage differentiation [103]. This study has clearly demonstrated the tRNA m^7G methylome in mammals and shown the critical nature of METTL1 and WDR and m^7G modification in regulation of stem cells and disease.

4. Perspective

Since m^7G was found in tRNA, the genes encoding tRNA m^7G methyltransferase have been identified in several organisms and amino acid residues key to the reaction mechanism have been identified [56,60,62,80]. In addition, primitive quality control systems, resulting from tRNA m^7G46 modification have been demonstrated [81,93]. Furthermore, the physiological functions of m^7G46 in tRNA have started to be determined over the past decade [99–103]. There seems to be little commonality between tRNA (m^7G46) methyltransferase, rRNA m^7G methyltransferase, and cap-m^7G methyltransferase as far as the active centers are concerned. Recently we have proposed a reaction mechanism for TrmB [62]. However, catalytic mechanism structural studies on AdoMet and/or the tRNA bound form of m^7G methyltransferases are still necessary to fully understand the catalytic machinery. Also, genes encoding the tRNA m^7G methyltransferase responsible for m^7G at position 49 in archaeal tRNA and for anticodon m^7G of mt tRNA or chloroplast tRNA have not yet been identified. From the viewpoint of m^7G molecular evolution, it is important that reaction mechanisms of m^7G methyltransferases from different substrates and various organisms are compared and analyzed.

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