Published online 18 January 2018

Nucleic Acids Research, 2018, Vol. 46, No. 7 ***e37***
doi: 10.1093/nar/gky013

Selective terminal methylation of a tRNA wobble base

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Received August 14, 2017; Revised December 19, 2017; Editorial Decision January 04, 2018; Accepted January 09, 2018

ABSTRACT

Active tRNAs are extensively post-transcriptionally modified, particularly at the wobble position 34 and the position 37 on the 3′-side of the anticodon. The 5-carboxy-methoxy modification of U34 (cmo^{5}U34) is present in Gram-negative tRNAs for six amino acids (Ala, Ser, Pro, Thr, Leu and Val), four of which (Ala, Ser, Pro and Thr) have a terminal methyl group to form 5-methoxy-carbonyl-methoxy-uridine (mcmo^{5}U34) for higher reading-frame accuracy. The molecular basis for the selective terminal methylation is not understood. Many cmo^{5}U34-tRNAs are essential for growth and cannot be substituted for mutational analysis. We show here that, with a novel genetic approach, we have created and isolated mutants of *Escherichia coli* tRNA^{Pro} and tRNA^{Val} for analysis of the selective terminal methylation. We show that substitution of G35 in the anticodon of tRNA^{Pro} inactivates the terminal methylation, whereas introduction of G35 to tRNA^{Val} confers it, indicating that G35 is a major determinant for the selectivity. We also show that, in tRNA^{Pro}, the terminal methylation at U34 is dependent on the primary m^{1}G methylation at position 37 but not *vice versa*, indicating a hierarchical ranking of modifications between positions 34 and 37. We suggest that this hierarchy provides a mechanism to ensure top performance of a tRNA inside of cells.

INTRODUCTION

Transfer RNAs (tRNAs) are fundamental for translation of the genetic code. Although these nucleic acids are transcribed with only four nucleotides (A, C, G, U), the diversity of the four building blocks is substantially expanded by post-transcriptional modifications, which enhance the structure and activity of the L-shaped molecules in all living cells (1). Collectively, more than 100 different chemical moieties have been introduced to modify tRNAs in all three domains of life (2). Each chemical moiety is created after transcription by an enzyme or a pathway of enzymes. Most of these modifying enzymes target position 34 of tRNA, the wobble position of the anticodon, or position 37, on the 3′-side of the anticodon, to produce chemical moieties that improve the quality of decoding. Although the cellular genomic space dedicated to genes for tRNA modification enzymes is large, few of these enzymes are understood at the mechanistic level. A critical barrier to progress is the lack of the ability to isolate tRNA molecules to study modifications, particularly when a tRNA is required for growth and cannot be readily changed with mutations for enzymatic analysis. Another critical barrier is the insufficient understanding of the inter-dependence between positions 34 and 37 when each harbors a distinct modification. Little is known whether the two modifications are independent of each other or whether one determines the activity of the other. Overcoming these critical barriers is an important step forward to address the biology of tRNA in living cells.

We focus on the cmo^5 modification of the U34 wobble base in Gram-negative tRNAs (3), which is associated with isoacceptors specific for six amino acids: Ala, Ser, Pro, Thr, Leu and Val (4,5). While the unmodified U34 can read all four nucleotides in bacteria (6–10), the cmo^5 modification improves the quality of reading each (11–15) while the additional s^2 modification restricts the reading specificity to A, G and less so U (16). The cmo^{5}U modification is synthesized *via* multiple enzymatic reactions (Figure 1A). First, U34 is hydroxylated at the 5-position to form 5-hydroxyU (ho^{5}U34) by an as-yet unknown enzyme (15). Second, ho^{5}U34 is converted to cmo^{5}U34 by the combined action of two *S*-adenosyl methionine (AdoMet)-dependent enzymes (17). Specifically, CmoA transfers the carbon dioxide of prephenate to the sulfonyl methyl of AdoMet to generate carboxy-*S*-AdoMet (Cx-AdoMet), and subsequently CmoB transfers the carboxy-methyl of Cx-AdoMet to the 5-hydroxy of ho^{5}U34 to synthesize cmo^{5}U34. Because prephenate is derived from chorismate, biosynthesis of cmo^{5}U is linked through chorismate to biosynthesis of

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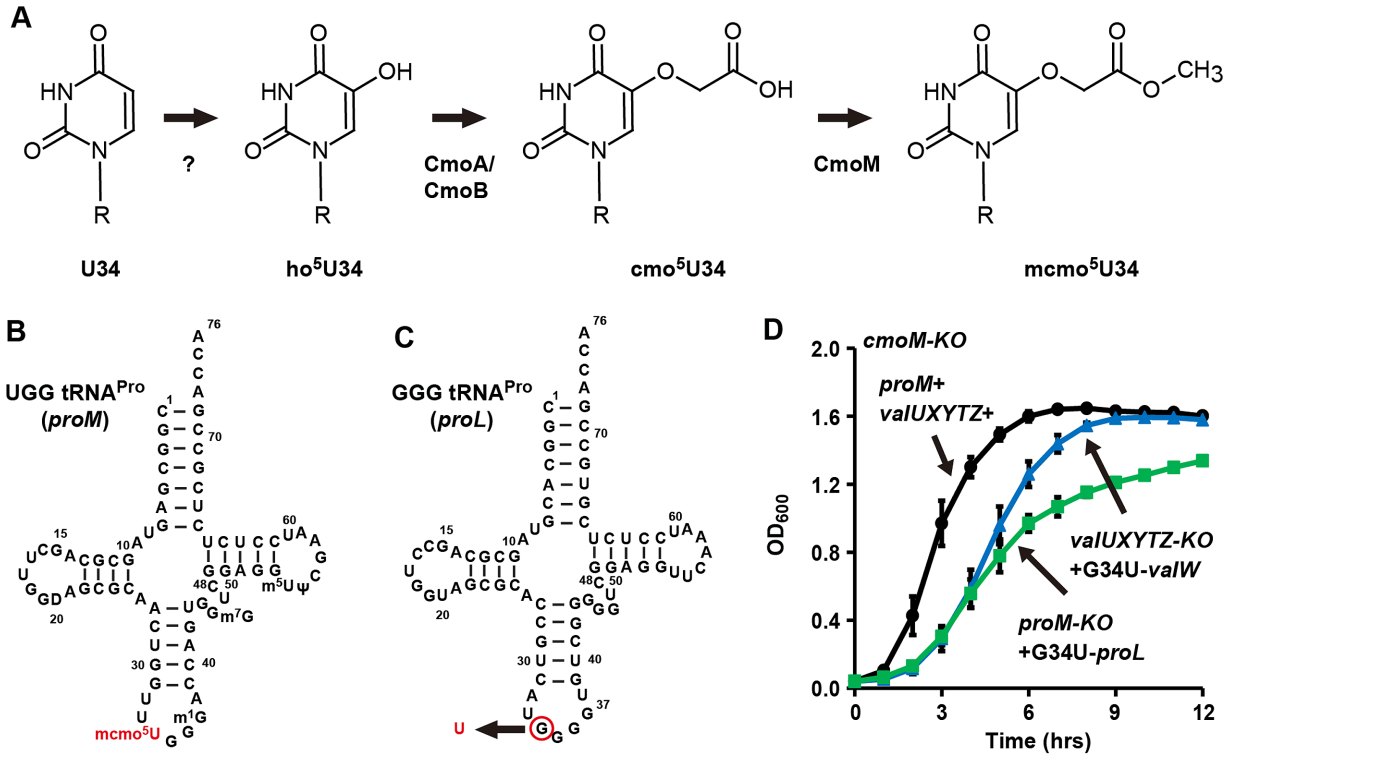


Figure 1. The maturation of mcmo^{5}U34 in *E. coli* tRNA. (**A**) Chemical structures in the pathway of modifying U34 to ho^{5}U34 to cmo^{5}U34 and to mcmo^{5}U34 catalyzed by enzymes. The symbol '?' indicates unknown. (**B**, **C**) Sequence and cloverleaf structure of *E. coli* UGG tRNA^{Pro} and GGG tRNA^{Pro} encoded by the *proM* and *proL* genes, respectively. The wobble base in each is shown in red and the G34U mutation in the *proL* gene to generate a chimeric tRNA is shown in an arrow. (**D**) Growth analysis of *E. coli* JM109 *cmoM-KO* cells, showing that expression of the maintenance chimeric tRNA^{Pro} in *proM-KO* cells was sufficient to promote growth (green), albeit slower relative to cells containing chromosomal *proM* (black), while growth of cells maintained by the chimeric tRNA^{Val} (blue) was only slightly slower relative to cells containing *valUXYTZ* genes on the chromosome (black). Growth of cells containing all tRNA genes (*proM*+, *valUXYTZ*+, black), or lacking *valUXYTZ* and maintained by G34U-*valW* (blue), or lacking *proM* and maintained by G34U-*proL* (green) are shown. Errors are standard deviation (SD), $n = 3$.

aromatic amino acids and vitamins (18,19). Interestingly, the carboxyl of cmo^{5}U34 in four of the six families of tRNA isoacceptors (Ala, Ser, Pro, Thr) is further methylated to mcmo^{5}U34 (Figure 1A) in a terminal methyl transfer reaction catalyzed by CmoM (5). The presence of the methyl ester in mcmo^{5}U34 further ensures reading frame accuracy by suppressing +1-frameshifts (5). However, the selectivity for four out of six families of tRNAs by CmoM is not understood at the molecular level, thus limiting our insight into the cmo^{5}U34 pathway.

The four tRNA families with the terminal methyl group in mcmo^{5}U34 share G35 in the middle position of the anticodon, whereas the two without the methyl group share A35 in common (Supplementary Figure S1). This raises the hypothesis that G35 is a major determinant for recognition by CmoM. In the crystal structure of the ribosomal 30S subunit in complex with an A-site-bound anticodon–stem–loop of tRNA^{Val}, which lacks the terminal methylation, the carboxylate of the unmethylated cmo^{5}U34 in tRNA^{Val} interacts with the N^6 of the neighboring base A35 to stabilize the wobble pairing with all four bases at the third position of the codon (14). If A35 is replaced with G35, the N^6 is replaced with an O^6, which would block the interaction. The addition of a methyl ester to the cmo^5 moiety in the form of mcmo^{5}U34 would neutralize the repulsion between the O^6 and the negative charge of the carboxylate, thus providing a basis for the hypothesis that the terminal methylation is necessary for stabilizing the pairing with anticodons that contain G35. However, testing and validating the hypothesis has not been done and the difficulty is associated with at least two reasons. First, the substrate for CmoM, cmo^{5}U34-tRNA, must be isolated from cells in a *cmoM*-knockout (*cmoM-KO*) background. An *in vitro* reconstitution of the cmo^{5}U34 substrate from a tRNA transcript is not yet possible due to the lack of information on the enzyme that synthesizes ho^{5}U34. Second, tRNA isoacceptors harboring cmo^{5}U34 are usually essential for cell growth (e.g. those for Ser, Leu, Pro and Thr) and cannot be readily made with a site-specific substitution inside of cells without compromising cell survival.

We show here the development of a genetic approach (Supplementary Figure S2) that enables isolation of mutants of *Escherichia coli* tRNA^{Pro} and tRNA^{Val} with a site-specific substitution at position 35 of the anticodon. This approach is robust and amendable to generate sufficient quantities of tRNA for detailed biochemical and mechanistic studies. It is broadly applicable to isolate mutants of other tRNA species that are essential for growth. Using this approach, we show that substitution of G35 in the UGG isoacceptor of tRNA^{Pro} (UGG tRNA^{Pro}) abolishes the terminal methyl transfer by CmoM, while introduction of G35 to the UAC isoacceptor of tRNA^{Val} (UAC tRNA^{Val}) con-

fers the activity, supporting the notion that G35 is a major determinant for tRNA recognition by CmoM. Additionally, using $tRNA^{Pro}$ isolated from this approach, we explore the inter-dependence of the primary m^1G methylation at position 37 present in this tRNA (20) and the terminal methylation at position 34. We show that the m^1G37 methylation is important for the terminal methylation at position 34 but not *vice versa*, providing new insight into the ranking and ordering of the two modifications in the cellular production of a functional tRNA.

MATERIALS AND METHODS

tRNA plasmids

All primers for creating tRNA plasmids are listed (Supplementary Table S1). The maintenance plasmid for expression of a chimeric tRNA was based on pACYC184. The gene for the G34U mutant of the isoacceptor GGG $tRNA^{Pro}$ (G34U-*proL*, Supplementary Figure S3) with the *lpp* constitutive promoter and the *rrnC* transcription terminator sequence was isolated from a derivative of pGFIB (21,22) as a PvuII fragment and cloned into the EcoRV site of pACYC. The G34U mutation was created with Quikchange (Agilent) using primers GGGtoUGG-F and GGGtoUGG-R. The gene for the G34U mutant of the isoacceptor GAC $tRNA^{Val}$ (G34U-*valW*, Supplementary Figure S4) was created by hybridization of a pair of oligos GACtoUAC-F and GACtoUAC-R, inserted into the EcoRI and PstI sites of pGFIB, isolated as a PvuII fragment with the *lpp* promoter and *rrnC* terminator sequence, and cloned into the EcoRV site of pACYC184. The *Bacillus subtilis* $tRNA^{Cys/GCA}$ (*BscysT*) was amplified from pTFMA (23) using primers BscysT-F and BsCysT-R and cloned into pGFIB at EcoRI and PstI sites. The PvuII fragment encoding the gene, the *lpp* promoter, and the *rrnC* terminator, was sub-cloned to pACYC184 at the EcoRV site to generate a *cysT-KO* maintenance plasmid.

The test plasmid for expression of a wild-type (WT) or a mutant tRNA was based on pKK223–3. The gene for UGG $tRNA^{Pro}$ (encoded by *proM*) or UAC $tRNA^{Val}$ (encoded by identical genes *valUXY* and *valTZ*) was cloned into the EcoRI and PstI sites. Anticodon mutants were created by Quikchange with primers UGGtoUAG-F and UGGtoUAG-R for $tRNA^{Pro}$ and primers UACtoUGC-F and UACtoUGC-R for $tRNA^{Val}$. The amber suppressor mutant of *E. coli cysT* on pGFIB was made by Quikchange using primers GCAtoCUA-F and GCAtoCUA-R, which changed the anticodon from GCA to CUA.

E. coli strains

All of the *E. coli* strains in this study are listed (Supplementary Table S2). The *cmoB-KO* locus of *E. coli cmoB-KO* strain (from Dr. Steven Almo) was transferred to the published *E. coli trmD-KO* strain maintained by *trm5* (24). After transfer, the kanamycin marker (kan^R) of the *cmoB-KO* locus was removed by the flippase recombinase (FLP) encoded in pCP20 (25). To construct the vehicle for expression of a mutant version of UGG $tRNA^{Pro}$ (Supplementary Figure S3), *E. coli* JM109 was used as the host and transduced with the P1 lysate of an *E. coli cmoM-KO* strain (Coli Genetic Stock Center, Yale University) and removed of the kan^R of the *cmoM-KO* locus by FLP. The resulting JM109-*cmoM-KO* strain was introduced with the maintenance plasmid expressing the chimeric G34U-*proL*. The strain was then deleted of the UGG $tRNA^{Pro}$ gene, using the λ Red recombinase of pKD46 (26) to catalyze homologous recombination at the *proM* locus with a *proM-kan*^{R} PCR fragment amplified by primers proMKO-F and proMKO-R from pKD4. The kan^R for selection of the resultant JM109-*cmoM-KO-proM-KO* strain was removed by FLP. The resultant strain was transformed with the test pKK223–3 plasmid with *proM* to generate the expression vehicle for a mutant version of *proM*.

To construct the expression vehicle of a mutant form of UAC $tRNA^{Val}$ (Supplementary Figure S4), the *E. coli* JM109-*cmoM-KO* strain above was introduced with the maintenance plasmid carrying the chimeric G34U-*valW*. *E. coli* JM109-*cmoM-KO* carries five genes for $tRNA^{Val}$ (*valUXY* and *valTZ*, each encoding the same sequence, and was made into two derivatives, one deleted of the cluster of *valUXY* (using primers valUXYKO-F and valUXYKO-R), and the other deleted of the cluster of *valTZ* (using primers valTZKO-F and valTZKO-R). The kan^R of the first resultant strain JM109-*cmoM-KO-valUXY-KO* was removed by FLP and the strain was transduced with the P1 lysate of the second resultant strain JM109-*cmoM-KO-valTZ-KO* to generate the JM109-*cmoM-KO-valUXYTZ-KO* strain. After removal of kan^R by FLP, the resultant strain was transformed with the test pKK223–3 plasmid carrying the gene for UAC $tRNA^{Val}$ as a vehicle for expressing a mutant version of the tRNA. To construct the *E. coli* $tRNA^{Cys/GCA}$(*cysT*)-*KO* (Supplementary Figure S5), the kan^R (amplified by PCR from pKD4) was used to target the *cysT* chromosomal locus in JM109 using primers cysTKO-F and cysTKO-R. After the resultant strain was introduced with a maintenance plasmid expressing *BscysT*, the *cysT* was removed by λ Red recombinase and the kan^R removed by FLP. This *cysT-KO locus*, together with the maintenance plasmid for expressing *BscysT* and a pGFIB-derived test plasmid for expressing the amber-reading form of *cysT*, was transduced into an *E. coli* XAC-1 strain with an internal amber codon in the chromosomal *lacZ* (21).

Growth assay

Strain JM109-*cmoM-KO-proM-KO* maintained by chimeric G34U-*proL*, strain JM109-*cmoM-KO-valUXYTZ-KO* maintained by chimeric G34U-*valW*, and strain JM109-*cmoM-KO-cysT-KO* maintained by *BscysT*, were cultured in LB medium overnight at 37°C. Cells were then inoculated into 25 ml LB at 1:100 at 37°C and the OD_{600} was monitored over time using Tecan Infinite M200 Pro plate reader (Tecan). The growth assay for strain JM109-*cmoM-KO-proM-KO* maintained by chimeric G34U-*proL*, harboring either pKK223–3-G35A-*proM* or an empty pKK223–3, was performed similarly except that the overnight culture was inoculated to 30 ml fresh LB to OD = 0.04 and 0.4 mM IPTG was added at $T = 2$ h. Overnight cultures of the two strains were also serially diluted and spotted on LB plates with Amp and Chl and 0.4 mM IPTG and incubated at 37°C.

X-gal assay

The *E. coli* strain XAC-1 *cysT-KO* maintained by pACYC184-*BscysT* was transformed with the pGFIB-*cysT* plasmid either with GCA or CUA anticodon, and streaked and grown on an M9 plate containing 20 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) at 37°C overnight. The amber suppression activity was monitored as the color development by the β-galactosidase-catalyzed hydrolysis of X-gal.

Recombinant proteins

The *E. coli cmoM* gene was amplified from *E. coli* MG1655 genomic DNA using primers cmoM-F and cmoM-R and cloned into pET28 (Novagen) at NdeI and NotI sites. This pET28-*cmoM* was transformed into *E. coli* BL21(DE3) and over-expressed for 4 h at 37°C in LB containing 0.1 mM IPTG. The dual 6xHis-tagged recombinant CmoM was purified with HisLink Protein Purification Resin (Promega) as described previously (5). The recombinant *E. coli* TrmD was prepared as described (27–29). The recombinant *E. coli* prolyl-tRNA synthetase (ProRS) and leucyl-tRNA synthetase (LeuRS) were purified as described (30).

Substrate tRNAs

All substrate tRNAs for this study were expressed from the *tac*-controlled pKK223–3 plasmid and isolated from *E. coli* cells. Each tRNA was pulled down from total RNA by an affinity resin coupled with an oligonucleotide complementary to a sequence of the target tRNA (24,31,32). For $tRNA^{Val}$, due to its partial sequence homology with UGC $tRNA^{Ala}$, two successive affinity purification steps were performed. The first used the affinity oligo Val-AP1 and the second used the affinity oligo Val-AP2 (Supplementary Table S1). The quality of UGG and UAG $tRNA^{Pro}$ in cmo^5U34- or $mcmo^5U34$-modified state is shown as a single band in denaturing gel analysis (Supplementary Figure S6A), indicating homogeneity.

WT $tRNA^{Pro}$ and $tRNA^{Val}$ were each purified from the over-expression plasmid pKK223–3 driven by the *tac* promoter in *cmoM-KO* cells. To isolate UGG $tRNA^{Pro}$ lacking m^1G37 but containing a distinct state of the wobble modification, we used *E. coli* strain MG1655 *trmD-KO* as the basis (24). The *trmD-KO* strain was made into *cmoB-KO* or *cmoM-KO* by P1 transduction and the kan^R was removed. Cells were then transformed with pKK223–3-*proM* and grown to saturation in an overnight culture with 0.2% (w/v) arabinose (Ara) to allow expression of *trm5*. Cells were then freshly diluted into Ara-free LB at 1:100 and grown for 3 h, followed by a second dilution into fresh Ara-free LB at 1:5 and growth for 2 h. After these two dilutions, cells were harvested and primer extension analysis showed that the cellular level of m^1G37-tRNA was below 5%.

Kinetic analysis

Methylation or aminoacylation was assayed at 37°C by monitoring the incorporation of [^{3}H-methyl] of AdoMet or [^{3}H]-amino acid to tRNA as acid precipitable counts (33–37). Aliquots were removed over time and acid-precipitable counts measured. Data for steady-state assays of V_o as a function of tRNA concentration were fit to the hyperbolic Michaelis–Menten equation [$y = m1 \times x/(m2 + x)$] (38), where $m1$ and $m2$ were k_{cat} and K_m, respectively. Data for single-turnover assays of a time course were fit to a single exponential equation [$y = m1 \times (1 - \exp(-m2 \times x))$] (38,39), where $m1$ and $m2$ were the plateau level and the k_{obs}, respectively. Data of k_{obs} as a function of enzyme concentration were fit to the hyperbolic Michaelis–Menten equation.

For CmoM methylation, steady-state assays of plateau levels were performed with 1.0 μM enzyme, 1.0 μM tRNA, and 20 μM [^{3}H-methyl]-AdoMet in a buffer of 50 mM HEPES–KOH (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, and 7 mM β-mercaptoethanol. Substrate tRNAs were isolated from a vehicle strain expressing *E. coli* WT or a mutant version of UGG $tRNA^{Pro}$ or UAC $tRNA^{Val}$. Single-turnover assays were performed with 1–16 μM enzyme, 1.0 μM tRNA, and 20 μM [^{3}H-methyl]-AdoMet in the same buffer. The substrate UGG $tRNA^{Pro}$ with m^1G37 was isolated from a vehicle strain expressing the WT tRNA, whereas the substrate UGG $tRNA^{Pro}$ without m^1G37 was isolated from the same vehicle strain with *trmD-KO* maintained by the Ara-controlled *trm5* on pACYC, which also expressed the *lpp*-controlled chimeric G34U mutant of GGG $tRNA^{Pro}$.

For TrmD methylation, steady-state assays were performed with 10 nM enzyme, 0.5–12.5 μM tRNA, and 28.9 μM [^{3}H-methyl]-AdoMet in a buffer of 100 mM Tris–HCl, pH 8.0, 24 mM NH_4Cl_2, 4 mM DTT, 0.024 mg/ml BSA, and 6 mM $MgCl_2$. The three states of UGG $tRNA^{Pro}$ were isolated from an *E. coli trmD-KO* strain (24) that expressed the tRNA from the pKK223–3 plasmid. The ho^5U34-state was isolated from the strain with additional *cmoB-KO* (with the kan^R removed), the cmo^5U34-state was isolated from the strain with additional *cmoM-KO*, and the $mcmo^5U34$-state was isolated from the *trmD-KO* strain.

For aminoacylation with Pro, the UAG variant of $tRNA^{Pro/UGG}$ was isolated from *E. coli* Pro vehicle strain (Supplementary Table S2). Plateau charging assays were conducted using 1.0 μM ProRS, 1.0 μM $tRNA^{Pro/UAG}$, 20 μM [^{3}H]-Pro in a buffer of 20 mM KCl, 50 mM HEPES pH 7.5, 4 mM DTT, 0.2 mg/ml BSA, 10 mM $MgCl_2$, and 2 mM ATP. For aminoacylation of the tRNA with Leu, LeuRS and [^{3}H]-Leu were used instead.

LC–MS/MS analyses

WT and mutant $tRNA^{Pro}$ species (3 μg), purified from cells, were digested with RNase T1 (50 U/μg tRNA) in a 220 mM ammonium acetate buffer at 37°C for 2 h. Upon completion, the samples were lyophilized and resuspended in 10 μl of the mobile phase A buffer (8 mM TEA/220 mM HFIP, pH 7.0). For liquid chromatography and mass spectrometry (LC–MS/MS) analysis, tRNA fragments from T1 digestion were separated in a Poroshell 120-EC-C18 column (1 × 50 mm, 2.7 μm particle size) using mobile phase A and mobile phase B (50% buffer A in methanol). The column oven was set at 60°C with the Thermo Surveyor HPLC attached to the Thermo LTQ-XL linear ion trap mass spectrometer. The LC gradient starts with 5–95% mobile phase B in 45 min at a flow rate of 60 μl/min. Post column equilibration is set for 10 min prior to the next injection. The MS oper-

ating parameters are: capillary temperature of 275°C, spray voltage of 3 kV and sheath, and auxiliary and sweep gases at 35, 14, 10 arbitrary units respectively. Data were recorded in negative mode, with a scan range from 600 to 2000 m/z. The product ion scans were obtained using collision induced dissociation at the normalized collision energy of 35%. All of the predicted tRNA sequences were obtained from the genomic tRNA database (http://gtrnadb.ucsc.edu/). The prediction of m/z and fragmentation of each was calculated by the Mongo Oligo Mass Calculator (http://mods.rna.albany.edu/masspec/Mongo-Oligo).

RESULTS AND DISCUSSION

A genetic approach

We chose *E. coli* UGG $tRNA^{Pro}$, encoded by the *proM* gene, as an example. This isoacceptor is a substrate for CmoM to convert cmo^5U34 to $mcmo^5U34$ (Figure 1B) (5). The other Pro isoacceptors in *E. coli* contain the GGG and CGG anticodon, respectively. Of the three, the UGG isoacceptor is essential for growth (15), the elimination of which would leave cells unable to efficiently translate the Pro codon CCA, ultimately leading to cell death. This growth-essentiality makes it impossible to introduce mutations to G35 to test recognition by CmoM. To overcome this limitation, we developed a genetic approach (Supplementary Figure S2), in which we created a plasmid-borne chimeric $tRNA^{Pro}$ to maintain cell growth, enabling us to remove the native *proM* gene from the chromosome while expressing a test version of the *proM* gene from a second plasmid for isolation of its tRNA product for enzymatic analysis. This approach consists of three key components. First, a chimeric $tRNA^{Pro}$ was expressed from the strong and constitutive *lpp* promoter in a pACYC-derived maintenance plasmid (21). The chimera was based on the sequence of the GGG isoacceptor (encoded by the *proL* gene, Figure 1C) but was made with the G34U mutation to carry the UGG anticodon for reading the Pro codon CCA. The remaining Pro codons would be read by the other two isoacceptors whose genes were intact on the chromosome. Second, the native *proM* was removed from its chromosomal locus in an operon containing tRNA genes *argX*, *hisR*, and *leuT* (Supplementary Figure S3). This was achieved in an *E. coli cmoM-KO* strain by λ Red recombination (26). The *cmoM-KO* strain was necessary as the host to produce tRNA species containing the cmo^5U34 wobble base but lacking the terminal methylation for evaluation as a substrate for CmoM. Third, the test version of *proM* was then expressed from pKK223–3 that was compatible with the maintenance pACYC. Expression of the test *proM* was driven from the IPTG-inducible *tac* promoter to produce high levels of the tRNA product for purification and kinetic analysis. In this 3-component system, expression of the maintenance chimeric $tRNA^{Pro}$ in *proM-KO* cells was sufficient to promote growth (Figure 1D), although the growth was slower relative to cells with *proM* intact on the chromosome. This establishes an *E. coli* vehicle strain that could be used to produce a mutant version of the *proM* tRNA from the test plasmid without losing cell viability.

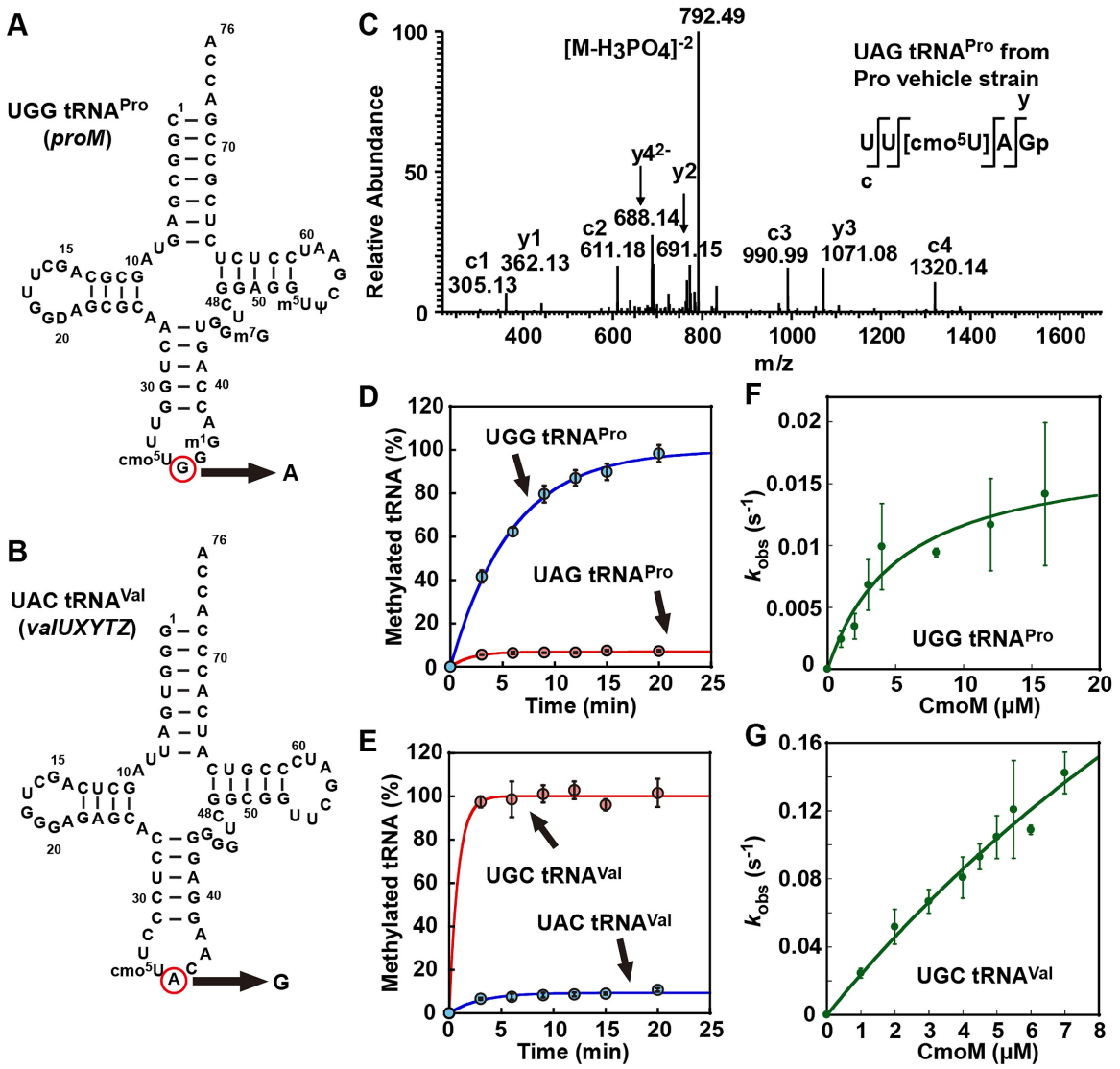
Using a similar genetic approach, we created an *E. coli* vehicle strain that was used to produce a mutant version of the UAC $tRNA^{Val}$ (Supplementary Figure S4). The WT isoacceptor is encoded in five genes of the same sequence on the chromosome (*valU*, *valX*, *valY*, *valT* and *valZ*), each of which produces a tRNA transcript that is modified to cmo^5U34 without the CmoM-catalyzed terminal methylation (5). In this vehicle strain, all of the isoacceptor genes were removed from the chromosome, while cell viability was maintained by expression of a chimeric $tRNA^{Val}$ based on the sequence of the *valW*-encoded GAC isoacceptor but made to contain the UAC anticodon. Growth of cells maintained by the chimeric $tRNA^{Val}$ was only slightly slower relative to cells containing *valUXYTZ* genes (Figure 1D). Expression from the test plasmid that harbored a mutant form of one of the *valUXYTZ* genes then provided a mechanism to isolate the mutant tRNA for enzymatic analysis.

We showed that this genetic approach was applicable to other single-gene tRNA species. For example, *cysT* is a single gene for GCA $tRNA^{Cys}$ in *E. coli*. Using the same genetic approach, we created a *cysT-KO* vehicle strain that was maintained viable by the homologous gene of *BscysT* in a maintenance plasmid and able to express the amber-reading version of *cysT* from a test plasmid (Supplementary Figure S5). The viability was consistent with our finding that *BscysT* tRNA is readily charged by the endogenous *E. coli* cysteinyl-tRNA synthetase (CysRS) (23). The amber-suppressor mutant of *E. coli cysT* was used to test its adaptor activity during live-cell protein synthesis. Indeed, the *cysT-KO* vehicle system, when introduced to strain XAC-1 harboring *lacZ* with an amber mutation, conferred a blue color on X-gal indicator plates (Supplementary Figure S5), indicating suppression of the amber mutation. These data demonstrate the adaptor activity of the amber-reading test $tRNA^{Cys}$ during protein synthesis in an *E. coli* strain where the essential single-gene *cysT* has been eliminated, validating the broad utility of our genetic approach.

G35 as the major determinant for recognition by CmoM

With the genetic approach in hand, we tested the importance of G35 as a determinant for tRNA recognition by CmoM. We created an *E. coli* vehicle strain that produced the G35A mutant of UGG $tRNA^{Pro}$ and a separate strain that produced the reciprocal A35G mutant of UAC $tRNA^{Val}$ (Figure 2A and B). Each mutant tRNA, as well as its WT counterpart (over-expressed from the pKK223–3 plasmid), was expressed in the *cmoM-KO* strain to abort the terminal methylation and to accumulate tRNA in the cmo^5U34 precursor state. Each was produced to high levels and purified to homogeneity (Supplementary Figure S6A) from binding to a complementary oligonucleotide on a solid support. As shown by LC-MS/MS, each purified tRNA contained cmo^5U34 in the precursor state, as exemplified by the UAG variant of $tRNA^{Pro}$ (Figure 2C, Supplementary Figure S6B), and was used as a substrate for CmoM.

Recombinant *E. coli* CmoM was over-expressed with a His-tag at both the N- and C-termini and purified to greater than 95% homogeneity. Using [^{3}H-methyl]-AdoMet as the methyl donor, we monitored the CmoM-catalyzed incorporation of [^{3}H-methyl] from the donor to tRNA as acid-precipitable counts on filer pads as we have shown with other AdoMet-dependent methyl transferases (27–29,38–40). Assays in a steady-state multiple-turnover condition



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	k_{chem} (s^{-1})	K_d (μM)	k_{chem}/K_d ($s^{-1}\mu M^{-1}$)
UGG tRNA^{Pro}	0.018 ± 0.003	5.3 ± 1.9	0.0034 ± 0.0013
UGC tRNA^{Val}	N. D.	N. D.	0.024 ± 0.003

Figure 2. G35 is the major determinant of tRNA recognition by CmoM. (**A**, **B**) Sequence and cloverleaf structure of *E. coli* UGG tRNA^{Pro} and UAC tRNA^{Val}. The substitution of position 35 in the anticodon of each is shown in an arrow. (**C**) The purified mutant UAG tRNA^{Pro} contains the precursor state cmo^{5}U34 as shown in LC-MS/MS. (**D**) Plateau methylation by CmoM is high for the WT UGG but low for the variant UAG of tRNA^{Pro}. (**E**) Plateau methylation by CmoM is low for the WT UAC but high for the variant UGC of tRNA^{Val}. Data for WT tRNAs are in dark blue, while those for mutant tRNAs are in red. Errors are SD, $n = 3$. (**F**) Titration of the single-turnover rate constant k_{obs} of methyl transfer to *E. coli* UGG tRNA^{Pro} as a function of CmoM concentration showed saturation kinetics. (**G**) Titration of the single-turnover rate constant k_{obs} of methyl transfer to *E. coli* UGC variant of tRNA^{Val} as a function of CmoM concentration showed no saturation. (**H**) Kinetic parameters of k_{chem}, K_d (tRNA), and k_{chem}/K_d derived from fitting the data of UGG tRNA^{Pro} in (**F**) to a hyperbolic equation. The k_{chem}/K_d value for the UGC variant of tRNA^{Val} was determined from fitting the data of k_{obs} at the enzyme concentration of 1 μM in (**G**) to a linear equation. Errors are SD, $n = 3$. N.D. = not determined. Assays were performed in single turnover conditions at 37°C, where the enzyme was 1–16 μM, tRNA was 1.0 μM, and [^{3}H-methyl]-AdoMet was 20 μM in a buffer containing 50 mM HEPES–KOH, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$ and 7 mM β-mercaptoethanol.

showed that the G35 base in the anticodon is indeed the major determinant for recognition and discrimination of tRNA by CmoM. While the WT UGG $tRNA^{Pro}$ was fully methylated by CmoM, the single G35A substitution abolished the methylation (Figure 2D). Conversely, while the WT UAC $tRNA^{Val}$ was an inactive substrate for CmoM, the single A35G substitution was sufficient to confer full methylation to this tRNA (Figure 2E). Thus, despite in different sequence contexts of $tRNA^{Pro}$ and $tRNA^{Val}$, the G35 base alone determines the methylation by CmoM.

To more closely evaluate the role of G35 in the methylation by CmoM, we determined the kinetic parameters of individual tRNA species (Figure 2F, G). CmoM is a member of the class I AdoMet-dependent methyl transferases (PDB: 4HTF), which use the Rossmann dinucleotide-fold to accommodate the methyl donor (41,42). We had shown that enzymes of the Rossmann-fold are rate-limited by the release of products (30,38,43), rather than by the chemistry involving the S_N2 nucleophilic attack on the sulfonium center of AdoMet. This kinetic signature indicates that the rate of chemistry is faster relative to the release of products and that, to probe the chemistry, kinetic assays must be performed in single-turnover assays to monitor chemistry, rather than in steady-state assays to monitor product release (38). We developed single-turnover assays for CmoM, where the enzyme was in excess of each tRNA to permit rapid equilibrium binding in one turnover, such that the rate constant k_{obs} of the turnover was not limited by binding but by the chemistry of methyl transfer (44). A titration of k_{obs} as a function of enzyme concentration then provided the basis to determine the saturating k_{obs} as k_{chem} (the intrinsic rate constant of methyl transfer) and the enzyme concentration that achieved half of the saturation kinetics as the thermodynamic dissociation constant K_d for the tRNA.

Analysis of k_{obs} as a function of CmoM concentration for the WT UGG $tRNA^{Pro}$ showed that all data points of single-turnover assays were fit to a hyperbolic equation without a lag phase (Figure 2F), supporting the notion that methyl transfer was performed in rapid binding equilibrium. The k_{obs} of each turnover was a composite term, representing all of the steps from binding to methyl transfer, including a conformational transition of the enzyme-tRNA-AdoMet complex that might have occurred before chemistry. The absence of a lag phase indicated that all steps leading to and including the chemistry were fast and that the kinetics was limited by the rate of methyl transfer. While we were unable to determine the kinetics of the UAG mutant of $tRNA^{Pro}$ or the WT UAC $tRNA^{Val}$, due to extremely low levels of activity, we were able to measure the kinetics of the UGC variant of $tRNA^{Val}$ (Figure 2G). Interestingly, the latter showed a linear increase of k_{obs} as a function of CmoM concentration, indicating that the substrate tRNA was non-optimal (45) and that a conformational transition of the enzyme-tRNA-AdoMet complex was necessary to adopt an active state. For example, this conformational transition might involve re-arrangement of the anticodon stem-loop of $tRNA^{Val}$, which differs from that of $tRNA^{Pro}$ and all other CmoM substrates by having a C30–G40 pair instead of a G30–C40 pair (Supplementary Figure S1). The kinetic distinction between the WT $tRNA^{Pro}$ and the active mutant of $tRNA^{Val}$ demonstrates that our single-turnover assays have the ability to discern subtle differences between substrates during methyl transfer.

The saturation kinetics of the WT UGG $tRNA^{Pro}$ showed that the single-turnover parameters for CmoM ($k_{chem} = 0.018 \pm 0.003\ s^{-1}$; K_d (tRNA) $= 5.3 \pm 1.9\ \mu M$, and $k_{chem}/K_d = 0.0034 \pm 0.0013\ s^{-1}\mu M^{-1}$, Figure 2H) are well within the range of other AdoMet-dependent tRNA methyl transferases (27,29,38–40). For the UGC variant of $tRNA^{Val}$, because partial saturation kinetics began to appear at 8 μM of the enzyme, fitting the data to a hyperbolic equation showed $k_{chem} \sim 0.66\ s^{-1}$, $K_d \sim 26\ \mu M$, and $k_{chem}/K_d \sim 0.025\ s^{-1}\mu M^{-1}$ (not shown). To independently validate the kinetics, we performed single-turnover assays to measure k_{chem}/K_d under conditions where the enzyme was sub-K_d such that the k_{obs} was a close representation of the efficiency. Kinetic analysis with the enzyme at 1 μM (~25-fold below the K_d) showed the k_{chem}/K_d of $0.024 \pm 0.003\ s^{-1}\mu M^{-1}$, Figure 2H), closely similar to the estimated value from the hyperbolic fit. This result reveals an increase of almost 7-fold efficiency for the mutant $tRNA^{Val}$ relative to the WT $tRNA^{Pro}$ (Figure 2H) and is consistent with the faster kinetics of the mutant tRNA to reach the plateau (Figure 2E). Thus, once the initial non-optimal enzyme-tRNA-AdoMet conformation was overcome in the mutant tRNA, the structure of the transformed complex was favorable for high efficiency of methyl transfer in a process driven by G35. These data support the hypothesis that the single G35 base is the major determinant of tRNA recognition by CmoM.

To validate the design principle of our genetic approach, which is particularly useful for tRNA species essential for cell survival, we tested the hypothesis that mutations in an essential tRNA would create cellular toxicity. Using UGG $tRNA^{Pro}$ as an example, where the UGG anticodon is a major determinant of charging of $tRNA^{Pro}$ by ProRS (46), we showed that the creation of the UAG anticodon mutation, which reads Leu codons CU[G/A], reduced specific charging with Pro to 90% and increased mis-charging with Leu to 10% (Supplementary Figure S6C). While charging was assayed *in vitro* with purified enzymes, the mutant UAG $tRNA^{Pro}$ was purified from *E. coli* cells lacking only *cmoM* but retaining genes for all other modification enzymes, indicating that the data obtained from the mutant tRNA should be relevant *in vivo*. Indeed, growth analysis showed that cells expressing the UAG variant of $tRNA^{Pro}$ had reduced viability relative to cells without it, even with expression of the maintenance UGG-*proL* gene (Supplementary Figure S6D, E). The reduced viability is consistent with the notion that the charged UAG $tRNA^{Pro}$ has delivered Pro to Leu codons CU[G/A], resulting in mis-translation during protein synthesis. This toxicity can only be identified in our genetic approach, where a basal level of viability is maintained, but not in an environment, where cell death occurs due to substitution of the natural UGG anticodon in the chromosome.

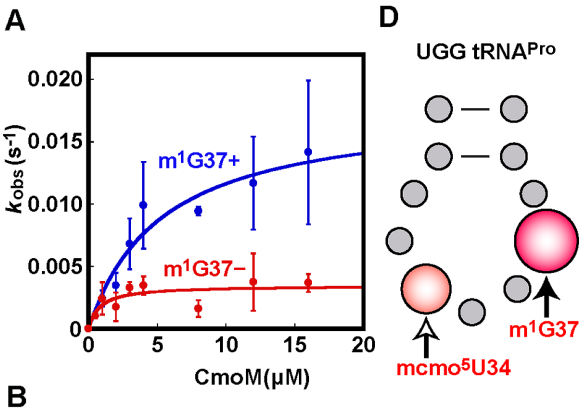
Dependence on m^1G37 for terminal methylation by CmoM

UGG $tRNA^{Pro}$ provides an opportunity to explore the inter-dependent relationship of the modifications at positions 34 and 37. In this tRNA, $mcmo^5U$ is a complex modification conserved at position 34 among γ-proteobacteria (5), whereas m^1G37 is a primary methylation (to the N^1 of

guanine) conserved across all bacterial species (both Gram-positive and Gram-negative) (47,48). Here we focused on the relationship between these two modifications. The terminal methylation is not essential for growth and its functional role in UGG tRNA^{Pro} has not been characterized, although it has an effect on reading-frame accuracy for another mcmo^{5}U34-containing tRNA (encoded by *alaT*, *alaU* and *alaV*, Supplementary Figure S1) (5). In contrast, the m^{1}G37 methylation is the major determinant in UGG tRNA^{Pro} for reading-frame accuracy and its elimination substantially increases the frequency of ribosomal shifts in both enzyme- and cell-based assays (24,32). Given that the two types of methylation occur in distinct chemical structure and space with distinct evolutionary distribution, we addressed the question of whether they are independent of each other or whether one is dependent on the other. Answering this question would shed light on the development of a functional UGG tRNA^{Pro}.

We showed that the presence of m^{1}G37 affected the terminal methylation level by comparing CmoM activity on UGG tRNA^{Pro} isolated with and without the primary methylation. The tRNA with m^{1}G37 was isolated from the *E. coli* vehicle strain described above, whereas that without m^{1}G37 was isolated from a derivative of the vehicle strain that also lacked the *trmD* gene. Because *trmD* is essential for growth (24), a simple knockout cannot be made. In the derivative, the chromosomal *trmD* was eliminated, but cell viability was maintained by the Ara-controlled expression of the human counterpart *trm5* (40) from the pACYC maintenance plasmid (Supplementary Figure S7) (24). Cells were grown to saturation in the presence of Ara to maintain the activity of *trm5* and 1% of these cells were then transferred to fresh media without Ara and grown for 3 h, followed by another transfer of 20% cells to fresh media and growth for 2 h. This serial dilution turned off *trm5* and depleted cellular m^{1}G37-tRNA to less than 5% of total tRNA (49) before isolation of UGG tRNA^{Pro}.

Single-turnover kinetic analysis of CmoM showed a 5-fold reduction in k_{chem} for the tRNA lacking m^{1}G37 versus with m^{1}G37 (Figure 3A, B), indicating a 5-fold reduction in the number of enzyme molecules engaged in methyl transfer per unit of time. Thus, the presence of m^{1}G37 is important for the action of CmoM. Although the tRNA lacking m^{1}G37 had a 6-fold more favorable K_d for the enzyme, resulting in an overall k_{chem}/K_d similar to that of the control, we note that the driver for the overall level of methylation is determined by k_{chem} in the context of a cell. This was based on the observation that the CmoM methylation to UGG tRNA^{Pro} progressively increases to the highest level in the stationary phase (5). The importance of m^{1}G37 for the level of terminal methylation at U34 by CmoM is supported by the crystal structure of the ribosomal 30S subunit with the anticodon–stem–loop of tRNA^{Pro} (50), showing that the anticodon loop would be disordered without m^{1}G37 but become organized when the methylation was in place. This ordering and organization of the anticodon loop helped to align the three anticodon bases to properly pair with the cognate codon. The dependence on m^{1}G37 for the action of CmoM suggests that the organization of the anticodon by the primary methylation enables the wobble base better recognized for the terminal methylation.



B

	k_{chem} (s^{-1})	K_d (μM)	k_{chem}/K_d (s^{-1} μM^{-1})
m^{1}G37+	0.018 ± 0.003	5.3 ± 1.9	0.0034 ± 0.0013
m^{1}G37–	0.0035 ± 0.0006	0.81 ± 0.61	0.0043 ± 0.0033

C

	U U ho^{5}U G G	U U cmo^{5}U G G	U U mcmo^{5}U G G
k_{cat} (s^{-1})	0.18 ± 0.07	0.24 ± 0.01	0.29 ± 0.01
K_m (μM)	1.8 ± 0.2	2.8 ± 0.4	2.7 ± 0.1
k_{cat}/K_m (s^{-1} μM^{-1})	0.10 ± 0.01	0.09 ± 0.01	0.11 ± 0.01

Figure 3. m^{1}G37 is required for the terminal methylation in mcmo^{5}U34 in *E. coli* UGG tRNA^{Pro}. (**A**) Titration of k_{obs} as a function of CmoM concentration for the terminal methylation of mcmo^{5}U34 in tRNA with (+) and without (–) m^{1}G37. Errors are SD, $n = 3$. (**B**) Kinetic parameters derived from the titration in (A). Errors are SD, $n = 3$. (**C**) Analysis of kinetic parameters of TrmD for the ho^{5}U34-, the cmo^{5}U34-, and the mcmo^{5}U34-state of the tRNA. The sequence and structure of the anticodon-stem loop of each state is shown and the status of the wobble modification (in red) was confirmed by LC–MS/MS previously (Supplementary Figure S6B). Note that the tRNA isolated from *wt* cells contained 40% mcmo^{5}U34 and 60% cmo^{5}U34 based on the ratio of peak areas of LC–MS/MS. (**D**) A model showing the hierarchical ranking of m^{1}G37 over mcmo^{5}U34 relative to the rest of the residues in the anticodon loop. In this ranking, the importance of each residue is shown by the size of the circle, showing that m^{1}G37 is required for the wobble modification but not *vice versa* and that the wobble modification is important for the tRNA decoding quality.

Conversely, we showed that the terminal methylation in mcmo^{5}U34 was unaffected by m^{1}G37. For broader impact, we explored not only the terminal methylation but also the two precursor states of the wobble modification. Using the *trmD-KO* strain that we constructed (24), which provided tRNA lacking m^{1}G37 for analysis of TrmD methylation, we isolated UGG tRNA^{Pro} from cells with the wobble base in three different states: the ho^{5}U34-state was isolated from the strain with additional *cmoB-KO*, the cmo^{5}U34-state from the strain with additional *cmoM-KO*, and the mcmo^{5}U34-state from the *trmD-KO* strain. Because the tRNA sequence in each case had no mutation, each was purified by over-expression of the *wt* gene from the pKK223–3 plasmid in the *trmD-KO* strain harboring additional gene-

knockout of choice. The modification status of each state was previously confirmed by LC-MS/MS in the strain expressing *trmD* (Supplementary Figure S6B). Kinetic analysis of TrmD was performed in a steady-state multi-turnover condition. This was appropriate for TrmD, which unlike CmoM is a non-Rossmann-fold enzyme and is rate-limited at the chemistry of methyl transfer (38). Because slow chemistry caused slow product release, steady-state assays that monitored product release (38) would faithfully report the chemistry events of TrmD. For the fully modified mcmo^{5}U34-state, the TrmD kinetic parameters k_{cat} (0.29 ± 0.01 s^{-1}) and K_m (2.7 ± 0.1 μM) (Figure 3C) are within a 2-fold range of those previously published for the unmodified transcript of the tRNA (38), indicating that post-transcriptional modifications are not critical for TrmD. Indeed, the k_{cat}, K_m and k_{cat}/K_m values for all three states of the tRNA were similar with each other (Figure 3C), supporting the notion that the TrmD activity does not depend on the status of the wobble modification.

Our results establish a hierarchical ranking of the TrmD-catalyzed m^{1}G37 over the modification state at the wobble base in UGG tRNA^{Pro} (Figure 3D). While we only studied the effect of m^{1}G37 on the terminal methylation of the wobble base, we suggest that the effect would be maintained throughout all states of the wobble modification, because the primary methylation is required to organize the anticodon structure to permit each modification step. Thus, m^{1}G37 is important not only for reading-frame accuracy of the tRNA, but also for initiating a complex modification process to the wobble base to improve the decoding quality of the latter. This new finding raises the possibility that the maturation of a functional UGG tRNA^{Pro} must have acquired m^{1}G37 before mcmo^{5}U34. Without the former to maintain the reading frame and cell survival (24), the latter would not form and the quality of tRNA decoding would be poor. Thus, the priority placement of m^{1}G37 over mcmo^{5}U34 has biological significance in that tRNA must support cell growth before it improves the decoding quality.

The interdependent relationship between tRNA positions 34 and 37 has been probed for two other cases. In one, the formation of C$_m$32 (2′-*O*-methylation of C32) and G$_m$34 (2′-*O*-methylation of G34) in tRNA^{Phe} is found to drive the conversion of m^{1}G37 to yW (wybutosine) (51). In the second, the synthesis of t^{6}A37 (threonylcarbamoyl adenosine) in tRNA^{Ile} is found to precede before aminoacylation by IleRS and possibly lysidinylation of C34 by TilS (52). The hierarchical ranking of the first case, placing positions 32 and 34 over 37, is opposite from our finding, whereas that of the second, placing position 37 over 34, is similar. To determine if there are rules governing the hierarchy, further studies are needed. For example, four other tRNA families with cmo^{5}U34 or mcmo^{5}U34 contain a different modification at position 37 (Supplementary Figure S1). These are m^{6}A37 (*N*^{6}-methyl-A37) for UAC tRNA^{Val} (53), ms^{2}i^{6}A37 (2-methylthio-*N*^{6}-isopentenyl-A37) for UGA tRNA^{Ser} (54,55), ct^{6}A37 (cyclic *N*^{6}-threonylcarbamoyl-A37) for UGU tRNA^{Thr} (56,57), and m^{1}G37 for UAG tRNA^{Leu} (20). Little is known about the hierarchical relationship between the two positions in the development of each tRNA. Based on our work here, we suggest that each tRNA has a priority placement of modifications between positions 34 and 37 to ensure the functional state of the tRNA in the context of a cell.

SUPPLEMENTARY DATA

Supplementary data are available at NAR online.

ACKNOWLEDGEMENTS

We thank Dr. Steven Almo for the *E. coli cmoB-KO* strain.

FUNDING

NIH [GM1081601 and GM114343 to Y.M.H.]; JSPS postdoctoral fellowship (to I.M.); Defense Threat Reduction Agency HDTRA [1-15-1-0033 to P.L.]. Funding for open access charge: NIH [GM081601 to Y.M.H., GM114343 to Y.M.H.].
Conflict of interest statement. None declared.

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