Biogenesis and growth phase-dependent alteration of 5-methoxycarbonylmethoxyuridine in tRNA anticodons

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ABSTRACT
Post-transcriptional modifications at the anticodon first (wobble) position of tRNA play critical roles in precise decoding of genetic codes. 5-carboxymethoxyuridine (cmo^5U) and its methyl ester derivative 5-methoxycarbonylmethoxyuridine (mcmo^5U) are modified nucleosides found at the anticodon wobble position in several tRNAs from Gram-negative bacteria. cmo^5U and mcmo^5U facilitate non-Watson–Crick base pairing with guanosine and pyrimidines at the third positions of codons, thereby expanding decoding capabilities. By mass spectrometric analyses of individual tRNAs and a shotgun approach of total RNA from Escherichia coli, we identified mcmo^5U as a major modification in tRNA^Ala^1, tRNA^Ser^1, tRNA^Pro^3 and tRNA^Thr^4; by contrast, cmo^5U was present primarily in tRNA^Leu^3 and tRNA^Val^4. In addition, we discovered 5-methoxycarbonylmethoxy-2′-O-methyluridine (mcmo^5U_m) as a novel but minor modification in tRNA^Ser^1. Terminal methylation frequency of mcmo^5U in tRNA^Pro^3 was low (≈30%) in the early log phase of cell growth, gradually increased as growth proceeded and reached nearly 100% in late log and stationary phases. We identified CmoM (previously known as SmtA), an AdoMet-dependent methyltransferase that methylates cmo^5U to form mcmo^5U. A luciferase reporter assay based on a +1 frameshift construct revealed that terminal methylation of mcmo^5U contributes to the decoding ability of tRNA^Ala^1.

INTRODUCTION
RNA molecules are frequently modified post-transcriptionally on their nucleobase and ribose moieties. These modifications carry qualitative information embedded in RNA molecules associated with various biological processes. To date, more than 100 species of modifications have been identified in various RNAs from all domains of life (1), the majority of which are found in tRNAs. tRNA modifications play critical roles in decoding properties and stabilization of tertiary structure (2,3). In particular, a wide variety of modifications occur at the first (wobble) position of the anticodon. These modifications play pivotal roles in modulating codon recognition and ensuring accurate translation of the genetic code (4).

In the classical wobble hypothesis proposed by Crick (5), uridine (U34) at the wobble position pairs with A and G at the third letter of the codon. In the decoding system of Mycoplasma species and mitochondria (6–9), however, U34 can recognize any of the four bases in a family box due to its conformational flexibility, a phenomenon termed ‘four-way wobbling’ (4), although efficiency of this type of wobbling strongly depends on the second and third letters of codons. To restrict decoding capability, tRNAs responsible for two codon sets ending in a purine (NNR) often contain 5-methyl-(2-thio)uridine derivatives [xm^5(s^2)U] at the wobble position (3,4), including 5-carboxymethylaminomethyl-(2-thio)uridine [cmnm^5(s^2)U] and 5-methylaminomethyl-(2-thio)uridine [mnm^5(s^2)U] in bacterial tRNAs, 5-methoxycarbonylmethyl-(2-thio)uridine [mcm^5(s^2)U] and its derivatives in eukaryotic cytoplasmic tRNAs and 5-taurinomethyl-(2-thio)uridine [tm^5(s^2)U] in mitochondrial tRNAs. Due to the conformational rigidity of xm^5(s^2)U modifications, which are largely fixed in the C3′-endo ribose pucker conformation (10), xm^5(s^2)U prefers to base-pair with A and G, thus preventing misreading of near-cognate...
codons ending in pyrimidine (NNY) (2,11). In addition, C5-substituent of \( \text{xm}^5\text{U} \) modification plays a critical role in stabilizing U-G wobble pairing at the A-site of ribosome (12,13).

By contrast, to expand decoding capacity in most bacterial species, 5-hydroxyuridine derivatives (\( \text{xo}^5\text{U} \)) are present at the wobble position of tRNAs responsible for recognizing family boxes. 5-carboxymethoxyuridine (\( \text{cmo}^5\text{U} \), also called uridine-5-oxy acetic acid) (Figure 1A) is present in tRNAs from Gram-negative bacteria including *Escherichia coli* and *Salmonella enterica* (3,4,14). \( \text{cmo}^5\text{U} \) was first reported as a minor nucleoside at the wobble position of *E. coli* tRNA\(^\text{Val}1\) (15,16). The chemical structure of \( \text{cmo}^5\text{U} \) was determined in 1970 (17). Subsequently, the presence of the methyl ester derivative of \( \text{cmo}^5\text{U} \), \( \text{mcmo}^5\text{U} \) was predicted by a series of *in vitro* studies (22–24). In Gram-positive bacteria, 5-methoxymethylene-\( \text{mcmo}^5\text{U} \) (18) was also reported as a major modification in tRNA\(^\text{Val}1\), tRNA\(^\text{Ala}1\), tRNA\(^\text{Ser}1\), tRNA\(^\text{Thr}4\), tRNA\(^\text{Pro}3\) and tRNA\(^\text{Leu}3\) (14,20). However, the exact frequency of these modifications in each tRNA has not been determined.

\( \text{cmo}^5\text{U} \) and \( \text{mcmo}^5\text{U} \) enable non-Watson–Crick base pairing at the wobble position of codons ending in pyrimidine (NNY) (2,11). In addition, \( \text{cmo}^5\text{U} \) is present in tRNA\(^\text{Pro}3\) from *S. enterica* (serovar Typhimurium) (21). The presence of the methyl ester derivative of \( \text{cmo}^5\text{U} \), 5-methoxycarbonylmethoxyuridine (\( \text{mcmo}^5\text{U} \), also called uridine-5-oxy acetic acid methyl ester) (Figure 1A) was predicted by an unidentified AdoMet-dependent methyltransferase, whereas \( \text{cmo}^5\text{U} \) in tRNA\(^\text{Val}1\) is not (22). Initially, the \( \text{cmo}^5\text{U} \) methyltransferase was predicted to be encoded by *supK* (23); however, *supK* was subsequently identified as *prfB* which encodes release factor 2 (RF2) (38). \( \text{cmo}^5\text{U} \) was also speculated to use its second function to methylate \( \text{cmo}^5\text{U} \) to generate \( \text{mcmo}^5\text{U} \) (31). However, it is now clear that CmoA is responsible for catalyzing SCM-SA formation (37). Thus, the gene responsible for the \( \text{cmo}^5\text{U} \) methyltransferase remains to be identified.

In this study, we isolated individual tRNAs from *E. coli* and analyzed the modification status of each tRNA by mass spectrometry. We found that \( \text{mcmo}^5\text{U} \) is present as a major modification in tRNA\(^\text{Ala}1\), tRNA\(^\text{Ser}1\), tRNA\(^\text{Pro}3\) and tRNA\(^\text{Thr}4\), whereas \( \text{cmo}^5\text{U} \) is primarily present in tRNA\(^\text{Leu}3\) and tRNA\(^\text{Val}1\). In addition, we discovered 5-methoxycarbonylmethoxy-2′-O-methyluridine (\( \text{mcmo}^5\text{Um} \)) (Figure 1A) as a novel derivative of \( \text{cmo}^5\text{U} \) in tRNA\(^\text{Ser}1\). Frequency of terminal methylation of \( \text{mcmo}^5\text{U} \) in tRNA\(^\text{Pro}3\) was dependent on growth phase. Moreover, we identified the \( \text{cmo}^5\text{U} \) methyltransferase, which we named CmoM, that methylates \( \text{cmo}^5\text{U} \) to form \( \text{mcmo}^5\text{U} \) in the presence of AdoMet. This terminal methylation of \( \text{mcmo}^5\text{U} \) contributes to the decoding ability of tRNA\(^\text{Ala}1\).

**MATERIALS AND METHODS**

**Strains and plasmid construction**

The *E. coli* deletion strains \( \Delta \text{cmoB}:\text{Km}^r \), \( \Delta \text{trmL}:\text{Km}^r \), \( \Delta \text{cmoM} \) (\( \Delta \text{msmtA} \))::\( \text{Km}^r \), and their parental strain BW25113, were obtained from the National BioResource Project (NBRP), National Institute of Genetics, Japan (39). All strains were cultured in LB media at 37 °C.

To generate a vector for expression of recombinant CmoM with an N-terminal His6-tag, *cmoM* was polymerase chain reaction (PCR)-amplified from the BW25113 genome and inserted into the *NdeI*/NotI site of pET-28a (Novagen) to yield pET-cmoM-N-His6. To complement *cmoM*, *cmoM* with its 5′ flanking region (including the promoter) was PCR-amplified and cloned into the low-copy plasmid pMW118 (Nippon Gene) to yield pMW–*cmoM* (psmtA). Point mutations were introduced in pMW–*cmoM* by QuikChange™ site-directed mutagenesis (Agilent Technologies). For dual-luciferase reporters, the fusion gene

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Figure 1. 5-carboxymethoxyuridine (cmo5U) and E. coli tRNAs. (A) Chemical structures of 5-carboxymethoxyuridine (cmo5U, left), 5-methoxycarbonylmethoxyuridine (mcmo5U, center) and 5-methoxycarbonylmethoxy-2′-O-methyluridine (mcmo5Um, right). (B) Secondary structures of E. coli tRNAAla1 (left) and tRNAser1 (right) with post-transcriptional modifications: 4-thiouridine (s4U), 2′-O-methylguanosine (Gm), dihydrouridine (D), 2′-O-methylcytidine (Cm), 2′-O-methyluridine (Um), 5-methoxycarbonylmethoxyuridine (cmo5U), 5-methoxycarbonylmethoxy-2′-O-methyluridine (mcmo5Um), 2-methylthio-N6-isopentenyladenosine (ms2i6A), 7-methylguanosine (m7G), 5-methyluridine (m5U) and pseudouridine (Ψ). The position numbers of the residues (gray letters) are displayed according to the nucleotide numbering system (64). Pairs of gray triangles indicate the positions of cleavage by RNase T1 that generate RNA fragments containing the wobble positions. The sequence of E. coli tRNAAla1 is the same as that of tRNAAlaB (65).

of Renilla and firefly luciferases was PCR-amplified from pQE-Luc(+1) (40) using primers containing NcoI and XhoI sites, and then inserted into the corresponding site of pBAD/Myc-His (Invitrogen) to yield pBAD-RFLuc. Subsequently, the RF2 recoding site (32,41), including an SD-like sequence and GCG as a test codon, was introduced by PCR into the linker region between the two luciferases to yield pBAD-RFLucGCG. Two variants in which the test codon was replaced by UCG (pBAD-RFLucUCG) and GG (pBAD-RFLucGG) were generated by QuikChange™ site-directed mutagenesis. All constructs used in this study were verified by Sanger sequencing. The primers used in this study are listed in Supplementary Table S1.

RNA extraction and tRNA isolation

Total RNA from each E. coli strain was extracted by phenol in acidic condition (42). Individual tRNAs were isolated by reciprocal circulating chromatography, as described previously (42,43). The 5′-terminal ethylcarbamate ammodifed DNA probes used in this method are listed in Supplementary Table S1.

Mass spectrometry of tRNA modifications

For RNA fragment analysis, isolated tRNA (1.25 pmol) was digested at 37°C for 1 h in 12.5 μl of a solution containing 20 mM NH₄OAc (pH 5.3) and 125 U RNase T1. The digested RNA was mixed with 12.5 μl of 0.1 M triethylamine-acetate
Isolated tRNA^{Ser}1 (110 pmol) was digested at 37 °C for 3 h in 15 μl of a solution containing 20 mM trimethylamine-HCl (TMA-HCl) (pH 7.0), 0.05 U of nuclelease P1 and 0.1 U of BAP. The digested RNA (100 pmol) was adjusted as 50 μl of 90% acetonitrile and subjected to HILIC/ESI-MS using Q Exactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific) equipped with a Dionex UltiMate™ 3000 LC System (Thermo Fisher Scientific) using a ZIC-chHILIC column (3 μm, 2.1 × 150 mm, Merck Millipore). For ribonucleotide analysis, a reverse genetic approach combined with RNA-MS (44), nucleosides of total RNAs obtained from knockout strains were subjected to RPC-ESI/MS using Q Exactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific) using a ZIC-cHILIC column (3.2 mm, 250 mm, GL Science).

Shotgun analysis of tRNA fragments by RNA-MS

Total RNA of each strain cultured in 2–20 ml of LB medium was extracted with TRIzol™ (Life Technologies). Then, 50–250 μg of total RNA was dissolved in 800 μl of 3 M NH4OAc (pH 5.3), mixed with 640 μl (0.8 vol) of isopropanol at room temperature and centrifuged at 15,000 rpm for 10 min to precipitate long RNAs including rRNAs. The supernatant was collected and precipitated with ethanol. Depletion of rRNA was verified by denaturing PAGE. The resultant small RNA fraction (50 ng) was digested for 1 h at 37 °C with RNase T1 (125 U) in 20 mM NH4OAc (pH 5.3), and the digested RNA (20 ng) was subjected to capillary LC coupled to nanoESI/MS as described above.

Mutation study of CmoM by genetic complementation

The E. coli ΔcmoM strain was transformed with psmtA, its mutant derivatives or an empty vector (pMW118), and the functional importance of the mutations was assessed by monitoring restoration of mcmo5U in the transformants. Transformants were cultured overnight at 37 °C in 2 ml of LB medium containing 100 μg/ml ampicillin. Total RNA was subjected to the shotgun analysis as described above to detect the anticodon-containing fragment of tRNA^{Pro}. Modification frequency was determined by calculating the intensity ratio of mass chromatograms between UmUmcmo5UGp (m/z 683.567, z = 2) and UmUmcmo5UGp (m/z 690.575, z = 2).

Preparation of recombinant protein

E. coli BL21 (DE3) transformed with pET-cmoM-N-His6 was cultured at 37 °C to an OD600 of ≈0.7, supplemented with 0.1 mM IPTG and cultured at 37 °C for an additional 4 h. Cells were harvested and disrupted by sonication in lysis buffer consisting of 50 mM HEPES-KOH (pH 7.5), 100 mM KCl, 10 mM MgCl2, 1 mM dithiothreitol and 0.2 mM PMSF. Purification of recombinant protein was performed basically as described (42,46) using a HisTrap column (GE Healthcare) with a linear gradient of imidazole (25–500 mM). The purified protein was dialyzed in lysis buffer, added with glycerol to f. c. 30% and stored at −20 °C.

In vitro reconstitution of mcmo5U

tRNA^{Ser}1, a substrate for CmoM, was isolated from the ΔcmoM strain. In vitro methylation was performed for 1 h at 37 °C in a 10 μl reaction mixture containing 50 mM HEPES-KOH (pH 7.5), 100 mM KCl, 10 mM MgCl2, 7 mM 2-mercaptoethanol, 1 mM AdoMet, 10 pmol of tRNA^{Ser}1 and 1 pmol of recombinant CmoM. After the reaction, tRNA was extracted with acidic phenol and chloroform, followed by ethanol precipitation. The tRNAs were digested with RNase T1 and subjected to LC/MS as described above.

Luciferase reporter assay

The luciferase reporter assay was performed essentially as described (40). E. coli wild-type, ΔcmoM and ΔcmoB strains were transformed with the series of dual-luciferase reporters described above. Each transformant was precultivated at 37 °C in 2 ml LB medium containing 100 μg/ml ampicillin overnight. The preculture (1%) was inoculated to 2 ml of LB medium containing 100 μg/ml ampicillin and 100 μM arabinose to induce expression of the reporter. When the OD600 reached 0.3–0.7, 1 ml aliquot was centrifuged, and the pellet was resuspended in 200 μl of lysis buffer [10 mM HEPES-KOH (pH 7.5), 100 mM NaCl, 10 mM MgCl2, 7 mM 2-mercaptoethanol, 400 μg/ml lysozyme]. Cell lysates were prepared by the freeze-thaw method (47) and cleared by centrifugation. The reporter assay was carried out on 5 μl of lysate using GloMax™ 96 Microplate Luminometer (Promega) with the Dual-Luciferase™ Reporter Assay System (Promega). The Fluc luminescence signal was normalized against the RLuc luminescence signal.

RESULTS

Modification status of individual tRNAs analyzed by mass spectrometry

Because the methyl ester of mcmo5U is easily hydrolyzed during preparation and handling of tRNAs (22), little information is available regarding the presence of mcmo5U in individual tRNAs. To determine which tRNA species contain this modification, we employed reciprocal circulating chromatography (RCC) (43) to isolate six tRNA species (tRNA^{Ala1}, tRNA^{Leu3}, tRNA^{Pro3}, tRNA^{Ser1}, tRNA^{Thr4} and tRNA^{Val1}) (Figure 1B) predicted to contain mcmo5U or mcmo2U from E. coli cells harvested at stationary phase. Each individual tRNA was digested with RNase T1 and subjected to capillary liquid chromatography (LC)/nanoelectrospray ionization mass spectrometry (MS) to precisely analyze its post-transcriptional modifications (44). The RNA fragment containing the wobble modification was detected as multiply-charged negative
Figure 2. Mass spectrometric analysis of individual tRNAs isolated from stationary-phase *E. coli*. (A) Mass chromatograms of RNase T1-digested fragments containing cmo5U and its derivatives from tRNAAla1 (left panels) and tRNASer1 (right panels) isolated from stationary-phase *E. coli*. Top and middle panels: extracted-ion chromatograms (XIC) for doubly-charged negative ions of cmo5U34-containing fragments (CUUcmo5UGp of tRNAAla1, MW 1660.18, m/z 829.08; UCmUcmo5UGp of tRNASer1, MW 1674.19, m/z 836.09) and mcmo5U34-containing fragments (CUUmcmo5UGp of tRNAAla1, MW 1674.19, m/z 836.09; UCmUmcmo5UGp of tRNASer1, MW 1688.21, m/z 843.10), respectively. Bottom left and bottom right panels: XICs for doubly-charged negative ions of Um32/mcmo5U34-containing fragment of tRNAAla1 (CUmUmcmo5UGp, MW 1688.21, m/z 843.10) and mcmo5Um34-containing fragment of tRNASer1 (UCmUmcmo5UmGp, MW 1702.22, m/z 850.10), respectively. The peaks marked with an asterisk represent Um32/cmo5U-containing fragment (CUUmcmo5UGp, MW 1674.19, m/z 836.09) in tRNAAla1 and mcmo5Um-containing fragment (UCmUmcmo5UmGp, MW 1688.21, m/z 843.10) in tRNASer1. The RNA fragments containing unmodified C32 are also present in tRNASer1, but they are not described here due to high frequency of Cm32 in tRNASer1 isolated from stationary-phase *E. coli*. (B) Modification frequencies of cmo5U and its derivatives at position 34 in six species of tRNAs isolated from stationary-phase *E. coli*. Relative composition of each modification was calculated from the peak area ratio of mass chromatograms of RNase T1-digested fragments containing mcmo5Um34 (red), mcmo5U34 (green), cmo5U34 (blue) or U (gray). (C) Collision-induced dissociation (CID) spectrum of a fragment of *E. coli* tRNASer1 containing mcmo5Um34. The doubly-charged negative ion of the RNase T1-digested fragment containing mcmo5Um34 (m/z 850.10) was used as the precursor ion for CID. The product ions were assigned as described previously (66). Sequences of parent ion and assigned product ions are described on the upper left side of this panel. (D) UV chromatogram of tRNASer1 isolated from stationary-phase *E. coli*. (E) CID spectrum of mcmo5Um nucleoside. The protonated mcmo5Um (MH+, m/z 347.11) was used as the precursor ion for CID. The N-glycoside bond cleaved to generate the base-related ion (BH2+) and other product ions are assigned on the chemical structure.
ions (Figure 2A and Supplementary Figure S1) and further probed by collision-induced dissociation (CID) to determine the position of each modification (Supplementary Figure S2). Frequencies of the wobble modifications in these six tRNA species were calculated from the peak areas of mass chromatograms (Figure 2B). The wobble modifications of tRNA^Ala1, tRNA^Pro3, tRNA^Ser1, and tRNA^Thr4 consisted of ≈80% mcmo5U and ≈20% cmo5U. By contrast, over 90% of tRNA^Leu3 and tRNA^Val1 molecules contained cmo5U, whereas the remaining 10% contained mcmo5U or unmodified U. In tRNA^Ala1, we observed partial modification (≈11%) of 2'-O-methyluridine (Um) at position 32 (Figure 2A), which was confirmed by CID analysis (Supplementary Figure S2A).

**Discovery of mcmo5Um as a novel modification**

At the wobble position of tRNA^Ser1, we found a novel minor modification with a molecular mass 14 Da larger than that of mcmo5U (Figure 2A). CID analysis of this fragment (Figure 2C) revealed a specific product ion (a-B4) lacking the 5-methoxycarbonylmethoxyuracil-base (mcmo5U-base), strongly suggesting that the 2' hydroxyl group of the ribose was methylated. To confirm this result, we analyzed total nucleosides of tRNA^Ser1 and detected a proton-adduct (MH+) of this minor nucleoside (m/z 347) (Figure 2D) which we then probed by CID analysis (Figure 2E). We clearly detected a base-related fragment (BH2+) with m/z 201, which also appears in mcmo5U, confirming that the ribose portion is methylated. We also verified the absence of this modification in a knockout strain of trmL, which encodes a 2'-O-methyltransferase that targets position 34 (see Figure 4C). Taken together, we conclude that the novel modification found in tRNA^Ser1 is 5-methoxycarbonylmethoxy-2'-O-methyluridine (mcmo5Um) (Figure 1A,B). About 4.2% of tRNA^Ser1 molecules contain this modification (Figure 2A,B). Given that 2'-O-methylation stabilizes C3'-endo ribose pucker conformation (48), mcmo5Um is likely to allow tRNA^Ser1 to recognize UCG codon more efficiently than mcmo5U.

**Frequency of mcmo5U in cellular tRNAs estimated by shotgun approach**

Although mcmo5U was detected in the four tRNAs with a frequency of ≈80% (Figure 2B), it is possible that the methyl ester of mcmo5U was partially hydrolyzed during isolation of individual tRNAs by RCC. To exclude the effects of the inevitable hydrolysis of mcmo5U during tRNA isolation, we analyzed the wobble modifications in total tRNAs using a shotgun approach. In order to profile the huge number of RNA fragments derived from all species of tRNAs, we prepared crude E. coli tRNA fractions from different growth phases, digested them with RNase T1, and subjected the digested material to LC/MS (Figure 3A). Among the four tRNAs bearing mcmo5U, we clearly detected an anticodon-containing fragment of tRNA^Pro3 and tRNA^Thr4 containing mcmo5U or cmo5U, each of which has a unique molecular mass and does not overlap with other fragments. Judging from the peak area of mass chromatograms for UmUmUcmo5UGp (m/z 683.57) and UmUcmo5UGp (m/z 690.57) in tRNA^Pro3 isolated from cells in stationary phase, mcmo5U was present in tRNA^Pro3 with a frequency of 98% (Figure 3B). Similarly, tRNA^Thr4 isolated from cells in stationary phase contained nearly 100% mcmo5U (Figure 3B). These results suggest that about 20% of the cmo5U detected in tRNA^Pro3 and tRNA^Thr4 (Figure 2B) was generated by artificial hydrolysis of mcmo5U during tRNA iso-
Growth phase-dependent alteration of mcmoU in tRNAPro3

To investigate the effect of growth phase on mcmoU, we prepared total tRNA from *E. coli* cells harvested at various growth phases. The total tRNA was digested with RNase T1, and analyzed by LC/MS to monitor the RNA fragment containing mcmoU or cmoU derived from tRNAPro3 (Figure 3A). Based on the ratio of the two fragment peaks, we calculated the frequency of mcmoU over the course of *E. coli* cultivation (Figure 3B and Supplementary Figure S3A). In early log phase (1–2 h after inoculation), mcmoU was present in tRNAPro3 with a frequency of ≈30%, whereas cmoU was present in the remaining molecules. The mcmoU frequency gradually increased as growth proceeded, reaching nearly 100% in late log and stationary phase. By contrast, mcmoU frequency in tRNAThr4 was consistently high (>99%) in all growth phases (Figure 3B and Supplementary Figure S3B).

The shotgun analysis failed to detect specific fragments bearing mcmoU or cmoU derived from tRNAAla1 and tRNASer1, because the molecular masses of these fragments overlapped with those of other fragments. To investigate the modification status of these tRNAs in log phase, we isolated four tRNAs containing mcmoU from *E. coli* cells harvested in mid-log phase (OD600 = 0.4). Mass spectrometric analysis revealed that both tRNAAla1 and tRNASer1 contained mcmoU with frequencies of 82% and 84%, respectively (Supplementary Figure S4). Because 20% of mcmoU is hydrolyzed and converted to cmoU during isolation of tRNAs by RCC (Figure 2B), we concluded that both tRNAAla1 and tRNASer1 were fully modified with mcmoU in mid-log phase. Meanwhile, tRNAPro3 and tRNAThr4 contained mcmoU at frequencies of 34% and 80%, respectively (Supplementary Figure S4). These results are consistent with those observed in the shotgun analysis (Figure 3B). Based on these findings, we conclude that mcmoU content in tRNAPro3 is dependent on growth phase, whereas the other three tRNAs are fully modified with mcmoU during all phases of cell growth.

Identification of a gene responsible for terminal methylation of mcmoU

To identify the gene encoding the AdoMet-dependent methyltransferase that methylates cmoU to form mcmoU, we conducted a genome-wide screen to identify genes responsible for RNA modifications. The method we employed, ribonucleome analysis, uses a reverse genetic approach combined with RNA-MS (44). This approach has been used to successfully identify many genes responsible for tRNA/tRNA modifications among uncharacterized genes in *E. coli* (40,46,49–52) and Saccharomyces cerevisiae (53–56). Screening of *E. coli* knockout strains revealed that mcmoU (m/z 333) was completely absent in the ΔsmtA strain; instead, the level of cmoU (m/z 319) was higher than that in the wild-type (Figure 4A). When smtA was introduced on a plasmid (pSmtA) into the ΔsmtA strain, formation of mcmoU (m/z 333) was restored (Figure 4A). These data indicate that SmtA is a methyltransferase that produces mcmoU from cmoU.

Next, we isolated tRNAAla1 and tRNASer1 from the ΔsmtA strain, and analyzed their wobble modifications by LC/MS (Figure 4B,C). As expected, mcmoU-containing fragments were completely converted to cmoU-containing fragments in both strains.

As mentioned above, we discovered mcmoU (Figure 1A) as a minor modification in tRNASer1. We confirmed the absence of mcmoU in both ΔsmtA and ΔtrmL (Figure 4C). *trmL* encodes a 2′-O-methyltransferase responsible for 2′-O-methylation of cmnmU34 of tRNAPro3 and Cm34 of tRNALeu (57). Therefore, we conclude that mcmoU in tRNASer1 is generated by 2′-O-methylation of mcmoU by TrmL.

In vitro reconstitution of mcmoU mediated by CmoM

To determine whether SmtA actually has methyltransferase activity, we conducted in vitro reconstitution of mcmoU formation by recombinant SmtA. In this experiment, tRNASer1 bearing cmoU was isolated from the ΔsmtA strain and used as a substrate. We clearly detected mcmoU in the tRNASer1 only in the presence of both recombinant SmtA and AdoMet (Figure 5A). CID analysis of the anticodon-containing fragment confirmed the methylation occurred at the wobble position (Figure 5B). This result demonstrated that SmtA is an AdoMet-dependent methyltransferase that transfers a methyl group to cmoU34 of tRNAs to form mcmoU34. Based on the enzymatic activity, we renamed this gene CmoM (cmoU methyltransferase).

Characterization of CmoM

CmoM belongs to the Class I AdoMet-dependent methyltransferase (MTase) family, whose members contain a Rossmann-fold as a characteristic structural motif (Figure 6A) (58–60). The high-resolution crystal structure of *E. coli* CmoM (SmtA) (PDB ID:4HTF) revealed that CmoM forms a homodimer, and that each subunit contains one molecule each of AdoMet, sulfate, acetate and 2-mercaptoethanol as ligands (Figure 6C and Supplementary Figure S5). Based on this structure, we designed eight mutant cmoM constructs bearing single amino-acid alterations (Figure 6A). The mutated residues, which are conserved in cmoM homologs, are located at the catalytic site where AdoMet is bound (Figure 6C). The ΔcmoM strain was transformed with each of the mutant constructs, and total RNA extracted from each construct was digested with RNase T1 and subjected to LC/MS to detect the RNA fragment of RNAPro3 bearing mcmoU or cmoU (Figure 6B). The positive and negative controls behaved as expected: mcmoU was fully restored by wild-type cmoM, whereas no mcmoU was formed in cells transfected with an empty vector. Little or no mcmoU was observed in the ΔcmoM strain introduced by the mutant constructs R26A, D73A, W124A, Y150A, R209A, D213A and R246A, whereas mcmoU formation was partially restored by the Y247A mutant.

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As mentioned above, we discovered mcmoU (Figure 1A) as a minor modification in tRNASer1. We confirmed the absence of mcmoU in both ΔsmtA and ΔtrmL (Figure 4C). *trmL* encodes a 2′-O-methyltransferase responsible for 2′-O-methylation of cmnmU34 of tRNAPro3 and Cm34 of tRNALeu (57). Therefore, we conclude that mcmoU in tRNASer1 is generated by 2′-O-methylation of mcmoU by TrmL.

In vitro reconstitution of mcmoU mediated by CmoM

To determine whether SmtA actually has methyltransferase activity, we conducted in vitro reconstitution of mcmoU formation by recombinant SmtA. In this experiment, tRNASer1 bearing cmoU was isolated from the ΔsmtA strain and used as a substrate. We clearly detected mcmoU in the tRNASer1 only in the presence of both recombinant SmtA and AdoMet (Figure 5A). CID analysis of the anticodon-containing fragment confirmed the methylation occurred at the wobble position (Figure 5B). This result demonstrated that SmtA is an AdoMet-dependent methyltransferase that transfers a methyl group to cmoU34 of tRNAs to form mcmoU34. Based on the enzymatic activity, we renamed this gene CmoM (cmoU methyltransferase).

Characterization of CmoM

CmoM belongs to the Class I AdoMet-dependent methyltransferase (MTase) family, whose members contain a Rossmann-fold as a characteristic structural motif (Figure 6A) (58–60). The high-resolution crystal structure of *E. coli* CmoM (SmtA) (PDB ID:4HTF) revealed that CmoM forms a homodimer, and that each subunit contains one molecule each of AdoMet, sulfate, acetate and 2-mercaptoethanol as ligands (Figure 6C and Supplementary Figure S5). Based on this structure, we designed eight mutant cmoM constructs bearing single amino-acid alterations (Figure 6A). The mutated residues, which are conserved in cmoM homologs, are located at the catalytic site where AdoMet is bound (Figure 6C). The ΔcmoM strain was transformed with each of the mutant constructs, and total RNA extracted from each construct was digested with RNase T1 and subjected to LC/MS to detect the RNA fragment of RNAPro3 bearing mcmoU or cmoU (Figure 6B). The positive and negative controls behaved as expected: mcmoU was fully restored by wild-type cmoM, whereas no mcmoU was formed in cells transfected with an empty vector. Little or no mcmoU was observed in the ΔcmoM strain introduced by the mutant constructs R26A, D73A, W124A, Y150A, R209A, D213A and R246A, whereas mcmoU formation was partially restored by the Y247A mutant.
Figure 4. Reverse genetic approach identified a gene responsible for terminal methylation of mcmo$^5$U. (A) Nucleoside analyzes by LC/MS using reverse phase chromatography of total RNA from wild-type (left panels), ΔsmtA (middle panels) and ΔsmtA rescued with psmtA (right panels). Top panels: UV trace at 254 nm. Second and bottom panels: XICs for cmo$^5$U (m/z 319) and mcmo$^5$U (m/z 333), respectively. Intensity of each peak was normalized to that of cyclic t6A (m/z 395). (B) Mass chromatograms of RNase T1-digested fragments containing cmo$^5$U and its derivatives from tRNA$^{Ala_1}$ isolated from wild-type (left panels) and ΔsmtA (right panels) strains. Top, middle and bottom panels: XICs for doubly-charged negative ions of the cmo$^5$U34-containing fragments (CUUmcmo$^5$U, m/z 829.08), the mcmo$^5$U34-containing fragments (CUUmcmo$^5$U, m/z 836.09) and the Um32/mcmo$^5$U34-containing fragment (CUmUcmo$^5$U, m/z 843.10), respectively. The peak marked with an asterisk represents the Um32/cmcmo$^5$U-containing fragment (CUmUcmo$^5$U, m/z 836.09). (C) Mass chromatograms of RNase T1-digested fragments containing cmo$^5$U and its derivatives from tRNA$^{Ser_1}$ isolated from wild-type (left panels), ΔsmtA (middle panels) and ΔtrmL (right panels) strains. Top, middle and bottom panels: XICs for doubly-charged negative ions of the cmo$^5$U34-containing fragments (UCUmcmo$^5$U, m/z 836.09), the mcmo$^5$U34-containing fragments (UCUmcmo$^5$U, m/z 843.10) and the mcmo$^5$U34-containing fragment (UCUmcmo$^5$Um, m/z 850.10), respectively.
Figure 5. In vitro reconstitution of cmo5U methylation by recombinant CmoM. (A) *E. coli* tRNAs2l bearing cmo5U isolated from the ΔcmoM strain was incubated in the presence or absence of recombinant CmoM with or without AdoMet. Top and bottom panels: XICs for doubly-charged negative ions of the cmo5U34-containing fragments (UCmUcmo5UGp, \textit{m/z} 836.09) and the mcmo5U34-containing fragments (UCmUmcmo5UGp, \textit{m/z} 843.10), respectively. (B) A CID spectrum of RNase T1-digested fragment of *E. coli* tRNAs2l incubated in the presence of recombinant CmoM with AdoMet. The doubly-charged negative ion of the mcmo5U34-containing fragment (UCmUmcmo5UGp, \textit{m/z} 843.10) was used as the precursor ion for CID. The product ions were assigned according to the literature (66). Sequences of parent ion and assigned product ions are described upper left side in this panel.

data indicate that the highly conserved residues in the catalytic center are essential for normal methyltransferase activity.

Terminal methylation of mcmo5U contributes to the decoding process

To investigate the functional role of the terminal methylation of mcmo5U, we constructed dual-luciferase reporters based on the RF2 recoding system (32,41). Renilla luciferase (Rluc) was fused to firefly luciferase (Fluc) by a linker sequence bearing the +1 frameshift signal of the RF2 recoding site (Figure 7A). The original UGA codon at the frameshift site was replaced with GCG or UCG to examine the decoding abilities of tRNA\textsubscript{Ala} and tRNA\textsubscript{Ser}, respectively. The GCG codon is exclusively deciphered by tRNA\textsubscript{Ala} (mcmo5UGC), whereas the UCG codon is redundantly recognized by tRNA\textsubscript{Ser} (mcmo5UGA) and tRNA\textsubscript{Ser2} (CGA) (Supplementary Figure S6) (14). We also prepared a control reporter construct lacking the +1 frameshift site (zero frame) (Figure 7A). Each of these reporters was introduced into wild-type (WT), ΔcmoM and ΔcmoB strains. The decoding ability of the test codon at the frameshift site is reflected by the +1 frameshift activity. Because the +1 frameshift activity in this system is pro-
Figure 6. Characterization of CmoM. (A) Sequence alignment of CmoM homologs from six γ-proteobacteria, *Escherichia coli* (NP_415441.1), *Salmonella enterica* (NP_455477.1), *Aeromonas hydrophila* (YP_856903.1), *Vibrio cholerae* (NP_231353.1), *Pseudoalteromonas atlantica* (ABG40695.1), *Pseudomonas aeruginosa* (NP_253478.1), and two Actinobacteria, *Modestobacter marinus* (WP_014741474.1), *Streptomyces coelicolor* (WP_011028138.1). Identical or similar residues are shaded in black or gray, respectively. Red triangles indicate residues that are essential (filled) or non-essential (open) for generic complementation. Motifs I to VI are conserved in Class I AdoMet-dependent methyltransferases. (B) XICS for doubly-charged negative ions of the cmo5U34-containing fragment (black lines, *UmUmcmo5UGp*, m/z 683.57) and the cmo5U34-containing fragment (red lines, *UmUmcmo5UGp*, m/z 690.57) from tRNAPro3 in the ΔcmoM strain rescued by plasmid-encoded wild-type cmoM or its mutant derivatives. The peak marked with an asterisk represents unspecific peak. (C) Close-up view of the AdoMet-binding site in the crystal structure of *E. coli* CmoM (PDB ID: 4HTF) containing ligands, AdoMet, acetate and sulfate. Predicted hydrogen bonds between ligands and CmoM are indicated by red dotted lines.
Figure 7. Terminal methylation of mcmo5U contributes to GCG decoding. (A) Schematic depiction of the dual-luciferase reporter constructs based on the RF2 recoding system. SD, Shine-Dalgarno sequence. Renilla and firefly luciferases were fused with a linker containing the +1 frameshift signal of the RF2 recoding site. The frameshift target site was replaced with a GCG codon for tRNAAla1, a UCG codon for tRNASer1 or GG for zero frame (used as a control). (B) Relative pausing activity at the frameshift site with GCG (left), UCG (middle) or zero frame (right) was calculated based on relative Fluc activity normalized to Rluc activity in wild-type, ΔcmoM and ΔcmoB strains. Data are presented as means ± SD (n = 4). *, P < 0.01 versus control (Student’s t-test).

moted by the ‘hungry’ A-site, the ability of the cognate tRNA to decode the test codons can be estimated indirectly. The +1 frameshift activity was calculated from the Fluc signal against the Rluc signal (F/R value). No difference in the frameshift activity of the zero frame construct, used as a negative control, was observed in the three strains (Figure 7B). In the ΔcmoB strain, in which all mcmo5U/cmo5U should be converted to ho5U, we observed clear stimulation of +1 frameshift activity at both GCG and UCG codons (Figure 7B), indicating that the carboxymethyl group of cmo5U contributes to decoding of G-ending codons. This result is consistent with the previous reports (32, 61). On the other hand, in the ΔcmoM strain, we detected a slight but significant stimulation of frameshift activity at the GCG codon (Figure 7B), but not at UCG. This observation indicates that the terminal methyl group of mcmo5U is partially involved in GCG decoding by tRNAAla1. The absence of any reduction in UCG decoding in ΔcmoM strain can be explained by the fact that this codon is redundantly deciphered by tRNASer1 and tRNASer2; tRNASer2 might have compensated for the reduced decoding ability of hypomodified tRNASer1 in the ΔcmoM strain.

DISCUSSION

It is difficult to estimate the exact frequency of an RNA modification with unstable chemical structure, such as an ester group, in individual tRNAs, because such modifications are easily hydrolyzed during tRNA isolation (42). Although the presence of mcmo5U in cellular tRNAs was reported previously, the exact frequency of this modification in individual tRNAs remained unknown (14, 22). Using the shotgun approach, we showed here that mcmo5U is present in nearly 100% of tRNAPro3 and tRNAThr4 molecules isolated from stationary-phase E. coli (Figure 3B). This method can be applied to analysis of other RNA modifications with unstable chemical structures from various sources, including 5-methoxycarbonylmethyluridine (mcm3U), wybutosine (yW), cyclic N6-threonylcarbamoyladenosine (ct6A), glutamylqueuosine (GluQ) and their derivatives. In addition, we used RCC to estimate the fraction of mcmo5U hydrolyzed during tRNA isolation. Judging from the mcmo5U frequency (≈80%) in the isolated tRNAs, we concluded that ≈20% of mcmo5U was converted to cmo5U during tRNA isolation (Figure 2B), indicating that all four tRNAs (tRNAAla1, tRNASer1, tRNAPro3 and tRNAThr4) are fully modified with mcmo5U in stationary-phase E. coli.
E. coli. The four tRNAs containing mcmo5^U all have G35 at the second letter of the anticodon, and therefore specify NCN codons, implying that CmoM preferentially recognizes G35. However, because mcmo5^U was present (albeit at a low frequency, <10%) in tRNA\textsuperscript{Leu3} and tRNA\textsuperscript{Val1}, which do not have G35 (Figure 2B), this residue is not an essential determinant for CmoM.

By applying the shotgun approach to total tRNA, we observed growth phase-dependent alteration of mcmo5^U in tRNA\textsuperscript{Pro3} (Figure 3B). In all phases of cell growth, cmo5^U was fully incorporated into this tRNA. In early log phase, cmo5^U of tRNA\textsuperscript{Pro3} was partially modified by CmoM to yield mcmo5^U with a frequency of 30%. As growth proceeded, the level of mcmo5^U gradually increased, and the level of cmo5^U concomitantly decreased. At late log and stationary phases, tRNA\textsuperscript{Pro3} was fully modified with mcmo5^U. According to the GEO profile database (ID: 35525121 and 35543521) (62), the steady-state level of cmoM mRNA is temporarily elevated in early log phase, and is expressed at a constant in late log and stationary phases. Therefore, we speculate that hypomodification of mcmo5^U in tRNA\textsuperscript{Pro3} might be due to slow methylation by CmoM that fails to catch up with fast production of tRNA in early log phase; as growth rate decreases, mcmo5^U accumulates gradually. By contrary, tRNA\textsuperscript{Thr4} was fully modified with mcmo5^U in all growth phases (Figure 3B). In addition, a high level of mcmo5^U was also found in tRNA\textsuperscript{Ala1}, tRNA\textsuperscript{Ser1} as well as tRNA\textsuperscript{Thr4} isolated from log phase E. coli. These results imply that cmo5^U34 is a better substrate for CmoM in these three tRNAs than in tRNA\textsuperscript{Pro3}. The growth phase dependency of mcmo5^U in tRNA\textsuperscript{Pro3} might be involved in regulatory decoding of CCN codons in growth phase-specific gene expression. Further studies will be necessary to examine this speculation.

Because ΔcmoM exhibited no obvious growth phenotype (data not shown) (63), the functional role of the terminal methyl group of mcmo5^U may be limited. To characterize this modification, we employed a reporter assay based on the RF2 recoding system to estimate the ability to decode GCG and UCG codons in the presence or absence of cmoM (Figure 7B). The +1 frameshift activity at a GCG codon increased specifically in the absence of cmoM, indicating that mcmo5^U facilitates decoding of GCG by tRNA\textsuperscript{Ala1}. However, we observed no change in UCG decoding in the absence of cmoM, because the UCG codon is redundantly recognized by the other isoacceptor. Similarly, it is difficult to assess the decoding ability of other NCN codons in the absence of cmoM, because NCA codons are recognized exclu-
sively by Watson–Crick base pairs, and other NCN codons are redundantly deciphered by two isoacceptors (14). However, in light of our findings, it is reasonable to assume that the terminal methyl group of mcmo5U contributes to NCN decoding in general.

According to the crystal structure of the 30S ribosomal subunit in complex with ASL of tRNAVal bearing the cmo5UAC anticodon and its cognate codons (33), the carboxylate of cmo5U forms a hydrogen bond with the N6 amino group of A35 (the second letter of the anticodon). This interaction is one component of the intramolecular hydrogen bonding network that pre-structures the anticodon loop, so that cmo5U can pair with all four bases at the third letters of codons. We showed here that mcmo5U is primarily present in tRNAs with anticodons containing G35. When cmo5U is present in these tRNAs at the ribosome A-site, the carboxylate of cmo5U cannot form a hydrogen bond with G35. To make the matter worse, cmo5U might be destabilized due to electrostatic repulsion between the carboxylate and the O6 carbonyl oxygen of G35, both of which are negatively charged. The terminal methyl group of mcmo5U neutralizes the negative charge of cmo5U carboxylate, suggesting that mcmo5U is involved in stabilizing the wobble base in the anticodons containing G35.

The crystal structure of CmoM also reveals AdoMet and other ligands bound to the positively-charged surface, which might be involved in tRNA recognition (Supplementary Figure S5). Seven residues essential for mcmo5U formation, R26, D73, W124, Y150, R209, D213 and R246, which we identified in this study, reside near the ligand-binding site on the positively charged surface of CmoM (Figure 6C). R26 and D73 play a critical role in positioning AdoMet by forming hydrogen bonds. D73 is a conserved carboxylate in motif II of AdoMet MTase (Figure 6A) (58, 59). W124 may participate in AdoMet binding via a stacking interaction with the adenine base of AdoMet. Given that W124 is in close proximity to the methyl group of AdoMet, it might be involved in the interaction with the cmo5U base of tRNA and thereby facilitate the cmo5U methylation. R26 forms a network of hydrogen bonds with two other essential residues, R209 and Y247, along with a sulfate. R209 extends the network to D213, which interacts with R246. R246 and Y150 to form the binding site for an acetate. The functional roles of the sulfate and the acetate bound to the catalytic site remain unknown, but these ligands may act as mimics for the phosphate group of tRNA bound to CmoM.

Phylogenetic analysis revealed that cmoM is present in γ-proteobacteria, actinobacteria and a few species in other bacterial clades (Figure 6A). Because actinobacteria doesn't have homologs of cmoB, cmo5U is not predicted to be present in this organism, indicating that actinobacterial counterpart is not a functional homolog of cmoM. Consistent with this, two essential residues, W124 and Y150, in E. coli CmoM are not conserved in there organisms (Figure 6A). Thus, CmoM and mcmo5U are mainly distributed in γ-proteobacteria. Presence of cmoM homologs in Spirochaeta cellobiosiphila (Spirochaeta), Zetaproteobacteria bacterium (ζ-proteobacteria) and Paenibacillus sophorae (Firmicutes) indicates horizontal gene transfer of cmoM from γ-proteobacteria to these species.

In the biogenesis of cmo5U and mcmo5U (Figure 8), six tRNA species (tRNAAla1, tRNALeu3, tRNAPro3, tRNASer1, tRNAThr4 and tRNAVal1) first undergo hydroxylolation at C5 of the uracil base in U34 to yield ho5U34; the enzyme and substrate involved in this process are unknown. Next, ho5U34 is further modified by CmoB using SCM-SAHA as a substrate to yield cmo5U34. SCM-SAHA is generated from AdoMet and prephenate in a reaction catalyzed by CmoA. For four tRNA species (tRNAAla1, tRNAPro3, tRNASer1 and tRNAThr4), cmo5U34 is methylated by CmoM in the presence of AdoMet to yield mcmo5U34. Only in tRNASer1, small portion of mcmo5U34 is further methylated by TrmL to yield mcmo5Um34. Alternatively, cmo5U34 could be first converted to cmo5Um34, then to mcmo5Um34. Growth phase-dependent alteration of mcmo5U34 takes place in tRNAPro3, implying a possible mechanism of translational control mediated by the regulatory decoding efficiency of CCN codons.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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REFERENCE

1. Machnicka, M.A., Milanowska, K., Osman Oglou, O., Purta, E., Kurkowska, M., Olekowik, A., Januszewski, W., Kalinowski, S., Dunin-Horkawicz, S., Rother, K.M. et al. (2013) MODOMICS: a database of RNA modification pathways–2013 update. Nucleic Acids Res., 41, D262–D267.
2. Yokoyama, S. and Nishimura, S. (1995) In: Soll, DRUL (ed). tRNA: Structure, Biosynthesis, and Function. American Society for Microbiology, Washington, D.C., pp. 207–224.
3. Björk, G. (1995) In: Soll, DRUL (ed). tRNA: Structure, Biosynthesis, and Function. American Society for Microbiology, Washington, D.C., pp. 165–205.
4. Suzuki, T. (2005) In: Grosjean, H (ed). Fine-Tuning of RNA Functions by Modification and Editing. GmbH & Co. KG, Springer-Verlag Berlin and Heidelberg, Vol. 12, pp. 23–69.
5. Crick, F.H. (1966) Codon–anticodon pairing: the wobble hypothesis. J. Mol. Biol., 19, 548–555.
6. Barrell, B.G., Anderson, S., Bankier, A.T., de Bruijn, M.H., Chen, E., Coulson, A.R., Drouin, J., Eperon, I.C., Nierlich, D.P., Roe, B.A. et al. (1980) Different pattern of codon recognition by mammalian mitochondrial tRNAs. Proc. Natl. Acad. Sci. U.S.A., 77, 3164–3166.
7. Inagaki, Y., Kojima, A., Bessho, Y., Hori, H., Ohama, T. and Osawa, S. (1995) Translation of synonymous codons in family boxes by Mycoplasma capricolum tRNAs with unmodified uridine or adenosine at the first anticodon position. J. Mol. Biol., 251, 486–492.
8. Suzuki, T., Nagao, A. and Suzuki, T. (2011) Human Mitochondrial tRNAs: Biogenesis, Function, Structural Aspects, and Diseases. Annu. Rev. Genet., 45, 299–329.

9. Suzuki, T. and Suzuki, T. (2014) A complete landscape of post-transcriptional modifications in mammalian mitochondrial tRNAs. Nucleic Acids Res., 42, 7346–7357.

10. Yokoyama, S., Watanabe, T., Murao, K., Ishikura, H., Yamazumi, Z., Nishimura, S. and Miyazawa, T. (1985) Molecular Mechanism of Codon Recognition by tRNA Species with Modified Uridine in the First Position of the Anticodon. Proc. Natl. Acad. Sci. U.S.A., 82, 4905–4909.

11. Agris, P.F., Soll, D. and Seno, T. (1973) Biological function of 2-mercaptouridine in Escherichia coli glutamic acid transfer ribonucleic acid. Biochemistry, 12, 4331–4337.

12. Kirino, Y., Yasukawa, T., Ohta, S., Akira, S., Ishihara, K., Watanabe, K. and Suzuki, T. (2004) Codon-specific translational defect caused by a wobble modification deficiency in mutant tRNA from a human mitochondrial disease. Proc. Natl. Acad. Sci. U.S.A., 101, 15070–15075.

13. Murao, K., Saneyoshi, M., Harada, F. and Nishimura, S. (1970) Nucleic Acids Research, 2016, Vol. 44, No. 2.

14. Murao, K., Saneyoshi, M., Harada, F. and Nishimura, S. (1984) Nucleotide sequence of E. coli B frameshift site. Nucleic Acids Res., 16, 2258–2268.

15. Yaniv, M. and Barrell, G.B. (1969) Nucleotide sequence of E. coli B tRNA-Val. Nature, 222, 278–279.

16. Harada, F., Kimura, F. and Nishimura, S. (1969) Nucleotide sequence of valine tRNA 1 from Escherichia coli B. Biochim. Biophys. Acta, 198, 590–592.

17. Kuchino, Y., Yabusaki, Y., Mori, F. and Nishimura, S. (1984) Tyrosine tRNA from Salmonella typhimurium. Nucleic Acids Res., 12, 657–662.

18. Ishikura, H., Yamada, Y. and Nishimura, S. (1971) Structure of uridine tRNA from Escherichia coli. Isolation of mutants resistant to amino acid analogs. Biochim. Biophys. Acta, 228, 471–481.

19. Williams, R.J., Nagel, W., Roe, B. and Dudock, B. (1974) Primary structure of E. coli alanine tRNA: relation to the yeast phenylalanyl-tRNA synthetase recognition site. Biochim. Biophys. Acta, 354, 16–24.

20. Vera, S., Bouaziz, G.H. and Surette, M.G. (2006) Aminoacyl-tRNA synthetase distribution is influenced by the global transcriptional regulator ArcB. Mol. Microbiol., 60, 1215–1221.

21. Kuchino, Y., Morinobu, T., Morii, F. and Nishimura, S. (1984) Nucleotide sequences of three proline tRNAs from Salmonella typhimurium. Nucleic Acids Res., 12, 1559–1562.

22. Pope, W.T. and Reeves, R.H. (1978) The identification of tRNA substrates for the supK tRNA methylase. Nucleic Acids Res., 5, 1041–1058.

23. Pope, W.T. and Reeves, R.H. (1978) Purification and characterization of a tRNA methylase from Salmonella typhimurium. J. Bacteriol., 136, 191–200.

24. Lesiewicz, J. and Dudock, B. (1977) In vitro methylation of E. coli alanine tRNA with homologous E. coli methylase. Fed. Proc., 36, 705.

25. Murao, K., Hasegawa, T. and Ishikura, H. (1976) Uridine-5-oxyacetic acid: a new minor constituent from E. coli valine tRNA. Nature, 267, 601–604.

26. Phelps, S.S., Malkiewicz, A., Agris, P.F. and Joseph, S. (2004) Modified Nucleotides in tRNA Lys and tRNA Val Are Important for Translation. J. Mol. Biol., 338, 439–444.

27. Samuelsson, T., Elias, P., Lustig, F., Axberg, T., Fölsch, G., Akesson, B. and Lagerkvist, U. (1980) Aberrations of the classic codon reading scheme during protein synthesis in vitro. J. Biol. Chem., 255, 4583–4588.

28. Takemoto, T., Takeishi, K., Nishimura, S. and Ukitani, T. (1973) Transfer of valine into rabbit haemoglobin from various isoaccepting species of valyl-tRNA differing in codon recognition. Eur. J. Biochem., 38, 489–496.

29. Kimura, S., Miyauchi, K., Kang, B.I., Ikeuchi, Y., Thiaville, P.C., Crecy-Lagard, V. and Suzuki, T. (2015) A cyclic form of N6-threonylcarnosine as a widely distributed tRNA modification in Escherichia coli 16S rRNA. Nucleic Acids Res., 38, 1341–1352.

30. Kamakari, Y., Kondo, Y.H., Bjork, G.R., Ikeda, H. and Nakamura, Y. (1988) Chromosomal location and structure of the operon encoding peptide-chain-release factor 2 of Escherichia coli. Proc. Natl. Acad. Sci. U.S.A., 85, 5620–5624.

31. Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K.A., Tomita, M., Wanner, B.L. and Mori, H. (2006) Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol. Syst. Biol., 2, 20060008.

32. Sakaguchi, Y., Miyauchi, K. and Suzuki, T. (2010) Fine-tuning of the ribosomal decoding center by conserved methyl-modifications in the Escherichia coli 16S rRNA. Nature, 498, 123–126.

33. Curran, J.F. (1993) Analysis of effects of tRNA:message stability on frameshift frequency at the Escherichia coli RF2 programmed frameshift site. Nucleic Acids Res., 21, 1837–1843.

34. Bjork, G.R. (1980) A novel link between the biosynthesis of aromatic amino acids and transfer RNA modification in Escherichia coli. J. Mol. Biol., 140, 391–410.

35. Hagervall, T.G., Jonsson, Y.H., Edmondson, C.G., McCloskey, J.A. and Bjork, G.R. (1990) Chorismate, a key metabolite in modification of tRNA. J. Bacteriol., 172, 252–259.

36. Byrne, R.T., Whelan, F., Aller, P. and Bird, L.E. (2013) S-Adenosyl-L-carboxymethyl-L-homocysteine: a novel cofactor found in the putative tRNA-modifying enzyme Cm0. Acta Crystallogr. D Biol. Crystallogr., 69, 1090–1098.

37. Kim, J., Xiao, H., Bonanno, J.B., Kalyanaraman, C., Brown, S., Tang, X., Al-Obaidi, N.F., Patksovsky, Y., Babbitt, P.C., Jacobson, M.P. and et al. (2013) Structure-guided discovery of the metabolic carboxy-SAM that modulates tRNA function. Nature, 498, 123–126.

38. Kimura, S. and Suzuki, T. (2010) Fine-tuning of the ribosomal decoding center by conserved methyl-modifications in the Escherichia coli 16S rRNA. Nucleic Acids Res., 38, 1341–1352.

39. Curran, J.F. (1993) Analysis of effects of tRNA:message stability on frameshift frequency at the Escherichia coli RF2 programmed frameshift site. Nucleic Acids Res., 21, 1837–1843.

40. Miyazawa, T., Kato, J., Nishimura, A. and Suzuki, T. (2006) Nucleoside Analysis by Hydrophilic Interaction Liquid Chromatography Coupled with Mass Spectrometry. Methods Enzymol., 506, 19–28.

41. Kimura, S., Miyauchi, K., Ikeuchi, Y., Thiaville, P.C., Crescent-Lagar, V. and Suzuki, T. (2014) Discovery of the beta-barrel-type RNA methyltransferase responsible for N6-methylation of N6-threonylcarnosine in tRNAs. Nucleic Acids Res., 42, 9350–9365.

42. Hirabayashi, N., Sato, N.S. and Suzuki, T. (2006) Conserved Loop Sequence of Helix 69 in Escherichia coli 23 S RNA Is Involved in A-site tRNA Binding and Translational Fidelity. J. Biol. Chem., 281, 17203–17211.

43. Kawai, G., Yamamoto, Y., Kamimura, T., Masugi, T., Sekine, M., Hata, T., Inomi, T., Watanabe, T., Miyazawa, T. and Yokoyama, S. (1992) Conformational rigidity of specific pyrimidine residues in tRNA arises from posttranscriptional modifications that enhance steric interaction between the base and the 2′-hydroxyl group. Biochemistry, 31, 1040–1046.

44. Ikeuchi, Y., Shigii, N., Kato, J., Nishimura, A. and Suzuki, T. (2006) Mechanistic insights into sulfur relay by multiple sulfur mediators
50. Ikeuchi, Y., Kitahara, K. and Suzuki, T. (2008) The RNA acetyltransferase driven by ATP hydrolysis synthesizes N4-acetylcytidine of tRNA anticodon. *EMBO J.*, **27**, 2194–2203.

51. Soma, A., Ikeuchi, Y., Kanemasa, S., Kobayashi, K., Ogawara, N., Oto, T., Kato, J., Watanabe, K., Sekine, Y. and Suzuki, T. (2003) An RNA-modifying enzyme that governs both the codon and amino acid specificities of isoleucine tRNA. *Mol. Cell*, **12**, 689–698.

52. Kimura, S., Ikeuchi, Y., Kitahara, K., Sakaguchi, Y. and Suzuki, T. (2012) Base methylations in the double-stranded RNA by a fused methyltransferase bearing unwinding activity. *Nucleic Acids Res.*, **40**, 4071–4085.

53. Noma, A., Ishitani, R., Kato, M., Nagao, A., Nureki, O. and Suzuki, T. (2010) Expanding role of the jumonji C domain as an RNA hydroxylase. *J. Biol. Chem.*, **285**, 34503–34507.

54. Noma, A., Kirino, Y., Ikeuchi, Y. and Suzuki, T. (2006) Biosynthesis of wybutosine, a hyper-modified nucleoside in eukaryotic phenylalanine tRNA. *EMBO J.*, **25**, 2142–2154.

55. Noma, A., Sakaguchi, Y. and Suzuki, T. (2009) Mechanistic characterization of the sulfur-relay system for eukaryotic 2-thiouridine biogenesis at tRNA wobble positions. *Nucleic Acids Res.*, **37**, 1335–1352.

56. Noma, A., Yi, S., Katoh, T., Takai, Y. and Suzuki, T. (2011) Actin-binding protein ABP140 is a methyltransferase for 3-methylcytidine at position 32 of tRNAs in Saccharomyces cerevisiae. *RNA*, **17**, 1111–1119.

57. Benitez-Páez, A., Villarroya, M., Douthwaite, S., Gabaldón, T. and Armengod, M.-E. (2010) YibK is the 2′-O-methyltransferase TrmL that modifies the wobble nucleotide in Escherichia coli tRNA(Leu) isoacceptors. *RNA*, **16**, 2131–2143.