Unexpected Accumulation of ncm$^5$U and ncm$^5$s$^2$U in a trm9 Mutant Suggests an Additional Step in the Synthesis of mcm$^5$U and mcm$^5$s$^2$U

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Unexpected Accumulation of ncm5U and ncm5s2U in a trm9 Mutant Suggests an Additional Step in the Synthesis of mcm5U and mcm5s2U

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Abstract

Background: Transfer RNAs are synthesized as a primary transcript that is processed to produce a mature tRNA. As part of the maturation process, a subset of the nucleosides are modified. Modifications in the anticodon region often modulate the decoding ability of the tRNA. At position 34, the majority of yeast cytosolic tRNA species that have a uridine are modified to 5-carbamoylmethyluridine (ncm5U), 5-carbamoylmethyl-2'-O-methyluridine (ncm5Um), 5-methoxycarbonylmethyl-uridine (mcm5U) or 5-methoxycarbonylmethyl-2-thiouridine (mcm5s2U). The formation of mcm5 and ncm5 side chains involves a complex pathway, where the last step in formation of mcm5 is a methyl esterification of cm5 dependent on the Trm9 and Trm112 proteins.

Methodology and Principal Findings: Both Trm9 and Trm112 are required for the last step in formation of mcm5 side chains at wobble uridines. By co-expressing a histidine-tagged Trm9p together with a native Trm112p in E. coli, these two proteins purified as a complex. The presence of Trm112p dramatically improves the methyltransferase activity of Trm9p in vitro. Single tRNA species that normally contain mcm5U or mcm5s2U nucleosides were isolated from trm9Δ or trm112Δ mutants and the presence of modified nucleosides was analyzed by HPLC. In both mutants, mcm5U and mcm5s2U nucleosides are absent in tRNAs and the major intermediates accumulating were ncm5U and ncm5s2U, not the expected cm5U and cm5s2U.

Conclusions: Trm9p and Trm112p function together at the final step in formation of mcm5U in tRNA by using the intermediate cm5U as a substrate. In tRNA isolated from trm9Δ and trm112Δ strains, ncm5U and ncm5s2U nucleosides accumulate, questioning the order of nucleoside intermediate formation of the mcm5 side chain. We propose two alternative explanations for this observation. One is that the intermediate cm5U is generated from ncm5U by a yet unknown mechanism and the other is that cm5U is formed before ncm5U and mcm5U.

Introduction

Transfer RNAs are adapter molecules, which decode mRNA into protein and thereby play a central role in gene expression. The primary tRNA transcript is processed by different endo and exonucleases, and tRNA modifying enzymes to produce a mature tRNA [1,2,3]. In this maturation process, a subset of the four normal nucleosides adenosine (A), guanosine (G), cytidine (C) and uridine (U) are modified [2,3]. The modifications are introduced post-transcriptionally, and the formation of a modified nucleoside may require one or several enzymatic steps [2,3]. Of the 50 modified nucleosides so far identified in eukaryotic tRNAs, 25 are present in cytoplasmic tRNAs from S. cerevisiae [2,4,5]. In the anticodon region, especially in positions 34 (wobble position) and 37, nucleosides are frequently modified. Modified nucleosides in these positions are important for reading frame maintenance and efficient decoding during translation [2,3]. In yeast, there are in total 42 cytosolic tRNA species, of which 11 have a uridine at position 34 modified to 5-carbamoylmethyluridine (ncm5U), 5-carbamoylmethyl-2'-O-methyluridine (ncm5Um), 5-methoxycarbonylmethyl-uridine (mcm5U) or 5-methoxycarbonylmethyl-2-thiouridine (mcm5s2U) [6]. The formation of these nucleosides requires addition of mcm or ncm side chains at the 5-position of the uracil moiety and a subset of these tRNAs also have a thio (s2) group at the 2-position of U34 or a methylation at the 2'-position of the ribose.

The common step in synthesis of ncm5 and mcm5 side chains at U34 in tRNAs requires at least 11 gene products (Figure 1). Deletion strains missing one of ELPI-ELP6, KTI11, KTI12, KTI14 or SIT4 genes, or both SAP185 and SAP190 genes completely lack the mcm5U, mcm5s2U and ncm5U nucleosides, whereas a kti13 deletion mutant show dramatically reduced levels of these
nucleosides [7,8]. In strains with these genes mutated, no intermediates of cm5U or cm5s2U have been detected, whereas s2U is detected in tRNAs normally containing mcm5s2U [7,8,9,10,11,12]. Thus, these gene products are required for an early step in synthesis of cm5 and ncm5 groups (Figure 1). The earliest intermediate in the synthesis of mcm5U and ncm5U that has been detected is cm5U, and there is evidence that it originates from a methyl group is added to cm5U by Trm9p/Trm112p complex in tRNA species that in their mature form should have a mcm5 side chain. The cm5U in other tRNA species is converted to ncm5U by an unknown enzyme. For tRNAs that should contain a s2 group, presence of a mcm5 or ncm5 side chain is a prerequisite for efficient thiolation.

The Elongator complex (Elp1-Elp6) and its potential regulators are required for the formation of cm5U. A methyl group is added to cm5U by Trm9p/Trm112p complex in tRNA species that in their mature form should have a mcm5 side chain. The cm5U in other tRNA species is converted to ncm5U by an unknown enzyme. For tRNAs that should contain a s2 group, presence of a mcm5 or ncm5 side chain is a prerequisite for efficient thiolation.

The Elongator complex (Elp1-Elp6) and its potential regulators are required for the formation of cm5U. A methyl group is added to cm5U by Trm9p/Trm112p complex in tRNA species that in their mature form should have a mcm5 side chain. The cm5U in other tRNA species is converted to ncm5U by an unknown enzyme. For tRNAs that should contain a s2 group, presence of a mcm5 or ncm5 side chain is a prerequisite for efficient thiolation.

The last step in formation of mcm5 side chain of U34 is a methyl esterification of cm5U [13], and requires Trm9p/Trm112p in yeast and ALKBH8/TRM112 in mammals [31,32,33]. We confirm that Trm112p is also required for the last step of mcm5 side chain formation at position 34 in a subset of tRNAs. In vivo, Trm112p is essential for the methyl esterification to mcm5U34, and in vitro Trm112p improves the methyltransferase activity of Trm9p. The observation that the major intermediates accumulating in trm9 and trm112 mutants are ncm5U and ncm5s2U and not the expected cm5U and cm5s2U raises the question; what is the order of intermediates formed in biosynthesis of the mcm5 side chain of U34?

Materials and Methods

Yeast strains, media and genetic procedures

Strains used in this report, except those from the yeast deletion collection (Open Biosystems), are listed in Table S1A. Yeast media, genetic procedures and yeast transformation have been described previously [34]. To construct Δmtq2::KanMX6 and Δtrm112::KanMX6 deletions, oligonucleotides (2104 and 2015, 1391 and 1392) in Table S1B containing 45nt sequence homology flanking the MTQ2 and TRM112 genes were used to amplify the KanMX6 cassette [35]. To delete TRM9, TRM11 and LYS9 in W303 strains, chromosomal DNA from the corresponding null mutants in the yeast deletion collection (Open Biosystems) were used as templates. The KanMX6 cassette together with 300–500 base pair flanking sequences to each gene were amplified with specific primers (1035 and 1036 for TRM9, 1950 and 1951 for TRM11, and 2059 and 2060 for LYS9) listed in Table S1B. The PCR products were introduced into diploid yeast strain UMY3104 and transformants were selected on YEPD plates containing 200 μg/ml Geneticin (G418). Transformants were sporulated and tetrads analysis verified a 2:2 segregation of mating type and G418 resistance. Deletions were confirmed by PCR. The double mutants Δtrm9ΔΔΔΔ, Δtrm11ΔΔΔΔ, Δtrm9ΔΔΔΔ Δlys9ΔΔΔΔ, Δtrm11ΔΔΔΔ Δlys9ΔΔΔΔ and Δlys9ΔΔΔΔ Δmtq2ΔΔΔΔ were generated by crossing the single mutants. The quadruplet mutant was generated in a cross between Δtrm9ΔΔΔΔ, Δtrm11ΔΔΔΔ, Δlys9ΔΔΔΔ and Δlys9ΔΔΔΔ Δmtq2ΔΔΔΔ.

Plasmid constructions

To generate the expression vector for the Trm9 protein, TRM9 gene was amplified by PCR using oligos 2015 and 2016 (Table S1B) and W303-1A genomic DNA as template. The PCR product was digested with BamHI and HindIII, and subcloned to the corresponding sites of the expression vector pRSF-Duet1 (Novagen), generating an in frame fusion with the histidine tag. To construct the Trm9p-TRM112 core-expression vector, the TRM112 gene was amplified from W303-1A genomic DNA using oligos 2013 and 2014 (Table S1B) and cloned into the pRSF-Duet1-TRM9 vector using NdeI and XhoI.

Protein purification

The expression vectors were introduced into BL21(DE3)pLysS competent cells. Overnight cultures of transformed cells were grown in LB media containing 50 μg/ml Kanamycin at 37°C. Cultures were diluted to OD600 0.05 and grown to OD600 0.5 at 37°C. Cultures were placed on ice for 10 minutes. IPTG was added to a final concentration of 120 μg/ml and protein expression was induced at 15°C overnight. Harvested cell pellets
were washed once by 0.9% NaCl and resuspended in breaking buffer (20 mM Tris pH 8.0, 10 mM imidazole, 150 mM NaCl, 0.2% NP-40, 2 mM β-mercaptoethanol) in the presence of proteinase inhibitor cocktail (Roche). Cells were broken by sonication and the cell extract was clarified by centrifugation at 16,000 g for 1 hour. The supernatant was mixed with TALON resin, equilibrated with breaking buffer and incubated at 4°C for 2 hours. The protein bound TALON resin was first washed with buffer 1 (20 mM Tris pH 8.0, 10 mM imidazole, 150 mM NaCl, 2 mM β-mercaptoethanol) and then with buffer 2 (20 mM Tris pH 8.0, 10 mM imidazole, 500 mM NaCl, 2 mM β-mercaptoethanol). Proteins were eluted with 330 mM imidazole and dialyzed overnight against storage buffer (25 mM Tris pH 8.0, 150 mM NaCl, 5 mM DTT, 10% glycerol) and kept at 4°C for future use.

### Methyltransferase reaction

In the methyltransferase reaction, 50 μl of 2X reaction buffer (200 mM Tris 7.5, 0.2 mM EDTA, 20 mM MgCl2, 20 mM NH4Cl) was mixed with 20 μl [3H]AdoMet (0.55 mCi/ml, Perkin Elmer) and 20 μg tRNA, incubated at 37°C for 5 minutes. The methyltransferase reaction was initiated by adding 10 μg Trm9p or Trm9p-Trm112p. Aliquots of the reaction was withdrawn at different time points and mixed with 1 ml of 5% ice cold trichloroacetic acid (TCA). The tubes were incubated on ice for 10 minutes and samples were vacuum filtered through nitrocellulose filter (Millipore 0.45 μm). The [3H] incorporation was measured using a Wallac 1409 scintillation counter. To analyze [3H] incorporation in total tRNA by HPLC, 200 μg of tRNA was used. After 30 minutes of methyltransferase reaction, 2.5 volume of 99% ice cold ethanol was added into the reaction and samples were kept at -20°C for 1 hour. The precipitate was collected by centrifugation and washed twice with 99% ice cold ethanol. The pellets were air dried and resuspended in 10% DTT in 1xTE buffer (pH 7.8). The [3H] incorporation was measured using a Wallac 1409 scintillation counter.

### Figure 2. trm9 and trm112 mutants are lacking the mcm5 side-chain in tRNA_{mcm5UCU} at wobble uridines.

HPLC analysis of modified tRNA nucleosides from wild-type (UMY3169, left panels), trm9::KanMX4 (Open Biosystems, middle panels) and trm112::KanMX4 (UMY3330, right panels). Arrows in red and black indicate expected retention time of mcm5U and cm5U, respectively. Arrow heads in red and black indicate expected retention time of m2G and ncm5U, respectively. (A) Part of the chromatogram between retention times 34 and 44 min is shown. (B) Part of the chromatogram between retention times 7 and 17 min is shown. The small peak in wild-type at 14 min represents an unrelated compound with a spectrum different from cm5U. The chromatograms were monitored at 254 nm.

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### Table 1. Relative amounts of various modified nucleosides in tRNA_{mcm5UCU} and tRNA_{mcm5UCU} isolated from wild type, trm9Δ and trm112Δ strains.

<table>
<thead>
<tr>
<th></th>
<th>tRNA_{mcm5UCU}</th>
<th>tRNA_{mcm5UCU}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cm5U/Y</td>
<td>cm5U/Y</td>
</tr>
<tr>
<td></td>
<td>mcm5U/Y</td>
<td>mcm5U/Y</td>
</tr>
<tr>
<td>WT</td>
<td>0.016</td>
<td>0.044</td>
</tr>
<tr>
<td>trm9Δ</td>
<td>0.051</td>
<td>0.199</td>
</tr>
<tr>
<td>trm112Δ</td>
<td>0.046</td>
<td>0.149</td>
</tr>
</tbody>
</table>

*Pseudouridine (Y) was used as the internal control. The numbers displayed are the ratios (modified nucleoside/Y). ND: not detected. The modified nucleosides cm5U, mcm5U, cm5Y and Y were monitored at 254 nm, and cm5s2U, ncm5s2U and mcm5s2U were monitored at 314 nm as thiolated nucleosides absorb well at this wavelength, while nonthiolated nucleosides do not.*

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centrifuged for 30 minutes in eppendorf tubes at maximum speed. The pellet was resuspended in MQ water, digested with nuclease P1 and analyzed by HPLC [36]. The [3H] incorporation was monitored by a flow scintillation analyzer (Packard Bioscience).

Single tRNA isolation
Yeast cells were grown in 2L YEPD at 30°C to OD600 = 1.5. Total tRNA was prepared as described [36]. Single tRNA species were isolated from total tRNA by hybridizing to biotinylated complementary oligonucleotides [36] and separated from total tRNA by attachment to streptavidin coated Dynabeads M-280 (Invitrogen). The single tRNAs were digested to nucleosides with nuclease P1 followed by bacterial alkaline phosphatase (BAP) treatment [0.2 M (NH₄)₂SO₄ pH 8.3], and analyzed by HPLC [37].

Results and Discussion
Trm112p is required for the methyl esterification of mcm5U and mcm5s²U
In a global analysis of protein complexes in yeast, Trm112p was found to interact with three methyltransferases Trm9p, Trm11p and Mtq2p [38,39,40,41]. In addition, Trm112p interacts with the saccharopine dehydrogenase Lys9p, the essential DEAH-box ATP-dependent RNA helicase Ecm16p and an essential component of the RSC chromatin remodeling complex.
Synthesis of mcm5 Side Chain in tRNA

Figure 5. Trm9p/Trm112p complex efficiently catalyzes the methyl incorporation into trm9 substrate tRNA. (A) SDS-PAGE analysis of histidine tagged Trm9p expressed alone or co-expressed with Trm112p and purified from E. coli. The gel was stained with Colloidal Blue (Invitrogen). Lane 1: Molecular weight standard (PageRuler prestained, Fermentas). Lane 2: Soluble fraction of extract from E. coli strains expressing Trm112p and histidine tagged Trm9p. Lane 3: Soluble fraction of extract from E. coli strains expressing histidine tagged Trm9p. Lane 4: Pellet from crude extract of E. coli strains expressing Trm112p and histidine tagged Trm9p. Lane 5: Pellet from crude extract of E. coli strains expressing histidine tagged Trm9p. Lane 6: Trm112p co-purified with histidine tagged Trm9 protein. Lane 7: Purified histidine tagged Trm9 protein. (B) [3H] methyl incorporation into tRNA as a function of time. Substrates were total tRNA preparations from strain UMY2067 (wild-type) and UMY3267 (trm9Δ). (■) and (▲) are methyl incorporation reactions into wild-type tRNA by using Trm9p or Trm9p/Trm112p as enzyme. (○) is methyl incorporation reaction into trm9 RNA by using Trm9p as enzyme. (C) The methyl incorporation into trm9 tRNA using Trm9p/Trm112p as enzyme (○), in addition to the reactions in (B).

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Shl1 [38,39,40,41]. The N'-Monomethylguanosine-10 (m1G10) methyltransferase Trm1lp, as well as the eRF1 methyltransferase MtbQp, has to be in complex with Trm1lp to be active [42,43]. Trm9p is required for the methyl esterification of modified uridine nucleosides, resulting in the formation of 5-methylcytosine and 5-methylcarbonylmethyls UUU [31,32]. The presence of cm5U supports the earlier suggestion to be the substrates [13,31,32,33]. Therefore, we also analyzed single tRNA species tRNA\textsubscript{mcm5-UCU}, tRNA\textsubscript{mcm5-s\textsubscript{2}UCU} and tRNA\textsubscript{mcm5-P\textsubscript{o}-UGG} from trm11Δ, lys9Δ and mtq2Δ strains. Trm1p and Trm112p are essential for formation of the m5U nucleoside [42]. Consistently, tRNA\textsubscript{mcm5-UCU} isolated from trm11Δ or trm112Δ strains does not have the m\textsuperscript{1}G modified nucleoside, whereas the same tRNA from wild-type has m\textsuperscript{5}G (Figure 2 and S1). In single tRNAs from lys9Δ and mtq2Δ strains, there was no notable change in modified nucleosides as assessed by HPLC analysis (Figure S1, data not shown). A deletion of the TRM112 gene causes a dramatic reduction in growth and a mtq2Δ strain also shows a clear reduction in growth, whereas trm11Δ, lys9Δ or trm12Δ strains show mild growth defects in YEPD medium at both 30°C and 37°C (Figure 4). We considered the possibility that strains with multiple null alleles of genes encoding Trm112p interacting proteins would show additive growth defects, possibly mimicking a trm11Δ null allele. Since two Trm112p associated proteins, Ecm16 and Shl1, are encoded by essential genes, we were only able to make strains with combinations of the trm11Δ, lys9Δ, trm12Δ, and mtq2Δ alleles. We first made the double mutants trm11Δ lys9Δ, trm11Δ trm12Δ, trm11Δ mtq2Δ, lys9Δ trm12Δ, mtq2Δ lys9Δ and mtq2Δ. No additive growth reduction was observed in any of the constructs at both 30°C and 37°C (Figure 4, data not shown), in contrast to the previously observed growth defect of the mtq2Δ mutant [33]. Further we made a trm11Δ lys9Δ trm12Δ, mtq2Δ quadruple mutant strain that grew like a mtq2Δ strain at both 30°C and 37°C (Figure 4). These data show that the poor growth of trm112Δ cells is not entirely caused by defects in tRNA modification, eRF1 methylation and dehydrogenase activity in the quadruple mutant. Possibly it is caused by reduced function of Ecm16p or Shl1p which might require the interaction with Trm112p to be fully active.

Trm112p/Trm9p complex efficiently incorporates methyl groups into trm9 substrate tRNA in vitro

Trm9p has been shown to catalyze the methyl esterification to mcm\textsuperscript{5}U and mcm\textsuperscript{s\textsubscript{2}}U in vitro [31]. We cloned the TRM9 gene into the expression vector pRSF to produce 6xHis-Trm9p recombinant protein in E. coli. We also made a pRSF vector construct, simultaneously expressing the 6xHis-Trm9p recombinant protein and a non-tagged Trm112p. When Trm9p was expressed alone, the majority of Trm9p recombinant protein was insoluble (Figure 3A), and the solubility of Trm9p dramatically improved when Trm112p was co-expressed with Trm9p. Purification of Trm9p by virtue of its 6xHis tag resulted in copurification of Trm112p (Figure 3A), indicating that Trm9p forms a stable complex with Trm112p.

Purified Trm9p and Trm9p/Trm112p complex was used to methylate total tRNA isolated from wild-type and a trm9 deletion strains in vitro. Saponification of total tRNA with sodium hydroxide leads to the production of cm\textsuperscript{5}U and cm\textsuperscript{s\textsubscript{2}}U from mcm\textsuperscript{5}U and mcm\textsuperscript{s\textsubscript{2}}U, and this method has previously been used to generate...
substrates for Trm9p or ALKBH8 [31,32]. However, saponification also efficiently degrades tRNA and we found that tRNA isolated from the trm9 deletion strain was a superior substrate in the methyl esterification assay (data not shown). To track methylation of tRNA substrates in vitro, S-adenosylmethionine containing a tritiated methyl donor group was used together with tRNA and purified enzyme. When total tRNA from wild type was used as a substrate, there was a small increase in incorporation of

Figure 6. HPLC analysis of total trm9 tRNA after methyl incorporation by using Trm9p/Trm112p as enzyme. (A–B) Part of the chromatogram between retention time 10 and 19 min is shown. The arrow in B indicates the expected retention time of cm5U. (C–D) Part of the chromatogram between retention time 34 and 45 min is shown. The arrow in C indicates the expected retention time of mcm5s2U. (E–F) Part of the chromatogram between retention time 26 and 52 min is shown. Open and closed arrowheads in G and H indicate the expected retention time of mcm5s2U and cm5U, respectively. Chromatograms in A–F were monitored at 254 nm and at 314 nm in G–H. The dashed line in D, F and H indicates the migration of isotope labeled nucleoside which overlaps with mcm5s2U and mcm5s2U, respectively. The Y axis to the left corresponds to absorbance units and the Y axis to the right shows the [3H] incorporation in cpm.

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radioactive methyl groups with time using either Trm9p or the Trm9p/Trm112p complex (Figure 5B). In contrast, use of total tRNA from the \( \text{trm9}^\Delta \) strain and Trm9p leads to a clear but modest increase in the incorporation of radioactive methyl groups (Figure 5B). Moreover, the incorporation of radioactive methyl groups was 20-fold more efficient using Trm9p/Trm112p over Trm9p alone (Figure 5C). Thus, Trm112p is required for Trm9p to methylate its substrate tRNA more efficiently \( \text{in vitro} \) and is a prerequisite \( \text{in vivo} \) as no mcm5 nucleosides are formed in a \( \text{trm112}^\Delta \) mutant (Figure 2, Table 1). In the reaction using tRNA from the \( \text{trm9}^\Delta \) strain and Trm9p/Trm112p, there was a rapid incorporation of \([3H]\) methyl groups in the first 5 minutes that entered to a plateau after 30 minutes (Figure 5C). The reduced incorporation was not due to enzyme inactivation with time as adding more enzyme at 30 minutes did not improve incorporation of radioactivity (data not shown).

Based on HPLC analysis, there is an accumulation of cm5U, ncm5U, and ncm5s2U in total tRNA from a \( \text{trm9}^\Delta \) strain compared with a wild-type strain [33] (data not shown). When tRNA isolated from a \( \text{trm9}^\Delta \) strain was used as substrate \( \text{in vitro} \), we observed a reduction of the cm5U nucleoside and appearance of mcm5U (Figure 6A-D, Table 2) consistent with cm5U being the substrate of Trm9 [31,32,33]. Furthermore, the relative amounts of ncm5U and ncm5s2U did not change after the methylation reaction, showing that these two nucleosides are not substrates of Trm9p/Trm112p under these conditions (Table 2) [33]. By using saponified tRNA, cm5s2U was suggested to be a substrate for Trm9p or ALKBH8/Trm112 [31,32]. However, cm5s2U was not detected in total tRNA isolated from \( \text{trm9}^\Delta \) or \( \text{trm112}^\Delta \) mutants [33]. In our analysis of \( \text{tmt9} \) total tRNA, we observed a very small peak migrating in the position of cm5s2U, which was absent after the methylation reaction (Figure 6G-H, Table 2). When \([3H]\)-CH\(_3\) was monitored by flow scintillation analyzer coupled to the HPLC, we found that the incorporated radioactivity migrated with retention times identical to those known for mcm5U and mcm5s2U nucleosides (Figure 6D, F and H). As the signal for the tentative cm5s2U is very weak, we cannot exclude the possibility that cm5s2U originated from another species. These observations are consistent with those shown by Kalhor and Clarke [31,32] and fully support the assertion that Trm9p is the methyltransferase catalyzing the formation of mcm5U from cm5U. Why and how cm5U and cm5s2U accumulates in tRNAs from strains lacking Trm9p or Trm112p, remains to be elucidated.

### Table 2. Relative amounts of various modified nucleosides of total tRNA isolated from the \( \text{trm9}^\Delta \) strain before and after methylation reaction.

<table>
<thead>
<tr>
<th></th>
<th>cm5U/Y</th>
<th>ncm5U/Y</th>
<th>mcm5U/Y</th>
<th>cm5s2U/Y</th>
<th>ncm5s2U/Y</th>
<th>mcm5s2U/Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before reaction</td>
<td>0.01752</td>
<td>0.04634</td>
<td>ND</td>
<td>0.00081</td>
<td>0.00571</td>
<td>ND</td>
</tr>
<tr>
<td>After reaction</td>
<td>0.00227</td>
<td>0.04707</td>
<td>0.01720</td>
<td>ND</td>
<td>0.00521</td>
<td>0.00132</td>
</tr>
</tbody>
</table>

Pseudouridine (Y) was used as the internal control. The numbers displayed are the ratios (modified nucleoside/Y). ND: not detected. The modified nucleosides cm5U, ncm5U, mcm5U and Y were monitored at 254 nm, and cm5s2U, ncm5s2U and mcm5s2U were monitored at 314 nm as thiolated nucleosides absorb well at this wavelength, while nonthiolated nucleosides do not.

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Alternative mechanisms in formation of the mcm5 side chain at wobble position

In \( \text{trm9}^\Delta \) or \( \text{trm112}^\Delta \) strains, the major species generated are ncm5U and cm5s2U instead of the expected cm5U or cm5s2U. According to the model proposed in Figure 1, Elongator complex is required for and might directly catalyze the formation of cm5U. In the presence of Trm9 and Trm112p, cm5U is rapidly converted to mcm5U in tRNAs destined to contain a mcm5U nucleoside. Those tRNAs destined to contain cm5U are not recognized by Trm9p/Trm112p and mcm5U is formed by an uncharacterized enzyme. In order to account for the presence of cm5U and

![Figure 7](image-url)
ncm^5^5U in tRNAs that normally should contain mcm^5^U and mcm^5^s^2U, one has to postulate that in the absence of Trm9p/Trm112p the uncharacterized enzyme responsible for amidation also recognizes these tRNA substrates (Figure 2). For tRNAs that should contain a s^2 group, the presence of a mcm^5^ side chain has been suggested to be a prerequisite for efficient thiolation [10,12]. We suggest that the presence of ncm^5^, but not cm^5^, in these tRNAs also promotes efficient thiolation, resulting in accumulation of ncm^5^s^2U (Table 1).

The observation that the major U_34 intermediates in tRNA^{ncm5UC} and tRNA^{ncm5s2UC} are cm^5^U and ncm^5^U in trm9 and trm112 mutants also supports an alternative model, i.e. ncm^5^U is generated before cm^5^U (Figure 7). Such a model would require a conversion of ncm^5^U to cm^5^U before the Trm9p/Trm112p complex finally can form cm^5^U. A similar mechanism has been described in Eubacteria that have mnm^5^ instead of mcm^5^ side chains and the first intermediate in its synthesis is cmm^5^U [45]. The bi-functional MnmC demodifies cmnm^5^Ut on m5U and thereby methylates nm^5^U to form mnm^5^U [45,46,47]. By analogy, the Trm9p/Trm112p complex may be involved in two reactions; deamination of ncm^5^Ut oc m5U, and then catalyzing formation of cm^5^U. The deaminase activity is not necessarily part of Trm9p or Trm112p. In the absence of Trm9p or Trm112p, ncm^5^U accumulates in tRNAs destined to contain mcm^5^s^2U, like tRNA^{Glu}. As postulated in model 1, the presence of an ncm^5^ side chain in these tRNAs promotes thiolation, generating ncm^5^s^2U. MnmC requires flavin adenine dinucleotide (FAD) as co-factor in the de-modification reaction and SAM in the methylation reaction. We performed an in vitro reaction with [3H]AdoMet in the presence or absence of FAD. We assumed if ncm^5^U is converted to cm^5^U in the presence or absence of FAD. We used the experiments: CC BH JTA ASB. Performed the experiments: CC BH JTA ASB. Analyzed the data: CC BH JTA ASB. Contributed reagents/materials/analysis tools: JTA ASB. Wrote the paper: the experiments: CC BH JTA ASB. Performed the experiments: CC BH JTA ASB. Analyzed the data: CC BH JTA ASB. Contributed reagents/materials/analysis tools: JTA ASB. Wrote the paper: CC JTA ASB.

References
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