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Trm4 and Nsun2 RNA:m^{5}C Methyltransferases Form Metabolite-Dependent, Covalent Adducts with Previously Methylated RNA

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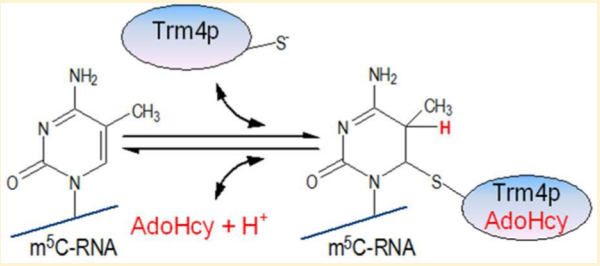
Supporting Information

ABSTRACT: Trm4p from *Saccharomyces cerevisiae* and its mammalian orthologue Nsun2 fabricate 5-methylcytosine (m^{5}C) in RNA molecules utilizing a dual-cysteine catalytic mechanism. These enzymes are now shown to form covalent complexes with previously methylated RNA. Enzyme linkage to methylated RNA requires *S*-adenosylhomocysteine (AdoHcy), and the removal of this metabolite results in the disassembly of preexisting complexes. The fraction of Trm4p linked to modified RNA is influenced by the AdoHcy concentration and by the pH of the solution, with maximal formation of Trm4p−RNA complexes observed in the pH range of 5.5−6.5. Four active-site residues critical for Trm4p-mediated tRNA methylation are also required for the formation of the denaturant-resistant complexes with m^{5}C-containing RNA. On the basis of these findings, it is proposed that formation of a covalent complex between dual-cysteine RNA:m^{5}C methyltransferases and methylated RNA provides a unique means by which metabolic factors can influence RNA. By controlling the degree of formation of the enzyme−RNA covalent complex, AdoHcy and pH are likely to influence the extent of m^{5}C formation and the rate of release of methylated RNA from RNA:m^{5}C methyltransferases. Metabolite-induced covalent complexes could plausibly affect the processing and function of m^{5}C-containing RNAs.



Enzymatic methylation of cytosine to form the 5-methylcytosine (m^{5}C) base occurs in DNA and RNA. Cytosine methylation in DNA is used by many eukaryotic organisms to mediate epigenetic control of gene expression and by some prokaryotic organisms to protect DNA from particular restriction enzymes. Likewise, the role of m^{5}C in RNA is multifaceted. The structural and metabolic stability of tRNA molecules is influenced by the conversion of cytosine to m^{5}C.[1−3] Protein translation in yeast is influenced by m^{5}C within the anticodon of tRNA$^{Leu(CAA)}$,[4] and m^{5}C at other positions within tRNA or the decoding region of rRNA may influence the translational process.[1,2,5] Recent studies have demonstrated the site specific presence of m^{5}C in mRNAs and additional noncoding RNAs,[6−8] generating evidence that the functions of m^{5}C may include the regulation of RNA processing and mRNA stability.[8,9]

It is likely that the majority of m^{5}C residues in RNA are produced by enzymes of the widely distributed Nol1/Nop2/Sun family of RNA:m^{5}C methyltransferases, although specific m^{5}C residues in RNA are formed by enzymes outside of this immediate enzyme family.[10,11] Two well-studied Nol1/Nop2/Sun enzymes are the yeast tRNA methyltransferase Trm4p and its mammalian orthologue Nsun2. Trm4p forms m^{5}C at multiple locations in the tRNA molecules of *Saccharomyces cerevisiae*[12] and plays a role in the organism's response to oxidative stress.[4] Nsun2 was initially thought to have a single tRNA target.[13] Instead, recent studies revealed that Nsun2 targets several tRNAs at multiple sites, mRNAs, and noncoding RNAs other than tRNA.[6−8,14] Nsun2 has been identified as a downstream mediator of Myc-induced proliferation and a component of the mitotic spindle.[15,16] The protein plays a role in tissue differentiation,[17,18] and defects in human Nsun2 cause an autosomal recessive form of intellectual disability.[19−22]

Enzymes of the Nol1/Nop2/Sun family contain a substantial region of conserved amino acids, which includes several short motifs shared widely with enzymes containing Rossman folds, including DNA:m^{5}C and RNA:m^{5}U methyltransferases.[23] The RNA:m^{5}C, DNA:m^{5}C, and RNA:m^{5}U methyltransferases all utilize covalent catalysis, but the location of the responsible cysteine residue varies.[24] Covalent catalysis is mediated by a cysteine within motif IV of DNA:m^{5}C methyltransferases, but the functionally analogous cysteine is located in motif VI of RNA:m^{5}U and RNA:m^{5}C methyltransferases.[24−26] A unique characteristic of the Nol1/Nop2/Sun family is that motifs IV and VI each contain a conserved cysteine and both are required for completion of the catalytic cycle. For Trm4p, Cys^{310} within motif VI forms the covalent adduct between the enzyme and RNA,[27] with the same role being mediated by Cys^{321} of Nsun2. The release of methylated RNA requires the motif IV cysteine (Cys^{260}) of Trm4p and Cys^{271} of Nsun2. The chemical role proposed for the motif IV cysteine is extraction of a proton from C5 of the methylated base to mediate double bond re-formation within the target base, thus aiding the release of RNA

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from the enzyme.[28,29] Mutation of the motif IV cysteine in either Trm4p or Nsun2 results in the accumulation of enzyme−RNA complexes.[8,16,28,30] Structural studies of confirmed and putative Nol1/Nop2/Sun methyltransferases have shown the motif IV and VI cysteines to be spatially close within the active site, despite their separation in the primary sequence.[29,31−37]

The conservation of the dual-cysteine active site in the NOL1/NOP2/Sun family of RNA:m^{5}C methyltransferases is intriguing because DNA:m^{5}C and RNA:m^{5}U methyltransferases perform pyrimidine methylation using a single active-site cysteine. C5 methylation of cytosine in RNA does not specifically require a dual-cysteine enzyme, as shown by the formation of m^{5}C at position 1962 of 23S rRNA in *Escherichia coli* by RlmI (YccW), a Rossman fold single-cysteine enzyme with a conformation similar to that of RNA:m^{5}U methyltransferases.[38] Another consideration is that amino acids other than cysteine can act as a base for proton extraction; for example, the RNA:m^{5}U methyltransferases TrmA and RumA utilize a glutamic acid residue as the proton-extracting base.[39,40] Therefore, adjacent cysteines are not required for pyrimidine methylation in general or specifically for cytosine methylation in RNA. Adjacent cysteines are likely to be sensitive to oxidation, resulting in reversible inhibition due to disulfide bond formation. However, this may also protect NOL1/NOP2/Sun enzymes from irreversible oxidative damage, because disulfide bond formation can reverse oxidative damage to a cysteine thiol and disulfides are less likely to interact with reactive oxygen species than thiols or thiolates.[41] Therefore, protection from irreversible forms of oxidation may be one reason to conserve the dual-cysteine mechanism.

In this report, we present evidence that enzymes in the Nol1/Nop2/Sun family form covalent adducts with previously methylated RNA. The metabolite AdoHcy is required for the formation of covalent adducts with methylated RNA, and previously formed complexes disassemble if AdoHcy is removed. The extent of complex formed is influenced by the concentration of AdoHcy and pH. Covalent complex formation with methylated RNA is rapid and depends upon the motif IV cysteine, as does the forward reaction. Metabolite-mediated covalent interaction with methylated RNA may provide mechanisms for regulating methyltransferase activity as well as RNA processing and function, thus providing another explanation for conservation of the dual-cysteine catalytic mechanism in Nol1/Nop2/Sun enzymes.

EXPERIMENTAL PROCEDURES

Trm4p Expression and Purification. Plasmids encoding wild-type Trm4,[28] C260S-Trm4p (PS-Trm4p), and C310S-Trm4p (TS-Trm4p)[30] or the D257A and K179M forms of Trm4p[23] were previously constructed. All forms of Trm4p were His$_6$-tagged and purified as reported previously.[23] Fractions containing Trm4p were supplemented with 1 mM ethylenediaminetetraacetic acid (EDTA) and stored in sealed vials flushed with high-purity nitrogen gas. For some experiments, Trm4p or C260S-Trm4p was further purified by ion exchange chromatography using buffer A [50 mM phosphate, 0.5 mM EDTA, and 5% glycerol (pH 7.0)] and buffer B (buffer A with 2 M NaCl). Chromatography utilized a 5 mm × 10 cm column of Bio-Rad Macro-Prep 50 CM weak cation exchange resin, equilibrated with buffer containing 150 mM NaCl (92.5% buffer A and 7.5% buffer B) using a fast protein liquid chromatography instrument. Samples eluted from a metal affinity column with elution buffer (50 mM phosphate, 300 mM NaCl, and 150 mM imidazole adjusted to pH 7.0) were diluted with an equal volume of buffer A and applied to the CM column at a flow rate of 1.25 mL/min. After unbound material had eluted, a linear gradient of buffer B was run, resulting in the peak elution of Trm4p at approximately 0.4 M NaCl (20% buffer B). Fractions from the CM column containing Trm4p were concentrated using Amicon Ultra centrifugal filter devices. The concentrated enzyme was diluted with buffer A to reduce the final salt concentration to 120−200 mM. Purified Trm4p was also stored in septum-sealed vials under high-purity nitrogen gas. Protein quantification utilized the Coomassie Protein Assay Reagent from Pierce, using cytochrome *c* as the standard.

Construction of the Mouse Nsun2 Expression Plasmid. Mouse Nsun2 IMAGE clones 5320805 and 4011674 carry overlapping portions of the Nsun2 coding region in the pCMV-SPORT6 plasmid.[42] The shared coding region contains a unique BglII site, so a BglII−NotI fragment of approximately 2230 bp was cut from the 3′ clone (4011674) and ligated into the 5′ clone (5320805), by restriction digestion and gel purification of the DNA fragments. This created pCMV-Nsun2, a pCMV-Sport6 plasmid, which contains the complete mouse Nsun2 coding sequence. To create a bacterial expression vector, pCMV-Nsun2 was digested with PvuI to split the ampicillin resistance gene to reduce the extent of cloning of the template plasmid. The Nsun2 coding region of the cut plasmid was then amplified via polymerase chain reaction (PCR) using primers Nsun2f and Nsun2r (primer sequences in Table S1 of the Supporting Information) in PCR mixtures containing 5% dimethyl sulfoxide, because of the high GC content of the 5′ primer. The resulting 2287 bp PCR product was column purified and ligated into the pJET plasmid using the CloneJET kit. Following transformation of JM109 cells, colonies were selected for ampicillin resistance. After confirmation of a correctly sized insertion into pJET by restriction digestion, NdeI and NotI sites designed into the PCR primers were used to move the Nsun2 coding region from pJET-Nsun2 to the pET28b plasmid, forming pET-Nsun2. The NdeI site creates an in-frame fusion between the His$_6$-containing N-terminal tag encoded by pET28b and the inserted Nsun2 coding sequence.

Expression and Purification of Mouse Nsun2. Turner BL21(DE3) cells that carried the pRARE2 plasmid were transformed with pET-Nsun2. Transformed cells were grown in MDAG liquid medium or on MDAG plates to prevent premature expression.[43] For Nsun2 expression, the transformed cells were grown in Luria Broth at 35 °C to an OD$_{600}$ of 0.4 prior to the addition of 75 μM isopropyl β-D-thiogalactoside (IPTG). This level of IPTG optimized the amount of soluble Nsun2. Two hours after the addition of IPTG, the cells were harvested by centrifugation and cells were frozen as a pellet at −80 °C. The His$_6$-tagged Nsun2 was then purified on metal affinity resin as described for Trm4p.[23]

MTAN Expression and Purification. The MTA/AdoHcy nucleosidase (MTAN, EC 3.2.2.9) encoded by the *pfs* gene of *E. coli* was expressed in a His$_6$-tagged form using the expression plasmid reported by Lee et al.[44] The expressed enzyme was metal affinity purified as described for Trm4p.[23]

Trm4p Substrate RNA Preparation. Methylated yeast RNA (tRNA$_{m5C}$) was isolated from strains of *S. cerevisiae* that express TRM4 (YPW16 or AA0718), and RNA lacking m^{5}C (tRNA$_C$) was isolated from a TRM4 deletion strain (YPW17 or AA0708). For most Trm4p experiments, a small RNA

preparation was prepared by ion exchange chromatography of total RNA on DEAE cellulose as previously described.[23]

Nsun2 Substrate RNA Preparation. Human sequence tRNA-Asp was prepared by *in vitro* transcription using the MEGAshortscript T7 transcription kit. The DNA template was generated by PCR amplification of human DNA using primers Asp-IT-F(T7) and Asp-IT-R that were previously designed for this purpose (primer sequences in Table S1 of the Supporting Information).[7] Transcribed RNA was purified by alcohol precipitation. Small methylated RNA was prepared by the isolation of total RNA from mouse liver using TRIzol reagent, according to the manufacturer's instructions. Large RNA molecules were precipitated from the total RNA with 2.5 M LiCl,[45] and then soluble RNAs were concentrated by ethanol precipitation, following dilution of LiCl to a concentration of 0.5 M.

Complex Formation Assay. Complexes were formed in reaction mixtures containing 4−5 μg of enzyme, 4−5 μg of RNA, 1 mM magnesium acetate, 100 mM sodium chloride, 5 mM DL-dithiolthreitol, and 8 units of RNasin, in a volume of 100 μL. Buffering was commonly provided by 100 mM 3-morpholinopropanesulfonic acid (MOPS) at pH 6.8. Incubations were conducted at 30 °C for Trm4p and 35 °C for Nsun2 and stopped by the addition of sodium dodecyl sulfate (SDS) sample buffer. Free enzyme and enzyme−RNA complexes were resolved by SDS−polyacrylamide gel electrophoresis (PAGE). For pH studies, the Trm4p enzyme solution was diluted 6−10-fold with 1× buffer at the pH used for the assay, to minimize the effect of purification buffer on the final pH. Citrate-phosphate buffers (8×) at various pH values were prepared by mixing appropriate ratios of 300 mM citric acid and 600 mM Na_2HPO_4, such that the desired pH was obtained upon dilution to 1×.

SDS−PAGE. Near-neutral-pH SDS−PAGE utilized buffers that had pH values 1.3 units lower than those of typical SDS−PAGE systems. The lower-pH gels contained an 8 to 12% gradient of acrylamide and were buffered with 72 mM Tris adjusted to pH 7.5 and included 0.1% SDS. The low-pH stacking gel contained 3.5% acrylamide, 0.1% SDS, and 72 mM bis-tris adjusted to pH 5.5, with HCl. The separating gel was polymerized with ammonium persulfate and TEMED, and the stacking gel was photopolymerized using riboflavin (5 μg/mL) and TEMED. The upper reservoir buffer for the low-pH system was 40 mM HEPES, adjusted to pH 7.5 with phosphoric acid, and contained 0.1% SDS. The lower reservoir buffer lacked SDS and was buffered with 72 mM Tris, adjusted to pH 7.5 with HCl. Electrophoresis used a constant current of 35 mA.

Many of the gels presented utilized typical Laemmli SDS−PAGE conditions, containing either 7.5% acrylamide or a gradient of 8 to 12% acrylamide. The gel solution was buffered with 375 mM Tris, adjusted to pH 8.8, and included 0.1% SDS. Stacking gels contained 3.5% acrylamide and 0.1% SDS, in 125 mM Tris adjusted to pH 6.8. The proteins were resolved at a constant current of 40 mA, using electrode buffer containing 25 mM Tris, 192 mM glycine, and 0.1% SDS. Completed gels were either stained or fixed, with staining done in 0.2% Brilliant Blue R dissolved in a solution containing 22.5% methanol, 7.5% acetic acid, and 10% trichloroacetic acid, and then destained in 22.5% methanol, with 7.5% acetic acid. Destained gels were dried between sheets of clear gel drying film, according to the manufacturer's instructions. For complex formation rate analysis, dried gels were scanned at 600 pixels per in. and Trm4p−RNA bands were quantitated by analysis of the scanned image with UN-SCAN-IT gel version 6.1. Correction for differences in background staining between the gels was made on the basis of blank regions of the gels. Gels were prepared for fluorography by being fixed in a 2-propanol/water/acetic acid mixture (25:65:10) for 30 min and then soaked in 75 mL of Amersham's Amplify fluorographic reagent for 20 min, prior to being dried with a heated vacuum drier. Dried gels were exposed to preflashed Fugi Super RX film at −80 °C.

RESULTS

Observation of Size-Shifted Forms of Wild-Type Trm4p. Studies were initiated to determine whether zebularine acts as a mechanism-based inhibitor of RNA:m^5C methyltransferases as do the cytosine analogues 5-fluorocytosine and 5-azacytidine.[7,24,27] When zebularine is converted to the deoxy form and incorporated into DNA, DNA:m^5C methyltransferases become trapped in covalent complexes with the analogue containing DNA.[46−49] Similar complexes could form with RNA:m^5C methyltransferases, because mammalian cells incorporate substantially more zebularine, the ribonucleoside form of 2-pyrimidinone, into RNA than into DNA.[50]

Trm4p and *S*-adenosylmethionine (AdoMet) were incubated with tRNA preparations from control and zebularine-treated *E. coli* cultures. The tRNAs from *E. coli* normally lack m^5C, but Trm4p can introduce this modification.[12] Near-neutral-pH SDS−PAGE was used to resolve free enzyme from any Trm4p−tRNA complexes formed, because of evidence that covalent complexes between 2-pyrimidione-containing DNA and DNA:m^5C methyltransferases are unstable at the high pH used for standard SDS−PAGE.[46] Once stained, the gel revealed that a very small fraction of Trm4p migrated as a larger form that displayed an apparent molecular mass consistent with covalent Trm4p−tRNA complexes. However, the extent of the Trm4p upshift with tRNA from control cells matched that seen with tRNA from drug-treated cells, indicating that zebularine incorporation was not the basis for the observed denaturant-resistant complexes (data not shown). We had not anticipated that native Trm4p would form sufficient covalent intermediates with natural RNA to be detected by a gel shift approach, so this observation was explored further in experiments without zebularine.

To confirm that the Trm4p upshift was not an artifact related to the use of heterologous tRNAs, production of denaturant-resistant complexes was attempted using yeast tRNAs. Methylcytosine deficient $tRNA_C$ was isolated from a TRM4 deletion strain of *S. cerevisiae* and incubated with Trm4p and AdoMet. This resulted in the upshift of a fraction of the Trm4p upon analysis by near-neutral-pH SDS−PAGE (Figure 1, lanes 1−3). When either the RNA (Figure 1, lanes 4−6) or the AdoMet preparation (Figure 1, lanes 7−9) was deleted, no upshift was detected. The apparent AdoMet requirement for upshift could indicate that the cofactor is necessary for RNA binding by Trm4p or that cytosine methylation is a prerequisite for the formation of denaturant-resistant complexes, possibilities that are not mutually exclusive. *S*-Adenosylhomocysteine (AdoHcy) alone would not be anticipated to cause upshift if methylation is required, but it could potentially be a cofactor for RNA binding. However, no Trm4p upshift was observed when the enzyme was incubated with unmodified $tRNA_C$ and AdoHcy (Figure 1, lanes 10−12). Trm4p-mediated tRNA methylation converts AdoMet to AdoHcy, and 5-methylthioadenosine (MTA) or AdoHcy could be in the reaction mixtures

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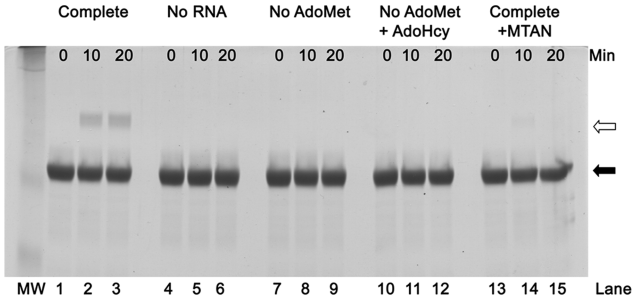

Figure 1. Observation of wild-type Trm4p–RNA complexes with unmethylated yeast tRNA. Complete reaction mixtures contained Trm4p, unmethylated yeast RNA ($tRNA_C$), 100 μM AdoMet (80%), and additional components as described in Experimental Procedures. Where indicated, 100 μM AdoHcy or 0.2 μg of MTAN/100 μL was included or AdoMet was deleted. Samples were heated to 65 °C for 5 min prior to being analyzed by SDS–PAGE, in a pH 7.5 gel containing an 8 to 12% gradient of acrylamide. Free Trm4p is indicated by a filled arrow, and the expected size for Trm4p–tRNA complexes is shown by an empty arrow. Incubation times are in minutes (Min).

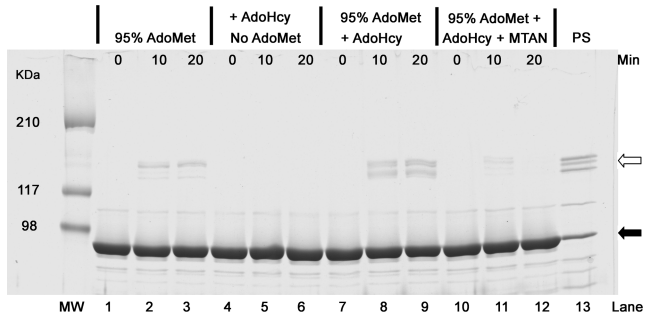

Figure 2. AdoMet and AdoHcy are needed in combination for maximal Trm4p upshift with unmethylated $tRNA_C$. Assay conditions described in Experimental Procedures were used. Where indicated, 100 μM AdoMet (95%), 50 μM AdoHcy, or 0.2 μg of MTAN was included. Complex formation was analyzed using standard-pH SDS–PAGE and a 7.5% acrylamide gel. Lane 13 (PS) contains bacterially expressed C260S-Trm4p, which exhibits multiple Trm4p–tRNA bands between 120 and 150 kDa (empty arrow) and some free C260S-Trm4p protein (filled arrow). Incubation times are in minutes (Min).

as a result of contamination or breakdown of the 80% AdoMet preparation. Thus, caution was warranted with regard to the cofactor requirement for upshift. To determine whether AdoMet is sufficient for the observed upshift, MTA/AdoHcy nucleosidase (MTAN) was added to a set of reaction mixtures to specifically degrade any AdoHcy or MTA present or produced by methylation, while leaving AdoMet intact. Addition of MTAN all but eliminated formation of the Trm4p–tRNA complex (Figure 1, lanes 13–15). In the presence of MTAN, a slight upshift was just detectable at 10 min, but no indication of upshifted Trm4p remained after incubation for an additional 10 min (Figure 1, lanes 14 and 15, respectively). The inhibitory effect of MTAN indicates a role for AdoHcy or MTA in the formation or stability of the observed complexes, despite the inability of AdoHcy alone to cause any upshift of Trm4p in combination with m^5C deficient tRNA.

AdoHcy Increases the Extent of Trm4p Upshift with Unmethylated tRNA. To more carefully examine the possible role of AdoHcy in the Trm4p upshift, higher-purity AdoMet was used in similar experiments. Incubation of Trm4p with $tRNA_C$ and 95% AdoMet resulted in a modest Trm4p upshift (Figure 2, lanes 1–3). As shown in Figure 1, AdoHcy alone caused no detectable Trm4p upshift in the presence of unmethylated RNA (Figure 2, lanes 4–6); however, the combination of 95% AdoMet and AdoHcy caused significantly more upshift of Trm4p than did the 95% AdoMet preparation alone (Figure 2, lanes 7–9). The upshift due to the combination of AdoMet and AdoHcy was largely blocked by the inclusion of MTAN (Figure 2, lanes 10–12), with the extent of upshift reduced to levels lower than that seen with the 95% AdoMet preparation alone. The complexes that did form in the presence of MTAN were again transitory, being just observable at 10 min and absent by 20 min (Figure 2, lanes 11 and 12, respectively). This experiment clearly indicates a role for AdoHcy in Trm4p upshift and confirms that AdoHcy alone is insufficient to cause Trm4p upshift with unmethylated tRNAc.

The apparent molecular mass of the upshifted Trm4p corresponds closely to that of covalent complexes formed by the C260S-Trm4p mutant upon expression in *E. coli* (Figure 2, lane 13).[30] In Figure 2, the Trm4p–RNA complexes are resolved into multiple distinct bands because of the use of standard-pH SDS–PAGE buffers rather than the near-neutral-pH buffer.

Trm4p Upshift Occurs in the Presence of Its Reaction Products. We proceeded to test the ability of Trm4p to form complexes in the presence of both reaction products. AdoHcy was found to cause a substantial upshift of Trm4p in the presence of methylated tRNA (Figure 3, lanes 10–12), and the

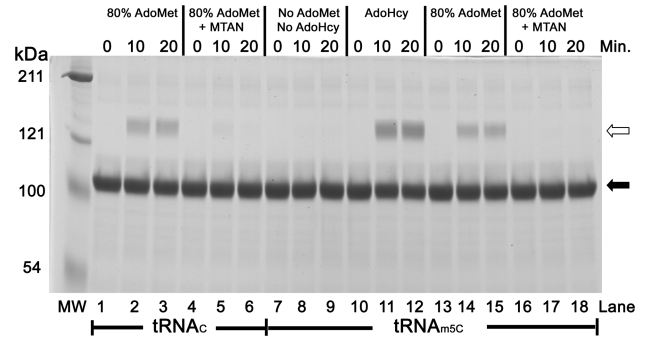

Figure 3. AdoHcy is sufficient for Trm4p complex formation with methylated tRNA. Assay conditions were as described in Experimental Procedures. When present, the concentrations of AdoMet (80%) or AdoHcy were 100 μM. Reaction mixtures containing MTAN had 0.2 μg of enzyme/100 μL. Free Trm4p is indicated by the filled arrow, and Trm4p–RNA complexes are marked by the empty arrow. Trm4 was incubated with unmethylated $tRNA_C$ (lanes 1–6) or methylated $tRNA_{m5C}$ (lanes 7–18). Reaction products were analyzed by SDS–PAGE in a pH 7.5 gel, with a gradient of 8 to 12% acrylamide.

upshift was prevented by AdoHcy deletion (Figure 3, lanes 7–9). The level of complexes formed with AdoHcy and methylated tRNA was greater than that produced by an 80% AdoMet preparation with either unmethylated tRNA (Figure 3, lanes 1–3) or methylated tRNA (Figure 3, lanes 13–15).

Further analysis revealed that the extent of Trm4p upshift caused by AdoHcy in the presence of methylated RNA is not further increased by the addition of 95% AdoMet (data not shown). In addition, MTAN completely prevents formation of a complex between Trm4p and methylated tRNA when either AdoHcy (data not shown) or 80% AdoMet was added (Figure

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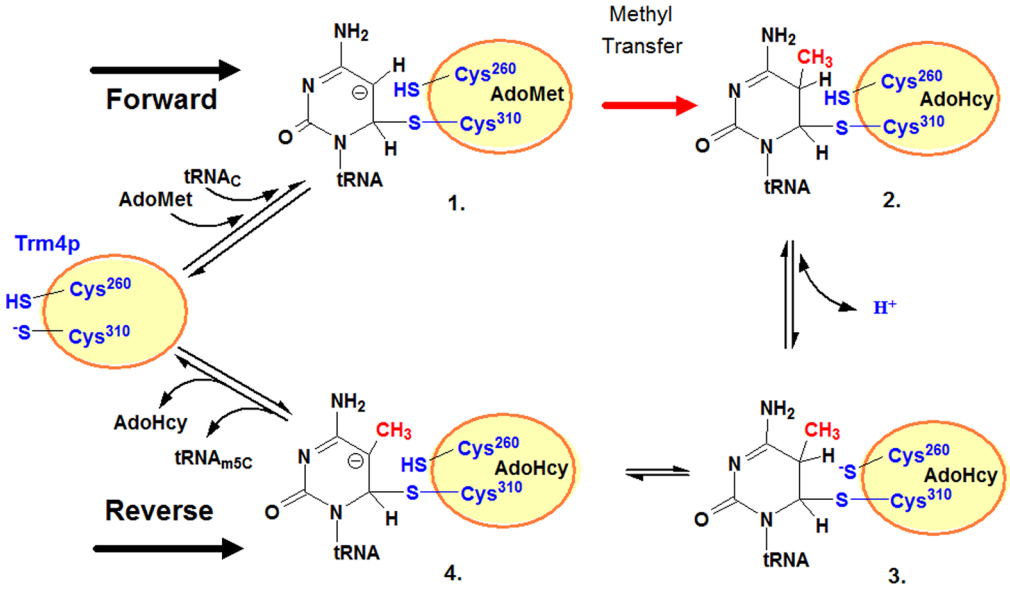

Figure 4. Proposed mechanism for forward and reverse covalent adduct formation by Trm4p. Denaturant-resistant covalent complexes are proposed to form by the Trm4p forward methylation reaction requiring $tRNA_C$ and AdoMet and by partial reversal utilizing AdoHcy and $tRNA_{m5C}$. Methyl transfer is anticipated to be an irreversible step in the scheme. The indicated order of substrate and product binding is speculative.

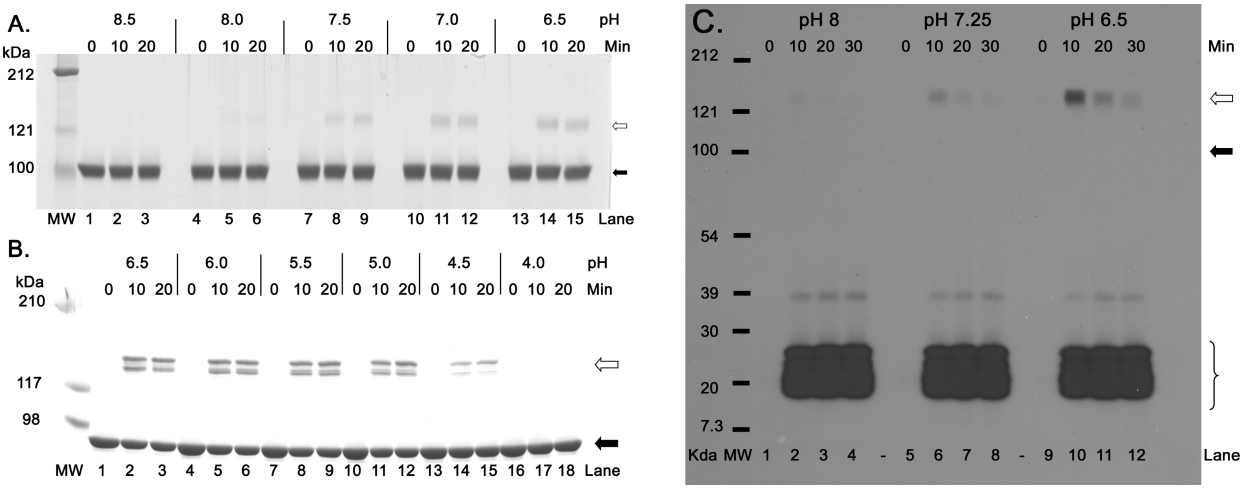

Figure 5. Solution pH influences the extent of complex formation and complex stability. Reaction mixtures for panels A and B contained methylated RNA and 100 μM AdoHcy. Assay conditions were as described in Experimental Procedures, except other buffers replaced MOPS. Free enzyme is identified with a filled arrow, and Trm4p–RNA complexes are identified with empty arrows. (A) Reaction mixtures were buffered to the indicated pH with 100 mM bis-tris propane. SDS samples were heated to 65 °C for 5 min, and complexes were analyzed by pH 7.5 SDS–PAGE. (B) Reaction mixtures were buffered to the indicated pH using citrate-phosphate buffers. Complex formation was analyzed by standard-pH SDS–PAGE using a 7.5% acrylamide gel. (C) Complexes were formed by the forward methylation reaction in asssays buffered with bis-tris propane that included 1 μg of Trm4p, 3 μg of unmethylated yeast RNA, and radiolabeled AdoMet in a volume of 120 μL. MTAN (0.1 μg) was added to eliminate AdoHcy formed by RNA methylation. At each time, a 25 μL aliquot was added to SDS sample buffer. Trm4p–RNA complexes (empty arrow) and methylated tRNA (bracket) were resolved by SDS–PAGE at pH 7.5. The fixed, Amplify-treated, and dried gel was exposed to preflashed film for 96 h. The filled arrow indicates the position of free Trm4p that is not radiolabeled.

3, lanes 16–18). As shown in Figures 1 and 2, the addition of MTAN allowed a minimal level of transitory complexes with unmethylated tRNA when reaction mixtures included AdoMet (Figure 3, lanes 4–6).

Trm4p Partial Reversibility Proposed. On the basis of the observed results, we propose that covalent complexes between Trm4p and tRNA can form by the forward AdoMet-dependent methylation of unmethylated tRNA and by reaction reversal where complex formation requires methylated tRNA and AdoHcy (Figure 4).

Thus, intermediate 2 in Figure 4 can be produced by the forward methylation reaction or by reaction of Trm4p with its products. Complete reversal of methylation is not predicted, as methyl group removal and the re-formation of AdoMet would be energetically unfavorable, but all steps other than the methyl transfer step are proposed to be reversible. Robust formation of denaturant-resistant complexes occurs when Trm4p is incubated with AdoHcy and methylated RNA. In this situation, MTAN totally blocks complex formation, because reverse complex formation is AdoHcy-dependent. The formation of denaturant-resistant complexes in the forward reaction requires

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AdoMet, indicating the important role of methylation for complex stability and the instability of the initial carbanion intermediate 1 (Figure 4). However, the level of complexes that accumulates when starting with unmethylated RNA is substantially increased by the presence of AdoHcy, with sources including AdoHcy produced by the methylation reaction, AdoHcy contamination of the AdoMet preparation, or intentionally added AdoHcy. The importance of AdoHcy for complex accumulation when starting with unmethylated RNA is revealed by the substantial inhibition of complex accrual seen in the presence of MTAN. Only very low levels of complexes are seen with unmethylated tRNA in the presence of MTAN, and these probably represent covalent complexes formed directly by methylation; their transient nature (Figure 1, lane 14, and Figure 2, lane 11) will be shown to be due to the removal of AdoHcy by MTAN.

The model in Figure 4 supports further predictions. Formation of denaturant-resistant complexes by the reverse reaction requires conversion of the unstable carbanion intermediate 4 to the protonated intermediate 3, the exact reversal of the role proposed for Trm4p Cys^{260} in the forward methylation reaction, where this amino acid is critical for RNA release. Cys^{260} is likely to act as an acid in the reverse direction and is anticipated to be critical for the formation of denaturant-resistant complexes with methylated RNA.

In addition to m^5C tRNA and AdoHcy, the methylation reaction produces a proton because of displacement by the incoming methyl group, and this proton is ultimately released to the solvent. For the forward reaction, proton extraction is modeled as occurring in two steps. The first step is the release of a proton from Trm4p to solvent, resulting in formation of the Cys^{260} thiolate. The second step involves extraction of a proton from the cytosine base by the thiolate, but additional steps could be involved depending on the access of the solvent to the active site and the possible participation of other ionizable groups. This model predicts that increasing proton concentrations would lead to greater accumulation of Trm4p−tRNA complexes, by shifting the equilibrium of partial reversal toward formation of the fully protonated intermediate 2 (Figure 4). Likewise, intermediate 2 formed by the forward reaction would be expected to be more stable under low-pH conditions.

The Extent of Complex Formation Is pH-Dependent. To test the hypothesis that increased proton concentrations would shift the equilibrium of partial reversal toward greater Trm4p−tRNA complex formation, the enzyme was incubated with $tRNA_{m5C}$ and AdoHcy in reaction mixtures buffered with bis-tris propane to pH values ranging from 8.5 to 6.5. The results shown in Figure 5A reveal a steady increase in the amount of enzyme that is upshifted as the pH decreases. There was slight but detectable formation of Trm4p−tRNA complexes at the highest pH of 8.5. Citrate-phosphate buffers were used to extend the pH range to pH 4 as shown in Figure 5B, revealing that complexes form at pH values as low as 4.5. These experiments confirm the anticipated effect of pH on the extent of complex formation by partial reversal and indicate that maximal Trm4p complex formation occurs in the pH range of 5.5−6.5.

Influence of pH on the Stability of Complexes Formed by Methylation. A tenet of the model in Figure 4 is that both the forward and partial reverse reactions can produce the protonated intermediate 2. The ability of reduced pH to push reaction reversal toward greater complex formation implies that low-pH conditions would stabilize complexes formed by the forward methylation reaction. To test this, radiolabeled covalent complexes were formed by methylation in reaction mixtures containing [*methyl*-^{3}H]AdoMet along with Trm4p and nonmethylated RNA. MTAN was included to remove AdoHcy produced by the reaction, thereby preventing reverse complex formation as methylated RNA accumulates. Radiolabeled intermediates and products were analyzed by SDS−PAGE after the reactions had been stopped with SDS sample buffer. Fluorography of the dried gel revealed a substantially greater level of ^{3}H-labeled Trm4p−RNA complexes as the pH decreased. At each pH, the amount of labeled complex also decreased with continuing incubation time, indicating gradual release of the methylated tRNA (Figure 5C). The substantially higher level of complexes observed at pH 6.5 relative to that observed at pH 7.25 or 8.0 is consistent with a decreased rate of release of methylated RNA from the enzyme as the pH decreases. Substantial enzyme turnover occurred at all pH values as indicated by the formation of free ^{3}H-labeled tRNA seen as a broad band near the bottom of the gel. Less labeled tRNA was formed at lower pH values, but this is not evident in the fluorogram shown, because the exposure time required for detection of radiolabeled Trm4p−tRNA complexes is beyond the linear exposure range for the free tRNA bands.

To further assess the effect of pH on the formation of methylated tRNA, the forward reaction was followed by measuring the transfer of radiolabel from [*methyl*-^{3}H]AdoMet to $tRNA_C$ in bis-tris propane buffers at pH values ranging from 8.5 to 6.5. A relatively steep drop in the level of tRNA methylation is observed as the pH decreases from 7.5 to 6.5, and a more modest decrease in activity occurs as the pH increases to pH 8.5 (Figure S1 of the Supporting Information).

Formation of the Trm4p−RNA Complex by Reaction Reversal Is Rapid. In initial studies, zero time samples had noticeable levels of Trm4p−RNA complexes despite the addition of SDS sample buffer within 5 s of enzyme addition, indicating a rapid rate of complex formation. To gain insight into the rate at which complexes form, a series of short reactions were performed. The results shown in Figure 6 confirm rapid complex formation, with upshift being nearly 50% complete at the first time point of 15 s. Complex formation reached maximal levels in approximately 2 min.

The Maintenance of Trm4p−tRNA Complexes Requires the Sustained Presence of AdoHcy. In the presence of AdoHcy, Trm4p−RNA complexes formed by reversal persist for at least 30 min without showing any reduction in intensity. If the complexes formed by AdoMet-dependent methylation and those formed with AdoHcy and previously methylated RNA are identical, then the latter should be able to disassemble. An anticipated equilibrium between free enzyme and enzyme−RNA complexes should be shifted toward free enzyme by the removal of AdoHcy. Therefore, MTAN was used to degrade AdoHcy in reaction mixtures containing preexisting complexes (Figure 7). In control incubations (Figure 7, lanes 1−3, 7−9, and 13−15), complexes remained at initial levels for the full chase period of 30 min, but there was a complete loss of complexes within 15 min for two of the three samples that received MTAN (Figure 7, lanes 4−6, 10−12, and 16−18). Therefore, complexes formed by the wild-type enzyme with methylated RNA and AdoHcy disassemble when AdoHcy is removed. At the highest pH (7.5), disassembly appeared slower than at pH 7.0 or 6.5. However, no delay has been observed at

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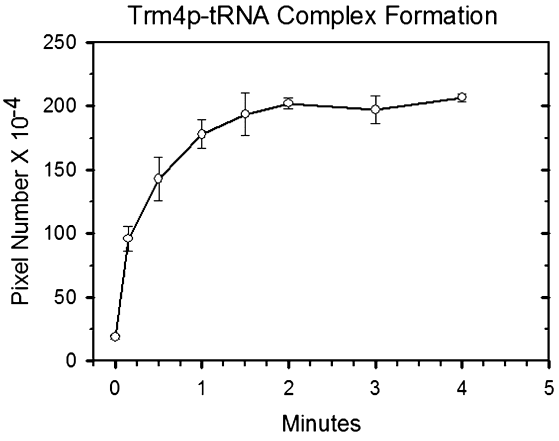

Figure 6. Time course of Trm4p−RNA complex formation. Complex formation was analyzed in triplicate samples for each time point using the assay conditions described in Experimental Procedures, with 100 mM bis-tris propane at pH 6.5 substituting for MOPS buffer. All reaction mixtures contained methylated RNA and 100 μM AdoHcy. Following pH 7.5 SDS−PAGE, Trm4p−RNA complexes were quantified using scanner images of the stained and dried gels as described in Experimental Procedures. The quantity of the complex is represented as pixel density, and the standard deviation is indicated by the error bars.

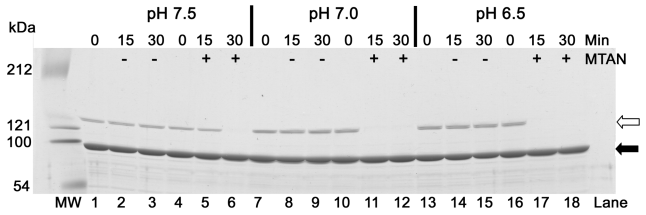

Figure 7. Maintenance of Trm4p−RNA complexes requires the continued presence of AdoHcy. Reaction mixes of 600 μL contained 4 μg of Trm4p and 4 μg of methylated RNA per 100 μL along with 100 μM AdoHcy. Salts, RNasin, and a reducing agent were present as indicated for the complex formation assays, but 100 mM bis-tris propane at the indicated pH replaced MOPS buffer. When MTAN was added, its concentration was 0.1 μg/100 μL. A 15 min preincubation at 30 °C allowed complex formation and was followed by the addition of 2 μL of water (−) or MTAN (+) to each incubation. Samples of 90 μL were removed and mixed with SDS sample buffer after the preincubation (0 min) and then 15 and 30 min (Min) after the additions. Complex stability was analyzed by standard-pH SDS−PAGE using an 8 to 18% gradient of acrylamide. Free Trm4 and Trm4p−RNA complexes are indicated by filled and empty arrows, respectively.

pH 7.5 in replicate experiments, so one possible explanation of the result is that the small volume of MTAN (2 μL) was not fully delivered to the pH 7.5 sample.

We anticipated that disassembly, like complex formation, would be relatively rapid, although the rate would depend on the speed of AdoHcy removal. Increasing the MTAN concentration was found to increase the rate of disassembly (Figure S2 of the Supporting Information). At the highest MTAN concentration used (3.08 μg/mL), 50% disassembly of Trm4p−RNA complexes required ~1 min but took 4 min at the lowest MTAN concentration (0.77 μg/mL).

Cysteine 260 Is Required for Complex Formation by the Reverse Reaction. On the basis of our model, we propose that Cys^{260} mediates the bidirectional conversion of the unstable carbanion intermediate 4 and the protonated dihydropyrimidine intermediate 3 (Figure 4). Therefore, Cys^{260} is anticipated to be critical for Trm4p−RNA complex formation by reaction reversal just as it is for RNA release in the forward methylation reaction.[28,30] We utilized the C260S-Trm4p mutant to test this hypothesis.[30] When C260S-Trm4p is expressed in *E. coli* BL-21 cells, approximately half of the mutant protein is isolated as protein−RNA complexes, but the remaining free enzyme can be purified by ion exchange chromatography.[30] Incubation of purified C260S-Trm4p with unmethylated RNA and an 80% AdoMet preparation results in an upshift much greater than that observed with the natural Trm4p and unmethylated RNA (Figure 8, lanes 1−3). In

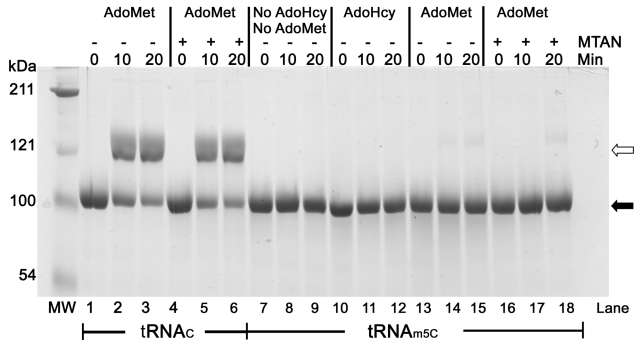

Figure 8. C260S-Trm4p forms complexes only by way of the forward reaction. The experimental design is identical to that in Figure 3, except for the use of C260S-Trm4p in place of wild-type Trm4p. Free C260S-Trm4p is indicated by the filled arrow, and C260S-Trm4p-RNA complexes are marked by the empty arrow. Incubations contained unmethylated $tRNA_C$ (lanes 1−6) or methylated $tRNA_{m5C}$ (lanes 7−18). Reaction products were analyzed by pH 7.5 SDS−PAGE using a gel containing an 8 to 12% acrylamide gradient.

contrast to the wild-type enzyme, formation of the C260S-Trm4p−RNA complex with unmethylated RNA was neither blocked nor inhibited by the inclusion of MTAN (Figure 8, lanes 4−6). These results are consistent with C260S-Trm4p having the ability to form covalent complexes by way of the forward methylation reaction while failing to release methylated RNA.[30]

Further samples were used to examine the ability of C260S-Trm4p to form complexes with previously methylated RNA. In the presence of methylated RNA, neither AdoHcy (Figure 8, lanes 10−12) nor AdoMet (Figure 8, lanes 13−15) caused significant upshift of C260S-Trm4p. This result is consistent with Cys^{260} being critical for formation of denaturant-resistant complexes by reaction reversal in addition to its previously observed role in RNA release during the forward reaction. Trace levels of complexes are seen when C260S-Trm4p is incubated with AdoMet and unmethylated RNA (Figure 8, lanes 15 and 18), but these complexes are likely to be the result of the forward reaction for reasons discussed below.

Additional Active-Site Mutations Block Partial Reversal. The methyltransferase activity of Trm4p is known to depend upon three additional active-site residues.[23,27,30] These include Cys^{310}, which is involved in covalent linkage to tRNA, as well as Asp^{257} and Lys^{179}. We examined the ability of the C310S, D257A, and K179M forms of Trm4p to form covalent complexes with methylated tRNA in the presence of AdoHcy. No detectable level of complex formation was observed with C310S-Trm4p (Figure 9A, lanes 4−6) or with the D257A and K179M forms of Trm4p (Figure 9B, lanes 4−9), but in both experiments, wild-type Trm4p showed strong complex

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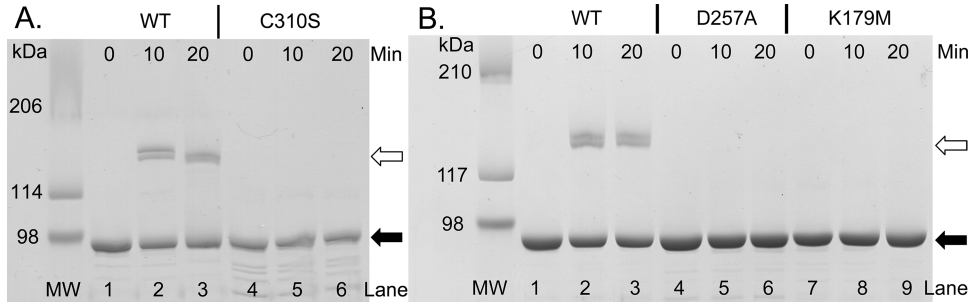

Figure 9. Trm4p C310S, D257A, and K179M mutants fail to form covalent complexes with methylated RNA. Reaction mixtures contained 5 μg of methylated RNA, 100 μM AdoHcy, and 5 μg of the indicated form of Trm4p. Assay conditions were as described in Experimental Procedures except that citrate-phosphate buffer at pH 6.0 was utilized. Free Trm4p (filled arrow) and Trm4p–RNA complexes (empty arrow) were resolved by SDS–PAGE in standard-pH gels containing 7.5% acrylamide. Incubation times are in minutes (Min). WT indicates wild-type Trm4p.

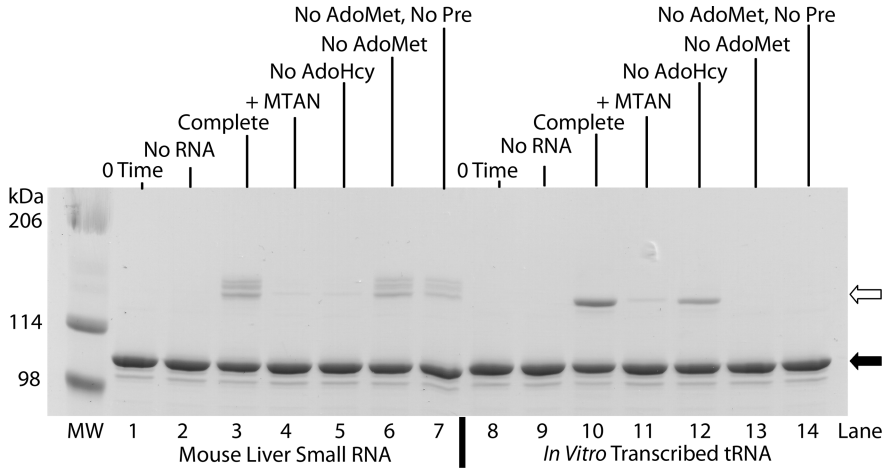

Figure 10. Accumulation of the Nsun2–RNA complex is methylation- and AdoHcy-dependent. Preincubation samples contained 5 μg of Nsun2 and 4 μg of the indicated RNA preparation in a volume of 87.5 μL, buffered to pH 7.5 with 100 mM Tris. When included, reaction mixtures contained 0.2 μg of MTAN, 100 μM AdoMet, or 100 μM AdoHcy. All samples contained 40 mM NH_4Cl, 100 mM NaCl, 1 mM magnesium acetate, 5 mM DL-dithiothreitol, and 8 units of RNasin. After 1 h at 35 °C, the pH was lowered to ~6.3 by the addition of 12.5 μL of citrate-phosphate buffer containing AdoHcy or buffer alone. Reactions were stopped with SDS sample buffer 20 min after the additions. Nsun2–RNA complexes (empty arrow) and free Nsun2 (filled arrow) were resolved by SDS–PAGE in a 7.5% acrylamide standard-pH gel. No preincubation with AdoMet was done for samples labeled "No Pre", only the 20 min reduced-pH incubation with AdoHcy.

formation. Therefore, Cys^{310}, Asp^{257}, and Lys^{179} are all critical for Trm4p reverse covalent complex formation just as they are for RNA methylation.[23,30]

Formation of Covalent Complexes with Methylated RNA May Be a General Characteristic of Nol1/Nop2/Sun Methyltransferases. To determine if other Nol1/Nop2/Sun family enzymes can form covalent complexes with methylated RNA, experiments were conducted with mouse Nsun2. Substrates included *in vitro*-transcribed $tRNA^{Asp}$ lacking m^5C and a small RNA preparation from mouse liver that contains methylated tRNA. To allow modification of unmethylated RNA, Nsun2 was preincubated with each RNA preparation and 100 μM AdoMet for 1 h at pH 7.5, followed by addition of AdoHcy and incubation for an additional 20 min at lower pH, to allow complex formation. Incubations were terminated by adding SDS sample buffer. This protocol resulted in the formation of Nsun2–RNA complexes with the unmethylated and methylated RNA preparations (Figure 10, lanes 3 and 10). Control samples showed that no bands corresponding to complexes were initially present in the enzyme preparation (Figure 10, lanes 1 and 8) and confirmed that RNA is required for the observed upshift (Figure 10, lanes 2 and 9).

The importance of AdoHcy for Nsun2 complex formation was demonstrated in two ways. The addition of MTAN strongly inhibited the formation of complexes with both RNA preparations indicating a critical role for AdoHcy in the accumulation of the complex, whether starting with methylated or unmethylated RNA (Figure 10, lanes 4 and 11). Similarly, not adding AdoHcy virtually eliminated Nsun2 complex formation with small methylated RNAs (Figure 10, lane 5), while AdoHcy deletion reduced complex formation with the *in vitro*-transcribed $tRNA^{Asp}$ (Figure 10, lane 12). The importance of prior RNA methylation for Nsun2 complex formation is demonstrated by the deletion of AdoMet. While AdoMet deletion has a minimal effect on complex formation with previously methylated small RNAs (Figure 10, lane 6), AdoMet omission blocked complex formation with *in vitro*-transcribed $tRNA^{Asp}$ (Figure 10, lane 13). Samples in the final lane for each type of RNA demonstrate that AdoHcy is sufficient for Nsun2 complex formation with methylated RNA, but not unmethylated RNA. For these samples, AdoMet was deleted, but AdoHcy was included. No 60 min preincubation was conducted, only the 20 min incubation at lower pH. Under these conditions, complexes formed with the small methylated

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RNA preparation (Figure 10, lane 7) but failed to form with the *in vitro*-transcribed $tRNA^{Asp}$ lacking m^5C (Figure 10, lane 14).

DISCUSSION

Complex Stability and Rate of Formation. The observed gel-shifted forms of native Trm4p were recognized as being an appropriate size to be Trm4p−tRNA complexes based on previous work with C260S-Trm4p (PS-Trm4p),[30] and this was confirmed by direct comparison (Figure 2 and Figure S3 of the Supporting Information). The properties of Trm4p−RNA complexes are consistent with covalent linkage, being resistant to denaturation by SDS and heat stable in SDS sample buffer at 65 °C (Figure S4 of the Supporting Information). Complexes gradually disassemble when they are heated to 100 °C in SDS sample buffer, but wild-type Trm4p complexes are as stable as those formed by the C260S-Trm4p mutant, supporting the covalent nature of complexes formed by the wild-type enzyme (Figure S4 of the Supporting Information). As this work proceeded, it became clear that the RNA complexes formed by wild-type Trm4p were stable when resolved using standard-pH SDS−PAGE buffers; therefore, many of the experiments utilized typical Laemmli buffers (pH 8.8) for electrophoresis, rather than the near-neutral-pH SDS−PAGE system developed to test for zebularine-based complexes. The standard-pH gel system resolves upshifted Trm4p and Nsun2 into multiple distinct bands when using mixed RNA substrates, so individual bands are likely to represent complexes formed with different RNA molecules.

Early indications of rapid complex formation were confirmed by rate studies. Significant levels of covalent complexes are formed in 15 s, and the level of complexes reached equilibrium in 2 min (Figure 6). Although the data provide a better indication of the reaction rate than longer incubations had, the lack of linearity in the early time points suggests that the reaction may be too rapid for accurate rate analysis using manual mixing techniques. A plausible alternate approach could be the use of a stopped-flow apparatus, which has allowed rate analysis of covalent complex formation for other RNA modification enzymes.[52] To accurately assess the initial state of Trm4p and Nsun2 in our experiments, SDS sample buffer was added to zero time samples prior to enzyme addition.

Role of MTAN in the Characterization of Complex Formation. The ability of MTAN to block all but a minor level of complex formation by wild-type Trm4p with unmethylated RNA was initially puzzling, because of a misconception that the observed complexes were formed strictly by the forward methylation reaction. However, investigation of MTAN's effect revealed that AdoHcy is necessary for substantial upshift when using initially unmethylated RNA and that AdoHcy is sufficient for maximal Trm4p upshift with methylated RNA. The AdoMet requirement for enzyme upshift with unmethylated RNA shows RNA methylation to be critical for formation of denaturant-resistant complexes, but the accumulation of complexes is largely AdoHcy-dependent, revealing that partial reversal is largely responsible for complex accumulation. By destroying AdoHcy, MTAN limits Trm4p upshift with initially unmethylated RNA to a minor level of short-lived complexes produced by the forward reaction (Figure 2, lanes 11 and 12, and Figure 3, lanes 5 and 6). This complex lability is related to the removal of AdoHcy by MTAN, as later shown by the disassembly of preformed complexes following MTAN addition (Figure 7 and Figures S2 and S3 of the Supporting Information). Therefore, wild-type Trm4p−RNA complexes are stable only in the presence of AdoHcy. No transient upshift of Trm4p was noted in reaction mixtures containing methylated RNA and MTAN, because complex formation in the reverse direction is AdoHcy-dependent and, therefore, is totally blocked by MTAN-mediated AdoHcy degradation (Figure 3, lanes 16−18).

The observed effects of MTAN appear to be entirely due to the degradation of AdoHcy. The other substrate for MTAN, MTA, is unable to cause complex formation with methylated tRNA (data not shown). MTAN does not degrade AdoMet, as shown by the ability of MTAN to increase the activity of AdoMet-dependent methyltransferases by removing AdoHcy, the common product and potent inhibitor of AdoMet-dependent methylation reactions.[51] Nonspecific inhibition or reversal of complex formation could potentially result from contamination of the MTAN preparation with RNase; however, the stability of C260S-Trm4p−RNA complexes in the presence of MTAN shows that the MTAN preparation lacks RNase activity (Figure 8, lanes 4−6, and Figure S3 of the Supporting Information).

Factors That Influence Complex Formation. The experiments shown in Figure 5 reveal that Trm4p reverse complex formation is influenced by proton concentration, as anticipated from the mechanism proposed in Figure 4. The extent of complex formation steadily increased as the pH decreased from 8.5 to 6.5 and then remained essentially constant between pH 6.5 and 5.5. The level of complex formation declined below pH 5.0, with no complex formation observed at pH 4.0. The failure to form complexes by partial reversal at pH 4.0 could be due to protein denaturation or result from a pH effect on the catalytic mechanism, such as weakened ability to form the Cys^{310} thiolate necessary for covalent interaction. It was also anticipated that covalent complexes formed by the forward methylation reaction would be stabilized by increased proton concentrations, and this effect is demonstrated by the experiment shown in Figure 5C. Slowed release of methylated RNA may partially or even substantially explain the reduced rate of the enzyme at lower pH values (Figure S1 of the Supporting Information), but other pH-influenced aspects of the mechanism could also contribute to the reduced reaction rate at low pH.

The extent of complex formation is also influenced by the concentration of AdoHcy. A gradual decrease in the level of complexes formed was observed as the AdoHcy concentration was reduced from 100 to 0.04 μM in reaction mixtures containing 0.63 μM Trm4p (5 μg/100 μL) (Figure S5 of the Supporting Information). A complete loss of observable complex formation occurs only once AdoHcy concentrations are below the concentration of the enzyme (Figure S5 of the Supporting Information). The formation of complexes at enzyme/AdoHcy ratios near one suggests that complexes would form at physiological levels of AdoHcy, although it is likely that the extent of complex formation would be influenced by the cellular AdoMet/AdoHcy ratio.

For Trm4p, the extent of complex formation is also influenced by the concentration of RNA (Figure S6 of the Supporting Information). As would be anticipated, the amount of the Trm4p−RNA complex increased with increasing levels of $tRNA_{m5C}$. However, the relative intensities of the two upshifted bands also changed as the RNA level increased, with a gradual shift from roughly equal intensity to the upper band being clearly the dominate form. This suggests that Trm4p has different affinities for various RNAs in the mixed RNA substrate.

Variability in the extent of complex formation for early experiments was at least partially due to inconsistent concentrations of AdoHcy, because it was present only as the result of AdoMet contamination or as a product of RNA methylation. Complex formation clearly depends on the specific incubation conditions used as we have shown in detail but is reproducible for a specific set of conditions. Analysis of complex formation under favorable conditions (100 μM AdoHcy, pH 6.0) results in 30−45% conversion of Trm4p to covalent complexes depending on how the fraction of Trm4p converted is calculated (Figure S7 and Table S2 of the Supporting Information).

Amino Acids Critical for Complex Formation. The experiment shown in Figure 8 uses C260S-Trm4p to confirm the critical role of Cys^{260} for RNA release in the forward direction (Figure 8, lanes 1−6) and to reveal its importance for reaction reversal (Figure 8, lanes 10−12). The differences between wild-type Trm4p and C260S-Trm4p can be seen by comparing Figures 3 and 8 that are identical in design. The key result for reaction reversal is the substantial complex formation by wild-type Trm4p in the presence of AdoHcy and $tRNA_{m5C}$ (Figure 3, lanes 10−12), compared to the complete failure of C260S-Trm4p to form complexes under the same conditions (Figure 8, lanes 10−12). A slight upshift of C260S-Trm4p is just detectable with methylated RNA, but only in reaction mixtures containing AdoMet (Figure 8, lanes 14, 15, and 18). This minor upshift is probably caused by the forward methylation reaction. The $tRNA_{m5C}$ preparation is not fully methylated, because it can serve as a methyl group acceptor in the methyltransferase assay of wild-type Trm4p, but the $tRNA_{m5C}$ isolated from cells expressing Trm4p accepts approximately one-tenth of the methyl groups as unmethylated $tRNA_C$ isolated from cells lacking Trm4p. Therefore, a small number of modifiable cytosines remain unmodified in the "methylated" $tRNA_{m5C}$ preparation, which allows C260S-Trm4p to form a low level of complexes by the AdoMet-dependent forward reaction.

The accumulation of C260S-Trm4p covalent complexes in the presence of MTAN, seen in lanes 5 and 6 of Figure 8, is clearly different from the strong MTAN inhibition of complex formation seen with the wild-type enzyme under the same conditions (Figure 3, lanes 5 and 6). This suggested that complexes formed by C260S-Trm4p would not be caused to disassemble by MTAN, and this was confirmed (Figure S3 of the Supporting Information). Therefore, AdoHcy removal results in total disassembly of complexes formed by wild-type Trm4p reversal (Figure 3 and Figure S3 of the Supporting Information), but loss of AdoHcy has no effect on the stability of complexes formed by the C260S-Trm4p mutant in the AdoMet-dependent forward reaction (Figure S3 of the Supporting Information).

Cys^{310} of Trm4p directly forms the covalent adduct with RNA during methylation,[27] and it is critical for Trm4p methyltransferase activity.[30] We anticipated that it would be required for reverse covalent complex formation, and this was shown to be correct (Figure 9A). It was less clear whether two additional Trm4p amino acids known to be required for tRNA methylation would be critical for reverse complex formation. On the basis of structural modeling, Asp^{257} and Lys^{179} were proposed to be part of the Trm4p active site and were shown to be critical for tRNA methylation by Trm4p.[23] The structure of RsmB first revealed that the corresponding amino acids (RsmB Asp^{322} and Lys^{260}) interact with enzyme-bound AdoMet,[29] so mutation of Asp^{257} and Lys^{179} is likely to interfere with cofactor binding. The experiment depicted in Figure 9B provides clear evidence that Asp^{257} and Lys^{179} are required for reverse complex formation, as no detectable complexes formed with either mutant. A similarly placed motif IV Asp is critical for uracil methylation by the m^5U methyltransferases TrmA and RumA in which the residue forms hydrogen bonds with the target base and bound cofactor.[39,53] No Nol1/Nop2/Sun structure has included bound RNA, so there is currently evidence only of the motif IV Asp having a role in cofactor binding in RNA:m^5C methyltransferases.

Implications of the Results for Nol1/Nop2p/Sun Methyltransferases. Experiments with Nsun2 duplicate the major findings obtained with Trm4p and indicate that reverse complex formation is likely to be a common feature of Nol1/Nop2/Sun RNA:m^5C methyltransferases. In addition, our results provide further evidence of the use of two cysteines in the catalytic mechanism of Nol1/Nop2/Sun methyltransferases and suggest another reason to conserve the dual-cysteine mechanism. The motif IV Cys-dependent formation of covalent complexes with previously methylated RNAs may allow Trm4 and Nsun2 to influence the processing or function of methylated RNA molecules, while the nuclear accumulation of methylated RNAs could mediate product inhibition of the methyltransferases. AdoHcy is a potent inhibitor of AdoMet-dependent methyltransferases, including Trm4p, but our finding of AdoHcy-dependent covalent complex formation increases the potential effects of AdoHcy from simply blocking methylation to blocking the function of previously methylated RNA molecules by causing covalent linkage to a methyltransferase. The interactions between a nuclear enzyme like Trm4p[54] and methylated tRNAs are quite feasible as it has been recognized that there is constitutive retrograde transport of tRNAs in yeast and mammals and accumulation of tRNAs occurs in the nucleus under certain conditions.[55]

Formation of Covalent Complexes with Methylated RNA Appears To Be Unique. Covalent complexes have been observed with other Rossmann fold RNA modification enzymes, as a result of enzyme mutation or incorporation of nucleotide analogues into RNA. For example, the RNA:m^5U-forming enzymes TrmA and RumA, from *E. coli*, modify U54 in tRNA and U1939 in 23S rRNA, respectively, using their single active-site cysteine located in motif VI to form the covalent adduct with the pyrimidine base.[39,53] TrmA and RumA can be trapped as RNA-linked complexes by using substrates containing 5-fluorouracil,[39,56] just as Trm4p and Nsun2 can be trapped by substrates containing 5-fluorocytosine or 5-azacytidine.[7,27] Covalent complexes also accumulate upon mutation of the glutamic acid that serves as the base required for proton extraction in TrmA (Glu^{358}) or RumA (Glu^{424}), blocking RNA release,[39,53] as seen with mutation of Cys^{260} of Trm4p[28,30] or Cys^{271} of Nsun2.[8,16] Although covalent trapping with TrmA and RumA is quite similar to that seen with Trm4p and Nsun2, the ability of wild-type TrmA and RumA to form covalent complexes with natural RNA differs from that of Trm4p and Nsun2. Covalent complexes are observed when either TrmA or RumA is incubated with unmethylated RNA in the absence of AdoMet, but the inclusion of AdoMet will prevent the accumulation of complexes by converting preformed complexes to free enzyme and methylated RNA.[39,56] The protein−RNA complexes are not an artifact of *in vitro* studies, as 30−45% of wild-type TrmA in *E. coli* cells is found covalently linked to tRNAs that are not fully modified.[40]

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Therefore, TrmA and RumA will form covalent complexes with unmethylated RNA in the absence of AdoMet, but Trm4p and Nsun2 fail to form denaturant-resistant complexes in this situation. As we have shown in this work, Trm4p and Nsun2 form covalent complexes in the presence of methylated RNA and AdoHcy, but there is no published evidence indicating any tendency of the RNA:m^{5}U methyltransferases to form covalent complexes with previously methylated RNAs.

DNMT2 provides another example of denaturant-resistant complex formation with natural oligonucleotides.[57] This Rossman fold enzyme is closely related to DNA:m^{5}C methyltransferases in sequence and structure.[10] Although DNMT2 has shown limited DNA methyltransferase activity, it readily methylates cytosine 38 of specific tRNAs.[10,58] The mechanism of RNA methylation is DNA methyltransferase-like, with Cys^{79} of motif IV being critical for the reaction in this enzyme that lacks cysteine in motif VI.[57,59] However, this rather enigmatic enzyme may not strictly be a "single-cysteine" enzyme. DNMT2 contains a second conserved cysteine near the active site in the TRD motif, and mutation of TRD Cys^{292} significantly reduces but does not eliminate enzyme activity.[59] Prior to the discovery that DNMT2 was a tRNA methyltransferase, the enzyme was shown to form denaturant-resistant complexes with deoxyoligonucleotides lacking 5-fluorocytosine, but other DNA methyltransferases are only trapped when target DNA sequences contain a cytosine analogue.[57] It is not known whether DNMT2 can form denaturant-resistant complexes with RNA or exactly how the methylation status of the target cytosine influences complex formation.

Clearly, the ability to accumulate covalent complexes is not limited to the RNA:m^{5}C methyltransferases of the NOL1/Nop2/sun family, but the ease of complex formation with previously methylated products may be unique to dual-cysteine RNA:m^{5}C methyltransferases. Therefore, when considering the biological role of RNA methylation, particularly m^{5}C formation, interactions between RNA and modification enzymes should be considered as a possible means of regulating RNA processing or function. More than 20 years ago, it was proposed that Michael adducts between proteins and pyrimidine bases might function as important intermediates that could regulate protein−RNA interactions.[60] On the basis of our results with Trm4p and Nsun2, it appears that this may in fact occur with enzymes of the Nol1/Nop2p/sun family. Our current efforts are targeted toward identifying biologically significant consequences of this interaction.

Finally, in regard to the studies that initiated these experiments, no clear evidence has been obtained, to date, that zebularine acts as a mechanism-based inhibitor of Nol1/Nop2/Sun RNA:m^{5}C methyltransferases, and thus, the significance of this drug to RNA m^{5}C methylation and RNA function remains unknown.

■ ASSOCIATED CONTENT

Supporting Information

Sequences of synthetic primers used for PCR of mouse Nsun2 and human tRNA^{Asp} (Table S1), influence of pH on Trm4p methyltransferase activity (Figure S1), effects of MTAN concentration on the complex disassembly rate (Figure S2), complexes formed by C260S-Trm4p do not disassemble in the presence of MTAN (Figure S3), thermal stability of Trm4p−RNA complexes in SDS sample buffer (Figure S4), influence of AdoHcy concentration on complex formation (Figure S5), effect of methylated RNA concentration on complex formation (Figure S6), extent of complex formation (Figure S7), and yields of Trm4p−RNA complexes calculated from Figure S7 (Table S2). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

AdoMet, *S*-adenosylmethionine; AdoHcy, *S*-adenosylhomocysteine; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl *β*-D-thiogalactoside; m^{5}C, 5-methylcytosine; MOPS, 3-morpholinopropanesulfonic acid; MTA, methylthioadenosine; MTAN, MTA/AdoHcy nucleosidase; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; tRNA$_{m5C}$, m^{5}C-methylated tRNA; tRNA$_C$, tRNA lacking m^{5}C.

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