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RNA Methyltransferases Utilize Two Cysteine Residues in the Formation of 5-Methylcytosine†

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ABSTRACT: Proteins that have sequence homology with known RNA m^5C methyltransferases contain two conserved cysteines, each of which lies within a sequence that bears similarity to a methyltransferase active site. Other enzymes that transfer a methyl group to carbon 5 of a pyrimidine nucleotide, such as the bacterial DNA m^5C methyltransferases, utilize their single conserved cysteine residue to form a covalent Michael adduct with carbon 6 of the pyrimidine ring during catalysis. We present a model for the utilization of two cysteines in catalysis by RNA m^5C methyltransferases. It is proposed that one thiol acts in a classical fashion by forming a covalent link to carbon 6 of the pyrimidine base, while the other cysteine assists breakdown of the covalent adduct. Therefore, alteration of the assisting cysteine is anticipated to stabilize the covalent enzyme−RNA intermediate. The model was conceived as a possible explanation for the effects of mutations that change the conserved cysteines in Nop2p, an apparent RNA m^5C methyltransferase that is essential for ribosome assembly and yeast viability. Evidence for the predicted accumulation of protein−RNA complexes following mutation of the assisting cysteine has been obtained with Nop2p and a known tRNA m^5C methyltransferase called Ncl1p (Trm4).

Several enzymes modify carbon 5 of pyrimidines including thymidylate synthase, DNA m^5C[1] methyltransferases, and the tRNA U54 methyltransferases. Each enzyme family has a conserved cysteine that is involved in covalent catalysis. The sulfur atom of the catalytic cysteine reversibly forms a covalent Michael adduct with carbon 6 of the pyrimidine ring, thereby activating carbon 5 for modification (*1*). The most recently recognized enzymes that appear to utilize this mechanism are the RNA m^5C methyltransferases (*2*). Enzymatic activity has been demonstrated for RsmBp from *Escherichia coli* and Ncl1p from *Saccharomyces cerevisiae*. The RsmB gene, initially thought to be two open reading frames (Fmu and Fmv) encodes an enzyme that specifically modifies cytosine 967 of 16S rRNA (*3*, *4*). Ncl1p, also called Trm4p, can form m^5C at four different positions in yeast tRNA (*5*). A considerable number of DNA sequences that encode RsmBp-related proteins have been identified by similarity searches, and the encoded proteins probably represent an extended family of RNA m^5C methyltransferases (*6*).

Nop2p and p120 are two of the proteins that share several motifs with RsmBp and Ncl1p (*6*). Nop2p is a nucleolar protein that is essential for the viability of *S. cerevisiae* (*7*). Reduced Nop2p expression limits yeast growth and decreases levels of mature 60S ribosomal subunits while altering rRNA processing (*8*). There is substantial identity between Nop2p and human p120, which is also called the proliferation-associated nucleolar antigen (*7*). In combination, the similarity to known RNA m^5C methyltransferases, the nucleolar location of human p120, and the role that Nop2p plays in ribosome formation suggest that Nop2p and p120 are enzymes that form 5-methylcytosine in the large rRNAs of the respective organisms.

A pair of conserved cysteines approximately 50 amino acids apart represent potential active site residues in the RNA m^5C methyltransferases (Figure 1), but studies with Nop2p and RsmBp seemed to be contradictory as to which residue acts as the active site nucleophile in covalent catalysis. The first Cys (PC-Cys) typically lies in a Pro-Cys-Ser tripeptide (Figure 1). The region surrounding the PC-Cys is quite conserved in the RNA m^5C methyltransferase family, and it even has similarity with motif IV of the DNA m^5C methyltransferases where a proline precedes the active site cysteine (*6*, *9*). Site-directed mutagenesis indicated an important role for the PC-Cys, because Nop2p in which the PC-Cys is replaced by either serine or alanine will not support yeast viability (*9*). The defect is recessive since expression of either of these Nop2p mutants does not reduce the growth of cells that also produce functional Nop2p. The second conserved Cys (TC-Cys) occurs within a Thr-Cys-Ser tripeptide sequence (Figure 1) that is not found in the DNA m^5C methyltransferases nor is it required for the essential function of Nop2p (*9*). Replacement of the TC-Cys by alanine results in a Nop2 protein that supports yeast viability and allows normal growth at 30 °C, although strains expressing only the TA-Nop2p mutant have a noticeably reduced growth rate at an elevated temperature (37 °C) (*9*).

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[1] Abbreviations: AdoMet, *S*-adenosyl-L-methionine; 5-FOA, 5-fluoroorotic acid; m^5C, 5-methylcytosine; IPTG, isopropyl β-D-thiogalactoside; KAN, kanamycin; Ni-NTA, nickel nitrilotriacetic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

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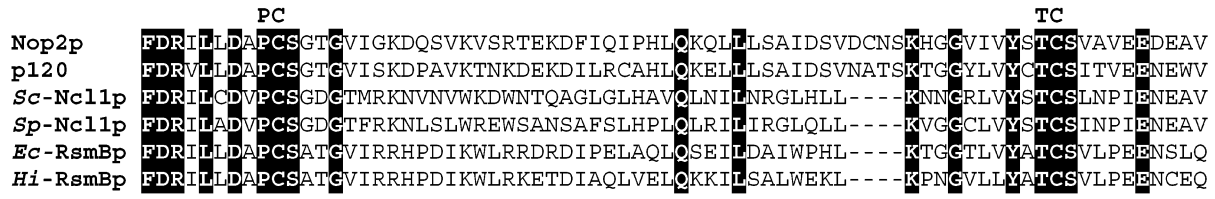

FIGURE 1: Conserved cysteines in the RNA m^{5}C methyltransferase family. Proteins in the RNA m^{5}C methyltransferase family have a conserved core area of approximately 270 amino acids (*6*). Sequences for a sample set of six proteins are aligned over a portion of that region to illustrate the conserved cysteines. Highlighted residues are identical in all six proteins. The PC-Cys and TC-Cys residues correspond to amino acids 424 and 478 of Nop2p, respectively. Confirmed RNA m^{5}C methyltransferases include RsmBp from *E. coli* (*Ec*-RsmBp) and Ncl1p from *S. cerevisiae* (*Sc*-Ncl1p). *Sp*-Ncl1p is an Ncl1p homologue from *Schizosaccharomyces pombe*, and *Hi*-RsmBp is the RsmBp homologue from *Haemophilus influenzae*. GenBank or SwissProt reference numbers for the amino acid sequences are as follows: Nop2p, P40991; human p120, P46087; *Sc*-Ncl1p, P38205; *Sp*-Ncl1p, NP_593189; *Ec*-RsmBp, P36929; and *Hi*-RsmBp, P44788.

Therefore, previous site-directed mutagenesis studies indicated a critical role for the PC-Cys in Nop2p function, consistent with covalent catalysis and limited functional significance for the TC-Cys despite its conservation in this protein family (*9*).

In sharp contrast with Nop2p, studies with the bacterial RsmB-encoded protein indicated that the TC-Cys is the catalytic residue in this enzyme (*2*). RsmBp loses all methyltransferase activity when the TC-Cys is changed to alanine, while partial activity remains if the PC-Cys is similarly altered (*2*). In addition, stable complexes between RsmBp and 5-fluorocytosine containing RNA were formed if the PC-Cys was converted to alanine but not when the TC-Cys was converted to alanine (*2*). Although DNA m^{5}C methyltransferases lack the TC-Cys, a motif like that containing the TC-Cys dipeptide is encoded by TrmA-related genes. TrmAp is the tRNA m^{5}U54 methyltransferase from *E. coli* where a conserved cysteine is the nucleophilic catalyst for uracil methylation (*2*). Therefore, both conserved cysteines in the RNA m^{5}C methyltransferase family are in regions that resemble a methyltransferase active site.

The PC-Cys in Nop2p could be critical because it is the residue involved in covalent catalysis, but the apparent conflict of active site identity between Nop2p and RsmBp led us to consider alternative explanations for the lethal effect of Nop2p PC-Cys mutations. Here we present a catalytic model for the RNA m^{5}C methyltransferases that utilizes both conserved cysteines. The TC-Cys is proposed to perform covalent catalysis while the PC-Cys plays an important role in product release. Experimental evidence in support of the proposal has been obtained with Nop2p and Ncl1p.

EXPERIMENTAL PROCEDURES

Materials and Service Providers. Synthetic oligonucleotides were purchased from Integrated DNA Technologies, and those used for site-directed mutagenesis were purified by polyacrylamide gel electrophoresis prior to use. Mutations were generated using the QuikChange kit from Stratagene. The pET28b plasmid was purchased from Novagen. *Bln*I was obtained from Boehringer Mannheim, but all other restriction enzymes were products of New England Biolabs. Gel-purified DNA restriction fragments were separated from low-melt agarose and concentrated using the Wizard PCR prep kit from Promega. T4 DNA ligase, the pGEM-T vector, and Wizard Plus Maxiprep kits for large-scale plasmid preparations were also purchased from Promega. Cobalt containing Talon affinity resin for His_6 protein purification was acquired from Clontech, and the Ni-NTA-coupled horseradish peroxidase His_6 detection reagent was obtained from Qiagen. Hemagglutinin-specific monoclonal antibody HA.11 was purchased from Covance Research Products Inc. (formerly Berkeley Antibody Co.). Peroxidase-conjugated goat anti-mouse antibodies were a product of Jackson ImmunoResearch Laboratories Inc. Luminol, RNase A, and general laboratory chemicals were products of Sigma Chemical Co. The X-ray film (Super RX) used for the detection of chemiluminescence was produced by the Fuji Photo Film Co. Protein molecular weight markers (broad range, prestained) were from Bio-Rad as were the Quantum Prep Miniprep kits used for small-scale plasmid preparations. Prime RNase inhibitor was purchased from 5 Prime → 3 Prime Inc. DNA sequence analysis was conducted by the Biochemistry Biotechnology Facility of the Indiana University School of Medicine in Indianapolis, IN, using a Perkin-Elmer Applied Biosystems 377 XL DNA sequencer.

Yeast Strains. YBH3 cells are *MATa, ade2, can1−100, his3−11,15, leu2−3, 112, trp1, ura3−1, nop2::LEU2* and carry plasmid pJPA40 which contains a 3 kb genomic DNA fragment that includes the NOP2 reading frame (YNL061W) (*8*). The YNOP2a strain was created from YBH3 by replacing pJPA40 with WpNOP2a by plasmid shuffling on 5-FOA containing media. The YKR46 strain was created from YNOP2a cells transformed with pBH46. After overnight growth of transformants in YEP-galactose, cells were plated at a dilution that gave approximately 100 colonies per dish. Replica plating on galactose-containing minimal media with appropriate supplements was used to recover colonies that retained only the pBH46 plasmid by identifying cells that required tryptophan but could grow without uracil supplementation. Except for NBC media (*9*), all yeast growth media were standard formulations.

Nop2p Mutation and Expression. Hemagglutinin-tagged forms of Nop2p (Table 1) were previously expressed following the insertion of a double-stranded oligonucleotide into the unique *Aat*II site near the 5′ end of the NOP2 gene (*9*). Further hemagglutinin-tagged constructs including WpHemREV, WpHemC424S, and WpHemC478A were generated by replacing the *Avr*II to *Nsi*I fragment of a previously hemagglutinin-tagged construct with the corresponding fragment from a plasmid containing one of the mutations. The WpHemREV plasmid encodes wild-type Nop2p but carries a silent change in the codon for Ser^{425} that is also present in the WpHemC424S and WpHemC424A constructs (*9*).

To generate the Nop2p double alanine mutant, the BptC424A plasmid that contains the C424A (PC to PA)

Table 1: Expression Plasmids Used

plasmid	marker	promoter	protein encoded	ref
Yeast Expression				
pJPA40	URA3	NOP2	Nop2p	*8*
pBH46	URA3	GAL10	Nop2p	*8*
WpNOP2a	TRP1	NOP2	Nop2p	*9*
WpC424A	TRP1	NOP2	PA-Nop2p	*9*
WpHemNOP2a	TRP1	NOP2	HMG[a]-tagged Nop2p	*9*
WpHemC424A	TRP1	NOP2	HMG-tagged PA-Nop2p	*9*
WpHemC424S	TRP1	NOP2	HMG-tagged PS-Nop2p	this study
WpHemC478A	TRP1	NOP2	HMG-tagged TA-Nop2p	this study
WpHemREV	TRP1	NOP2	HMG-tagged Nop2p	this study
WpDM5	TRP1	NOP2	PA·TA-Nop2p	this study
WpHemDM5	TRP1	NOP2	HMG-tagged PA·TA-Nop2p	this study
Bacterial Expression				
pRKNCL1	KAN	T7	His_6-Ncl1p	this study
pPANCL1	KAN	T7	His_6-PA-Ncl1p	this study

[a] HMG = hemagglutinin.

NOP2 mutation was subjected to site-directed mutagenesis with the oligonucleotides previously used to generate the C478A (TC to TA) mutation (*9*). The resulting plasmid (BpDM5) was identified by the presence of a new *Bgl*I site. The 295 base pair *Avr*II to *Nsi*I restriction fragment from BpDM5 containing both mutations was used to replace the same region of a yeast expression plasmid (WpNOP2a) to create WpDM5. The sequence of the inserted region was determined in both directions using the NOP2sp1 and NOP2sp2 primers (*9*) to confirm the appropriate changes and the absence of undesired alterations. A hemagglutinin-tagged version of the double alanine mutant was formed by replacing the 1.2 kb *Eco*RI to *Avr*II fragment of WpDM5 with the *Eco*RI to *Avr*II fragment of WpHemNop2a that includes the previously inserted nucleotides encoding the hemagglutinin tag (*9*).

Ncl1p Expression, Mutation, and Purification. The Ncl1p expression system was constructed essentially as reported (*5*) but with modifications that assist sequence analysis of site-directed mutants. The single *Bgl*II site in the pET28b vector was removed by cleavage with *Bgl*II followed by filling of the sticky ends using Klenow fragment and then closing the plasmid with T4 DNA ligase to form pRK28b. The NCL1 gene (YBL024w) was amplified from yeast genomic DNA using AGCTAGCATGGCTAGAAGAAAGAATTTCAAAAAAG as the forward primer and AGCGGCCGCTCAATTAGCAGCGCTAGGAGC as the reverse primer. The 2 kb PCR product was cloned into the pGEM-T vector to create pGEMNCL1h. The coding region was removed from pGEMNCL1h using the *Nhe*I and *Not*I sites designed into the PCR primers (underlined nucleotides) and inserted into *Nhe*I- and *Not*I-digested pRK28b to form pRKNCL1h. The *Nhe*I site places the NCL1 coding sequence in frame with the His_6 tag encoded by pRK28b. The second of two *Hin*dIII sites in the NCL1 gene was removed by site-directed mutagenesis of pGEMNCL1h with CAGCACGAAGCCTTAAAAGTTTGTTGG and its complementary oligonucleotide to generate pGEMNCL1. The resulting point mutation changes alanine codon 468 of the NCL1 coding sequence from ACT to ACC. This strategy places the codons for both conserved cysteines within a 399 bp DNA fragment flanked by unique *Bgl*II and *Hin*dIII sites. Replacement of the *Bgl*II to *Not*I fragment of pRKNCL1h with the corresponding fragment of pGEMNCL1 generated the final expression vector (pRKNCL1), which then also has the unique *Bgl*II and *Hin*dIII sites.

The PA-NCL1 mutant was made using the pGEMNCL1 plasmid, the NCL1PAf primer (CTGTGTGACGTTCCAGCTAGCGGTGATGGTACCATG), and its complementary oligonucleotide. Mutant plasmids were identified by screening for the creation of a new *Nhe*I site. The wild-type *Bgl*II to *Hin*dIII fragment of pRKNCL1 was then replaced by the mutated fragment to generate pPANCL1. The 399 bp region between the *Bgl*II and *Hin*dIII sites in pPANCL1 was confirmed to have only the desired changes by enzymatic sequence analysis in both directions using forward (TGAACCATCTGGTTTCGTTGTAGC) and reverse (TGTCATAGACAGGCCATTTGGA) primers that correspond to sequences outside of the *Bgl*II−*Hin*dIII fragment. NCL1p expression was induced with 1 mM IPTG in *E. coli* BL21-(DE3)-RIL Codon Plus cells (Stratagene) that carry extra copies of the rare Arg, Ile, and Leu tRNA genes to aid the expression of eukaryotic genes. His_6-tagged wild-type and mutant Ncl1p were purified using a batch procedure and Talon affinity resin. Extracts were prepared in 20 mM Tris, pH 8, 100 mM NaCl, and 5 mM benzamidine. Resin with bound proteins was washed with a similar buffer containing 500 mM NaCl followed by washes with the low salt extraction buffer. His_6 proteins were then released by batch elution with 20 mM Tris, pH 8, and 100 mM NaCl containing either 50 or 100 mM imidazole.

Electrophoresis, Western Blotting, and Detection. Yeast proteins were extracted in the presence of trichloroacetic acid essentially as described (*7*). All protein samples were resolved in 1.5 mm 8−18% polyacrylamide gels using previously reported buffers (*10*). The separated proteins were stained with Coomassie blue or tank transferred to nitrocellulose in buffer containing 25 mM Tris, 192 mM glycine, and 15% methanol using 100 mA constant current. Western blots were blocked in 25 mM Tris-HCl, pH 7.5, and 150 mM NaCl containing 0.25% gelatin and 0.25% nonfat dry milk. After blocking, His_6 proteins were detected using a 1/1000 dilution of the Ni-NTA reagent for 1 h followed by three washes with 20 mM Tris-HCl, pH 7.5, and 500 mM NaCl containing 0.05% Tween 20. The peroxidase activity associated with the Ni-NTA reagent was detected colorometrically with 4-chloro-1-naphthol. Signals generated by peroxidase-coupled antibodies were detected using luminol chemiluminescence. Luminol (5.5 mg) was dissolved in 250 μL of DMSO and then brought to a volume of 25 mL with 0.1 M Tris, pH 8.6. Just before use, 55 μL of 27.4 mM *p*-hydroxycinnamic acid in ethanol and 9 μL of 30% H_2O_2 were added. After equilibration of membranes with the luminol solution for 1 min, the blot was backed with filter paper moistened in the same mixture, and both layers were wrapped in plastic wrap for film exposure.

RESULTS

A Two-Cysteine Model for RNA m^5C Methyltransferases. RsmBp and Nop2p could use different cysteine residues for

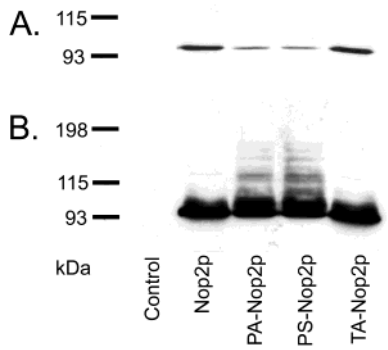

FIGURE 2: Nonfunctional Nop2p mutants form oversized complexes. The YBH3 strain was transformed with TRP1 plasmids that encode hemagglutinin-tagged forms of Nop2p or untagged Nop2p (control). Starter cultures were grown in minimal media to maintain the presence of pJPA40 and the TRP1 plasmid. For sample preparation, cells were grown to mid log phase in rich media (NBC) followed by extraction of proteins in the presence of trichloroacetic acid. Cellular proteins were resolved by SDS−PAGE and transferred to nitrocellulose. Hemagglutinin-tagged proteins were detected by incubation with the HA.11 monoclonal antibody (1/5000) followed by peroxidase-coupled rabbit anti-mouse secondary antibodies diluted 1/2000. The blot was equilibrated with luminol solution and exposed to film for 10 s (A) or 30 s (B). Panel B provides an estimated 24-fold greater intensity than panel A due to signal decay between exposures. All strains express untagged wild-type Nop2p encoded by the pJPA40 plasmid. The TRP1 plasmids used were WpNOP2a (control), WpHemRev (Nop2p), WpHemC424A (PA-Nop2p), WpHemC424S (PS-Nop2p), and WpHemC478A (TA-Nop2p).

covalent catalysis, but it seemed more likely that these related proteins would function in a similar fashion. Therefore, we sought a model that could explain the lethality of PC-Cys mutations if the TC-Cys functions as the site of covalent catalysis for Nop2p. A model was developed on the basis of our observation of large forms of Nop2p mutants and stable complexes previously seen with TrmAp. Longer exposures of western blots revealed higher molecular mass forms of Nop2p PC-Cys mutants that are much less abundant or absent with the wild-type protein and TA-Nop2p (Figure 2). PA-Nop2p and PS-Nop2p may be unstable, so the complexes could be ubiquitin conjugates, but there is at least one other possible explanation. In the absence of a methyl group donor, TrmAp will form protein−RNA complexes that appear to be covalent due to their stability to SDS−PAGE (*11*). Given the similarity between the regions around the active site cysteine of TrmAp and the TC-Cys of the RNA m^{5}C methyltransferases (*2*), we considered the possibility that protein−RNA complexes might explain the larger forms of Nop2p. The TC-Cys would be predicted to be the site of covalent linkage since it is the only remaining conserved cysteine in the PC-Cys mutants. The accumulation of RNA complexes when the PC-Cys is mutated would not only support the TC-Cys as the site of adduct formation but also indicate that the PC-Cys might assist release of RNA substrates from the TC-Cys as diagrammed in Figure 3. On the basis of this model we propose that the PC-Cys mutations of Nop2p are lethal due to their failure to release rRNA, its probable substrate, resulting in the disruption of ribosome formation and the observed fatal phenotype. Three predictions based on the model have been tested, and the results provide evidence in favor of the concept presented in Figure 3.

Evidence in Support of the Two-Cysteine Model. If the mechanisms of RsmBp and Nop2p are similar, then mutation of the TC-Cys to alanine should prevent methyl transfer by Nop2p as seen for RsmBp (*2*). Consequently, the viability of the TA-Nop2p mutant (C478A) implies that RNA methylation is not the essential role for Nop2p (*9*). Changing the TC-Cys to alanine should not only prevent methylation

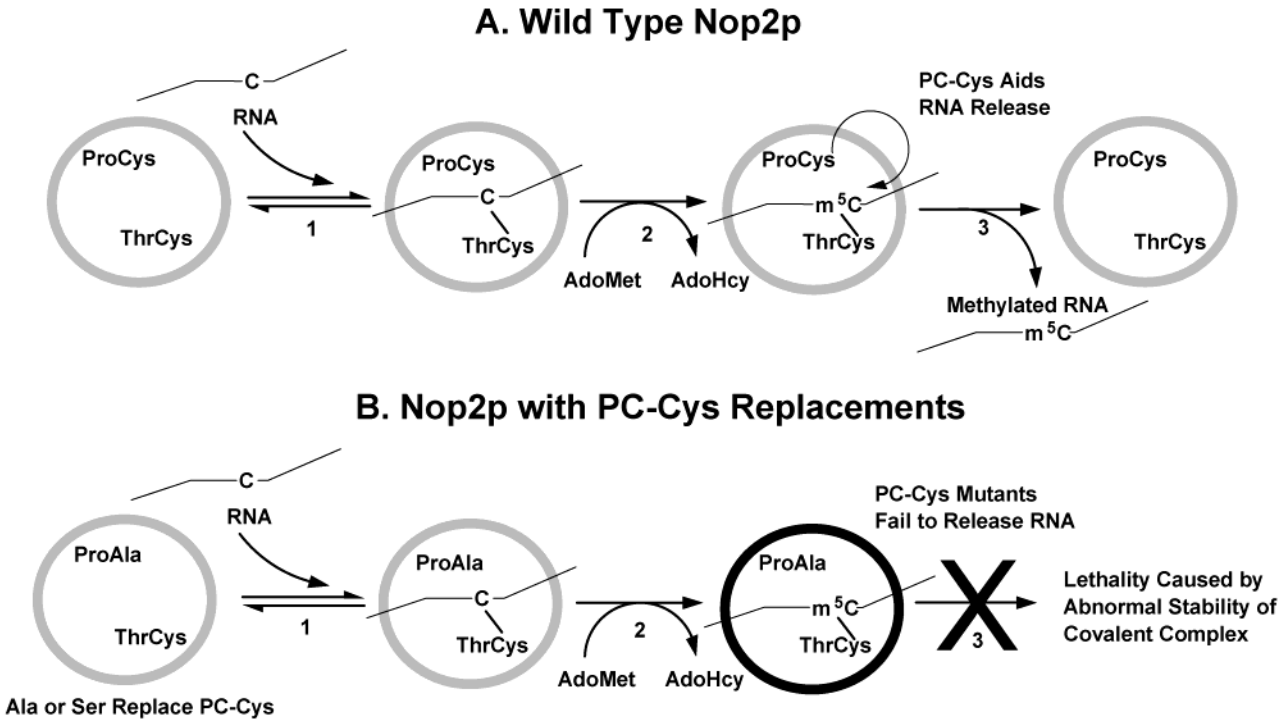

FIGURE 3: Proposed roles for conserved cysteines in RNA methylation. In wild-type Nop2p (A) an appropriate substrate RNA binds followed by formation of a covalent Michael adduct between carbon 6 of the pyrimidine ring and the TC-Cys. In step 2 methyl transfer from AdoMet occurs. In the third step, it is proposed that the PC-Cys participates either directly or indirectly in the release of methylated RNA. (B) Alteration of the PC-Cys is proposed to slow or prevent the release of methylated RNA.

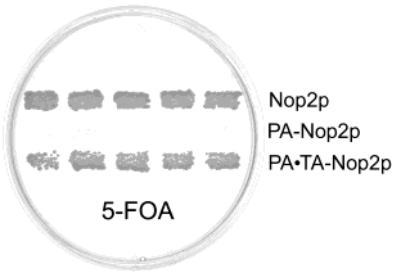

FIGURE 4: Test of PA•TA-Nop2p function by plasmid shuffling. YBH3 cells were transformed with TRP1 plasmids that encode wild-type Nop2p (WpNOP2a), the nonviable PA-Nop2p mutant (WpC424A), or the PA•TA-Nop2p double alanine mutant (WpDM5). Transformants were patched onto synthetic dextrose media containing the appropriate supplements and 1 mg/mL 5-FOA. Each patch contains cells from a single colony. Growth of cells to high density (dark color) will only occur when the URA3 plasmid carrying a wild-type NOP2 gene (pJPA40) is lost and Nop2p function is replaced by the NOP2 gene present in the TRP1 plasmid construct.

but also preclude the formation of any covalent complex. Therefore, the model leads to the prediction that changing the TC-Cys to alanine could restore viability to the PC-Cys mutants because no covalent complex would be formed in the first place, making the inability to release RNA substrates a mute issue. To test this possibility, we generated the PA•TA-Nop2p double mutant in which both conserved cysteines were changed to alanine and examined its ability to support yeast viability. Figure 4 shows that PA•TA-Nop2p supports yeast growth in the absence of wild-type Nop2p. When a strain (YDM5) that produces only the double alanine mutant was recovered from the 5-FOA selection, it was found to grow only slightly less rapidly than a corresponding control strain at either 30 or 37 °C (see figure in Supporting Information). An interesting aspect of the double mutant is that cells expressing it at 37 °C grow closer to the rate of wild-type cells than cells producing the TA-Nop2p single mutation that was previously shown to have a mild but reproducible growth defect at 37 °C (*9*) (see figure in Supporting Information).

A second prediction is that the double alanine mutant should not form the high molecular mass complexes observed with the lethal PC-Cys single mutants even though the original lethal PC-Cys to Ala mutation is retained. We also anticipated that greater amounts of complexes would be formed by the PA-Nop2p and PS-Nop2p single mutants if the cells no longer expressed wild-type Nop2p. To test these concepts, we created the YKR46 strain that carries a plasmid-borne NOP2 gene that is regulated by a galactose promoter. YKR46 cells were transformed with a control plasmid that lacks a Nop2p coding sequence (pRS314) and six plasmids that encode different forms of Nop2p under the control of the natural promoter (Figure 5, Table 1).

A culture of each transformed strain was grown for 12 h in glucose medium to extinguish expression of the GAL10-regulated untagged Nop2p while a second culture was grown in galactose medium to maintain expression of untagged Nop2p. New cultures were then initiated with the same carbon source and samples taken for protein analysis when the culture OD_{600} reached approximately 0.5. A Nop2p-specific monoclonal antibody (D68) was used to confirm the shutoff of galactose-regulated Nop2p in cells transformed with pRS314 (Figure 5, panels C and D). As anticipated,

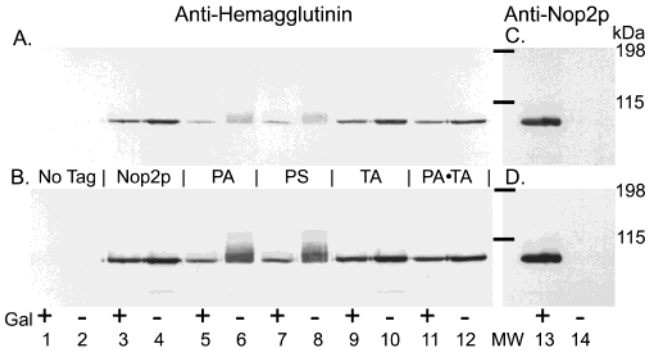

FIGURE 5: The PA•TA-Nop2p double mutant does not form a high molecular mass complex. YKR46 cells were transformed with TRP1 plasmids that allow expression of the forms of Nop2p indicated. Lanes: 1 and 2, untagged Nop2p (WpNOP2a); 3 and 4, tagged Nop2p (WpHemRev); 5 and 6, tagged PA-Nop2p (WpHemC424A); 7 and 8, tagged PS-Nop2p (WpHemC424S); 9 and 10, tagged TA-Nop2p (WpHemC478A); 11 and 12, tagged PA•TA-Nop2p (WpHemDM5). Cells in lanes 13 and 14 were transformed with pRS314 that lacks a Nop2p coding sequence. Cells were grown in synthetic media containing either galactose (Gal +) or glucose (Gal −) for 12 h to maintain or suppress expression of the GAL10 controlled wild-type Nop2p. YEP cultures containing either galactose or glucose were then inoculated to a starting OD_{600} of 0.15 using cells grown on the same carbon source. When cultures reached an OD_{600} of 0.5, the cells were harvested in the presence of TCA. Extracted proteins were resolved by SDS−PAGE and transferred to nitrocellulose. In panels A and B, hemagglutinin-tagged proteins were detected as in Figure 2. In panels C and D, Nop2p lacking a tag was detected using a 1/10000 dilution of the Nop2p-specific D68 monoclonal antibody (*23*) and a 1/2000 dilution of peroxidase-coupled rabbit anti-mouse secondary antibodies. In all cases, X-ray film was used to detect the luminol chemiluminescence. Panels B and D are 5- and 3-fold longer exposures of panels A and C, respectively. Prestained molecular mass markers were included in the indicated lane (MW).

these cells expressed high levels of Nop2p when grown on galactose, but Nop2p was not detectable after overnight growth on glucose (Figure 5, lanes 13 and 14). Examination of tagged proteins with a hemagglutinin-specific antibody in panels A and B of Figure 5 revealed that the loss of untagged wild-type Nop2p caused a substantial increase in the amount of high molecular mass complexes that are formed by PA-Nop2p and PS-Nop2p (Figure 5, lanes 6 and 8). Overexposure of the blot was not required to detect larger forms, but a longer exposure confirmed that large complexes occur only with the PC-Cys mutants. Especially significant is the lack of larger complexes with the PA•TA-Nop2p double mutant since it retains the PC-Cys to alanine mutation (Figure 5, lane 12). The high molecular mass bands are specifically related to Nop2p, because they are not detected in cells that express only untagged Nop2p (Figure 5, lanes 1 and 2). After overnight growth on glucose media, the pRS314 transformants lack Nop2p and show a substantially reduced growth rate as previously observed when Nop2p expression was stopped (*8*). Reduced growth is also noted for glucose-grown cells that express only the nonfunctional PA-Nop2 and PS-Nop2p mutants. In all cases, expression of the tagged forms of Nop2p increased following the shift to glucose media in response to the reduction of galactose-driven synthesis of Nop2p.

Finally, the model presented in Figure 3 predicts the accumulation of protein−RNA complexes when the PC-Cys is mutated. Efforts are in progress to confirm that the larger forms of PA-Nop2p and PS-Nop2p seen in Figures 2 and 5

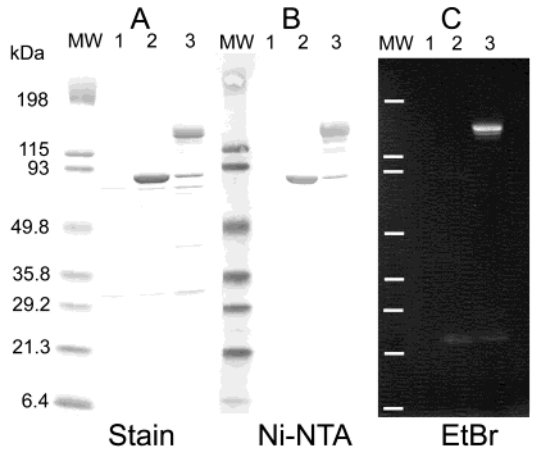

FIGURE 6: SDS−PAGE analysis of bacterially expressed Ncl1p and PA-Ncl1p. Affinity-purified His_6-tagged proteins were heated to 65° C for 10 min in the presence of SDS and then resolved by SDS−PAGE. Extracts were prepared from BL21(DE3) cells transformed with pRK28b that lacks an NCL1 gene (lane 1), pRKNCL1 that encodes Ncl1p (lane 2), or pPANCL1 that encodes PA-Ncl1p (lane 3). Proteins were detected by staining the gel with Coomassie blue (panel A) or by reaction of a western blot with the His_6-specific Ni-NTA-coupled horseradish peroxidase reagent and 4-chloro-1-naphthol (panel B). In panel C, nucleic acids were detected by staining with ethidium bromide (EtBr). The positions and sizes of prestained molecular mass markers (MW) are indicated for each panel. The EtBr staining bands just above the 21.3 kDa marker in lanes 2 and 3 of panel C copurify with Ncl1p and comigrate with bona fide bacterial tRNAs.

are protein−RNA complexes. However, clear evidence for such complexes was obtained with the yeast tRNA methyltransferase Ncl1p (Trm4). The Ncl1p coding sequence was cloned from *S. cerevisiae* and expressed in bacteria as described in Experimental Procedures. Purification of His_6-tagged Ncl1p by metal affinity chromatography produced a protein with an apparent molecular mass of 85 kDa, which is within 6% of the calculated size of 80 kDa for the 684 amino acid protein plus the 20 residue amino-terminal extension encoded by the plasmid that includes the His_6 tag. In addition, the expressed protein was confirmed to have methyltransferase activity (data not shown). Site-directed mutagenesis was then used to generate a mutant NCL1 gene in which alanine is encoded in place of the PC-Cys, and the resulting PA-Ncl1p was expressed and purified as indicated for the tagged wild-type Ncl1p. Analysis of the isolated proteins by SDS−PAGE revealed that only a small amount of PA-Ncl1p has the same apparent molecular mass as the wild-type Ncl1p. Most PA-Ncl1p migrates as a series of bands that have significantly larger apparent molecular masses, estimated to be in the range of 120−145 kDa (Figure 6, panel A). To provide evidence that these large proteins were PA-Ncl1p, the transfer of a second section of the gel was reacted with a His_6 reagent. Ni-NTA reacted strongly with these larger proteins but was unreactive with contaminating bacterial proteins, indicating that the large protein carries a His_6 tag as expected for PA-Ncl1p (Figure 6, panel B). Considering our model, this altered migration could result from linked nucleic acids, so an identical gel was stained with ethidium bromide, which revealed the comigration of nucleic acids with PA-Ncl1p, but neither the wild-type Ncl1p nor a control extract was significantly stained by the intercalating agent (Figure 6, panel C).

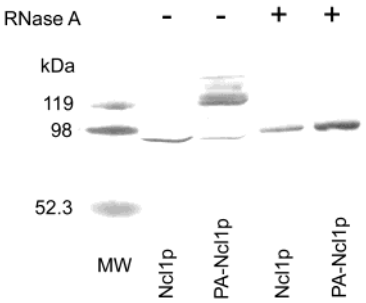

FIGURE 7: RNase A treatment of Ncl1p and PA-Ncl1p. His_6-tagged Ncl1p and PA-Ncl1p were affinity purified from bacterial extracts, and then 60 μL samples were incubated at 37 °C for 2.5 h with either 2 units of RNase inhibitor (−) or 10 μg of RNase A (+). Following addition of SDS sample buffer, the proteins were resolved by SDS−PAGE and transferred to nitrocellulose. The His_6-tagged proteins were detected using Ni-NTA-coupled horseradish peroxidase and 4-chloro-1-naphthol. The sizes of prestained molecular mass markers (MW) are indicated.

To provide evidence that the nucleic acid associated with PA-Ncl1p is RNA, samples of the proteins were subjected to digestion with RNase A. As can be seen in Figure 7, RNase treatment had little effect on the migration of wild-type Ncl1p; however, the mobility of the PA-Ncl1p was increased by this procedure, with several closely migrating bands all collapsing to a size that is indistinguishable from that of the wild-type protein. Virtually identical results were obtained when samples of the proteins were treated with sodium hydroxide; therefore, the increased size of the PA-Ncl1p is the result of an interaction with RNA that is stable to SDS−PAGE but sensitive to RNase and base hydrolysis.

DISCUSSION

Even though a critical role for the PC-Cys in Nop2p was revealed prior to the identification of related proteins as RNA m^{5}C methyltransferases, the similarity of the PC-Cys region to the active site motif of the DNA m^{5}C methyltransferases which contains a Pro-Cys active site dipeptide was noted (*9*). The identification of RsmBp and Ncl1p as RNA m^{5}C methyltransferases increased the apparent importance of the PC-Cys residue, especially as further similarities between the RNA and DNA m^{5}C methyltransferases were uncovered (*6*). Therefore, the discovery that the TC-Cys was the site of covalent catalysis in RsmBp was unexpected (*2*), and it raised an apparent conflict with the site-directed mutagenesis results obtained with Nop2p (*9*). Presuming that Nop2p is also a methyltransferase that uses a covalent catalytic mechanism, we needed to either explain why two related enzymes use different residues for covalent catalysis or rationalize why mutations that change a cysteine not directly involved in covalent catalysis are lethal. The model shown in Figure 3 was an attempt to do the latter by proposing a role for the conserved PC-Cys in the release of the covalent adduct.

The initial test of the model examined whether a mutation that converts the TC-Cys to alanine would restore the ability of a gene that already contained the lethal PC-Cys to alanine mutation to support yeast viability. We found that cells expressing only the PA·TA-Nop2p double mutant (YDM5 strain) were viable and that they exhibit a growth rate that is nearly the same as cells expressing wild-type Nop2p. These results show that the lethal effect of the PC-Cys to alanine

mutation requires the presence of the TC-Cys, an observation that is consistent with the proposed model. In addition, the viability of the double mutant provides strong evidence that RNA methylation is not the critical aspect of Nop2p function. This result appears to make Nop2p the latest addition to a growing list of RNA processing enzymes for which expression is essential for viability, even though the known enzymatic activity is dispensable. In addition to Nop2p, yeast examples are Cbf5p and Dim1p. Cbf5p appears to be the snoRNA-directed pseudouridine synthase that modifies rRNA (*12*), while Dim1p is the methyltransferase that modifies two adjacent adenosine residues near the 3′ end of 18S rRNA (*13*). Dim1p has been proposed to mediate quality control of ribosome formation (*14*), and the undefined essential functions of all three proteins could be related to ribosome assembly. Examples of essential proteins with nonessential RNA modification activities are also found in bacteria. In *E. coli*, RluDp forms three pseudouridines in the large rRNA (*15*), and TrmAp, as mentioned earlier, forms m^5U in tRNA (*16*), but in neither case is enzymatic activity essential. TrmAp seems to be an exception to a general theme of these proteins being involved in ribosome formation, but the observation of TrmAp in a complex with the small subunit rRNA indicates that even this tRNA-modifying enzyme could have some role in bacterial ribosome assembly (*17*).

Next, we demonstrated that the double alanine Nop2p mutant does not form high molecular mass complexes even though it retains the PC-Cys to alanine change. Compared to Nop2p itself, detection of mutant complexes requires long exposures when extracts from cells that also produce functional Nop2p are analyzed (Figure 2), but the quantity of complexes increased when wild-type Nop2p expression was stopped using a GAL-regulated construct and a shift to glucose media (Figure 5). However, no complexes were seen with PA·TA-Nop2p, even when it was the only form of Nop2p remaining in the cells (Figure 5, lane 12). The fact that the TC-Cys is needed for the formation of the high molecular mass complexes is consistent with it being the site of covalent catalysis in Nop2p as in RsmBp.

The most important prediction resulting from our model is that PC-Cys mutations should result in the accumulation of covalently linked enzyme−RNA complexes. The best evidence for such complexes was produced with bacterially expressed Ncl1p. Mutation of the PC-Cys to alanine clearly changes the interaction of Ncl1p with polynucleotides causing most of the bacterially produced PA-Ncl1p to have a substantially larger apparent size than wild-type Ncl1p. These larger forms of PA-Ncl1p can be reduced to the size of the wild-type Ncl1p by RNase A or base treatment, indicating that the protein is associated with bacterial RNA. There are currently two reasons to believe that the RNA associated with Ncl1p is tRNA. Yeast tRNAs are natural substrates for Ncl1p, but bacterial tRNAs are acceptable targets because they are modified when bacteria express the enzyme (*5*). RNA is also associated with wild-type Ncl1p, but it is released during SDS−PAGE, and the released RNA has been found to be the same size as authentic tRNA (data not shown). The conversion of the catalytic cysteine of bacterial DNA m^5C methyltransferases to glycine or alanine can increase the affinity of the enzyme for DNA to the point of being inhibitory to bacterial growth (*18*, *19*). Although changes in the affinity of Ncl1p for RNA may be involved, the ability of the PA-Ncl1p−RNA complexes to survive heating in the presence of SDS is more consistent with a covalent linkage than with increased binding affinity.

On the basis of the results obtained with Ncl1p, it is likely that the larger forms of Nop2p PC-Cys mutants are also protein−RNA complexes, but they are not large enough to contain the predicted substrate, rRNA. Therefore, they may contain fragments of rRNA or some other RNA altogether. The extraction and gel analysis methods used were not designed to isolate or resolve full-sized rRNA−protein complexes, and alternative methods are being developed to allow further characterization of the Nop2p complexes including the identification of the RNA molecule or molecules associated with Nop2p.

It is firmly established that the presence of a modified nucleotide such as 5-fluorocytosine or 5-azacytosine at the site of modification can stabilize the covalent intermediate formed by DNA (*1*, *20*, *21*) or RNA (*2*) m^5C methyltransferases. However, this work demonstrates that the alteration of a single amino acid residue within an RNA m^5C methyltransferase can stabilize the covalent intermediate with a natural polynucleotide. At least two proteins have been found to form denaturant-resistant complexes with natural nucleotides. Human Dnmt2 was recently shown to form SDS−PAGE-resistant complexes with normal DNA oligonucleotides (*20*). Dnmt2 belongs to a new eukaryotic subfamily of proteins related to the DNA m^5C methyltransferases; however, no Dnmt2 family member has been shown to have methyltransferase activity (*20*). As mentioned previously, TrmAp can form denaturant-resistant complexes with rRNA in vivo (*17*) and tRNA in vitro (*11*). It is of interest that proteins related to TrmAp (*22*) and to Dnmt2 (*20*) contain two conserved cysteines, but there is currently no evidence that either TrmAp or Dnmt2 utilizes their conserved cysteines as proposed for the RNA m^5C methyltransferases.

Bacterial DNA m^5C methyltransferases have only one conserved cysteine, making it clear that two are not chemically required for the methylation of a pyrimidine. Therefore, it is intriguing that two cysteines are used by the RNA m^5C methyltransferases. Covalent catalysis appears to be mediated by the TC-Cys, so what is the biochemical role of the PC-Cys in cytosine methylation? A direct role in RNA release could involve base catalysis, since a proton must be extracted from carbon 5 of the modified cytosine in order to re-form the pyrimidine C5−C6 double bond and release the covalently linked enzyme. A cysteine thiolate could potentially serve as the base for proton removal. This is an interesting hypothesis that would place two reactive cysteines in the RNA m^5C methyltransferase active site, but no direct evidence pertaining to the biochemical role of the PC-Cys has been obtained. Therefore, other possible functions for the PC-Cys cannot be excluded, such as the formation of a second Michael adduct to aid RNA binding, metal ion binding to stabilize protein structure or aid catalysis, or the formation of a disulfide bond that is critical for proper protein conformation.

In conclusion, our two-cysteine mechanism provides a reasonable and testable explanation for the observed lethality of Nop2p PC-Cys mutants and the seemingly contradictory results that had been obtained with Nop2p and RsmBp. The initial tests of the model reported here support the hypothesis

that RNA m^5C methyltransferases utilize both conserved cysteines during methyl transfer, with one residue forming a covalent adduct and the other having an important although undetermined role in the release of the covalent intermediate.

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SUPPORTING INFORMATION AVAILABLE

One figure concerning the growth rate analysis of yeast expressing the PA·TA-Nop2p double mutant. This material is available free of charge via the Internet at http://pubs.acs.org.

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