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Analysis of the Yeast Arginine Methyltransferase Hmt1p/Rmt1p and Its *in Vivo* Function

COFACTOR BINDING AND SUBSTRATE INTERACTIONS*

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Many eukaryotic RNA-binding proteins are modified by methylation of arginine residues. The yeast *Saccharomyces cerevisiae* contains one major arginine methyltransferase, Hmt1p/Rmt1p, which is not essential for normal cell growth. However, cells missing *HMT1* and also bearing mutations in the mRNA-binding proteins Npl3p or Cbp80p can no longer survive, providing genetic backgrounds in which to study Hmt1p function. We now demonstrate that the catalytically active form of Hmt1p is required for its activity *in vivo*. Amino acid changes in the putative Hmt1p *S*-adenosyl-L-methionine-binding site were generated and shown to be unable to catalyze methylation of Npl3p *in vitro* and *in vivo* or to restore growth to strains that require *HMT1*. In addition these mutations affect nucleocytoplasmic transport of Npl3p. A cold-sensitive mutant of Hmt1p was generated and showed reduced methylation of Npl3p, but not of other substrates, at 14 °C. These results define new aspects of Hmt1 and reveal the importance of its activity *in vivo*.

Post-translational modifications are frequently used by cells to expand the repertoire of proteins to control their activity temporally or spatially or to target their degradation. The methylation of the guanidino group of arginine residues was first recognized 30 years ago (1), but recently more work has focused on this modification. Protein arginine methyltransferases have been identified and cloned from many eukaryotes (2–6). In addition, the number of potential substrates for these enzymes has grown as genome sequencing projects have revealed numerous proteins containing the RGG motif common to substrates for arginine methylation, many of which are RNA-binding proteins (7, 8). Although it is not yet clear what effect methylation may have on the activity of such proteins, the yeast arginine methyltransferase Hmt1p has been shown to facilitate the export of at least two of its substrates from the nucleus, pointing to the importance of methylation in nucleocytoplasmic transport (9).

Arginine methyltransferases were initially identified biochemically in mammalian cell lysates by their ability to transfer a radiolabeled methyl group from *S*-adenosyl-L-methionine (SAM)[1] to histones, myelin basic protein, or the heterogeneous nuclear ribonucleoprotein A1 (10–14). In addition to monomethylated forms of these proteins, heterogeneous nuclear ribonucleoprotein A1 undergoes asymmetric dimethylation, resulting in N^G,N^G-methylarginine residues, whereas myelin basic protein is symmetrically methylated (N^G,$N^{G\prime}$-methylarginine) (3, 16, 11, 15). Sequence comparison of methyltransferases has revealed motifs common to enzymes that methylate different molecules (17); at least one of these motifs has been implicated in binding to their common co-factor, SAM (18). Novel proteins that contain these motifs have since been identified in general data base searches and in genetic screens (2–6, 19).

The first protein arginine methyltransferase gene in the yeast *Saccharomyces cerevisiae*, *HMT1* (heterogeneous nuclear ribonucleoprotein methyltransferase), was identified in a screen for genes that interacted with *NPL3*, which encodes an RNA-binding protein (4). The same methyltransferase gene, alternately called *RMT1*, was also found in a systematic search of the yeast genome for proteins containing methyltransferase motifs (3). *HMT1* is not an essential gene in yeast, but it is required in at least two separate genetic backgrounds: in strains with the temperature-sensitive *npl3–1* allele or in strains lacking the 80-kDa cap-binding protein gene *CBP80* (Δ*cbp80*) (4, 9). A search of ESTs for human homologs of *HMT1* revealed two human methyltransferase cDNAs, HRMT1L1 and HRMT1L2, and HRMT1L2 was shown to substitute for *HMT1* in *npl3–1* strains (19). The ability of a human arginine methyltransferase to function in yeast indicates that cellular mechanisms involving protein methylation are conserved throughout eukaryotes.

The *S. cerevisiae* Hmt1 protein has been shown to have methyltransferase activity *in vitro*, methylating a range of RGG-containing proteins including yeast proteins Npl3p, Hrp1p and Hrb1p, and human heterogeneous nuclear ribonucleoprotein A1 (4, 9, 20). A major poly(A) RNA-binding protein in yeast, Npl3p, contains 15 RGG motifs and has been implicated in many cellular processes including nuclear transport, ribosome biogenesis, and silencing (21–25). Hrp1p has three RGG motifs and is a component of cleavage factor 1, which is required for mRNA cleavage and polyadenylation (26). *In vitro* methylation of recombinant Hrp1p had no effect on its specific binding to the polyadenylation efficiency element; however, binding of Hrp1p to RNA inhibits its methylation by Hmt1p (27). Npl3p has been shown to be methylated *in vivo* (4, 20), and there are at least three other proteins that are substrates for Hmt1p (3, 28).

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[1] The abbreviations used are: SAM, *S*-adenosyl-L-methionine; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; PBST, phosphate-buffered saline with 0.25% Tween; MOPS, 4-morpholinepropanesulfonic acid; 5-FOA, 5-fluoro-orotic acid.

TABLE I
Yeast strains used in this study

Strain	Genotype	Source
PSY865	*MATα ade2 ade8 ura3 leu2 his3 lys1 Δhmt1::HIS3*	Ref. #4
PSY866	*MATα ade2 ade8 ura3 leu2 his3 lys1 Δhmt1::HIS3 npl3–1* +pPS1307	Ref. #4
PSY867	*MATα ade2 ade8 ura3 leu2 his3 lys1*	Ref. #4
PSY1191	*MATα ade2 ade8 ura3 leu2 his3 Δhmt1::HIS3 Δcbp80::HIS3* +pPS1307	Ref. #9
PSY1682	*MATα ade2 ade8 ura3 leu2 his3 lys1 hmt1-G68R::HIS3:LEU2*	This study
PSY1683	*MATα ade2 ade8 ura3 leu2 his3 lys1 hmt1-G68A::HIS3:LEU2*	This study
PSY1684	*MATα ade2 ade8 ura3 leu2 his3 lys1 hmt1–20::HIS3:LEU2*	This study
PSY1685	*MATα ade2 ade8 ura3 leu2 his3 lys1 hmt1–46::HIS3:LEU2*	This study
PSY1686	*MATα ade2 ade8 ura3 leu2 his3 lys1 hmt1-E18V::HIS3:LEU2*	This study
PSY1812	*MATα ade ura3 leu2 his3 lys trp1 npl3–27 Δhmt1::HIS3*	Footnote 2
PSY1825	*MATα ade ura3 leu2 his3 lys trp1 npl3–27 hmt1-G68R::HIS3:LEU2*	This study
PSY1826	*MATα ade ura3 leu2 his3 lys trp1 npl3–27 hmt1-G68A::HIS3:LEU2*	This study

TABLE II
Plasmids used in this study

Plasmid	Features	Source
pRS305	*LEU2* Amp^R vector	Ref. #45
pRS315	*CEN LEU2* Amp^R vector	Ref. #45
pRS316	*CEN URA3* Amp^R vector	Ref. #45
pRS425	*2μ LEU2* Amp^R vector	Ref. #46
pPS1305	*CEN LEU2 HMT1*	Ref. #4
pPS1307	*CEN URA3 HMT1*	Ref. #4
pPS1319	His-Hmt1 bacterial expression vector	Ref. #9
pPS1750	*CEN URA3 hmt1-G68R*	This study
pPS1751	*CEN URA3 hmt1-G68A*	This study
pPS1752	*CEN LEU2 hmt1-G68R*	This study
pPS1753	*CEN LEU2 hmt1-G68A*	This study
pPS1757	*CEN LEU2 hmt1–20*	This study
pPS1758	*CEN LEU2 hmt1–46*	This study
pPS1759	*CEN LEU2 hmt1-E18V*	This study
pPS1760	*LEU2 hmt1-G68R*	This study
pPS1761	*LEU2 hmt1-G68A*	This study
pPS1762	*LEU2 hmt1–20*	This study
pPS1763	*LEU2 hmt1–46*	This study
pPS1764	*LEU2 hmt1-E18V*	This study
pPS1765	His-Hmt1-G68R bacterial expression vector	This study
pPS1766	His-Hmt1-G68A bacterial expression vector	This study
pPS1767	His-Hmt1-E18V bacterial expression vector	This study
pPS1805	*CEN LEU2 hmt1–20 N-term. mutations*	This study
pPS1806	*CEN LEU2 hmt1–46 N-term. mutations*	This study
pPS1808	*CEN LEU2 hmt1–20 C-term. mutations*	This study
pPS1809	*CEN LEU2 hmt1–46 C-term. mutations*	This study

TABLE III
Oligonucleotides used in this study

Oligo	Sequence (5′-3′)[a]	Site
5′HMT1a	GAAGACATCCCATGTCCAGA	
3′HMT1a	CACGTTGCACAACCAAAGAC	
AM2	AATACCGGTAC**G**GCAACCGACGTCTAAA	*Kpn*I (−)
AM3	AATACCGGTA**G**CGCAACCGACGTCTAAA	*Kpn*I (−)
AM4	GCTCACCTTGCCGTTTCCAA	
AM7	CTTGTCTCTTGGCCGTCAA	
AM8	GTACACATATTCGCGTCAA	
AM9	CAGAAAAAACCAAGCTTAGTG**T**AAGCGAACAGCAC	*Hin*dIII (+)
AM10	GTGCTGTTCGCTT**A**CACTAAGCTTGGTTTTTTCTG	*Hin*dIII (+)
AM15	CCGACAAATTCATCCAAAGAAACATATGAGCAAGACAGCCG	*Nde*I (+)
AM16	CGGCTGTCTTGCTCATATGTTTCTTTGGATGAATTTGTCGG	*Nde*I (+)
AM17	GATCTACTCATATGCCTATACTAGGTTATTGG	*Nde*I (+)
HMT5′NDE	GGAATTCCATATGAGCAAGACAGCCG	*Nde*I (+)
HMT3′BAM	CGCGGATCCTTAATGCATTAAATAAGAACC	*Bam*HI (+)

[a] Introduced (+) or deleted (−) restriction sites are underlined, and boldface indicates nucleotide changes that result in amino acid substitutions.

Many known and potential substrates of arginine methyltransferases, including Npl3p, Hrp1p, and Hrb1p, are proteins that shuttle between the nucleus and the cytoplasm (9, 21, 26). The first evidence for the importance of *HMT1* in cellular processes was the finding that it is required for efficient export of Npl3p and Hrp1p from the nucleus (9). The role of Hmt1p in protein export is also specific; Hrb1p export is not inhibited by the deletion of *HMT1* (9).

Similar to Npl3p and Hrp1p, eukaryotic Cbp80p is involved in RNA processing and nuclear export of RNA-protein complexes (29–32). The lack of RGG tripeptides in Cbp80p, however, suggests that this protein is not likely to be a substrate for methylation. The lethality of Δ*hmt1* Δ*cbp80* strains may reflect the importance of methylation of Npl3p or other proteins involved in these processes in the absence of Cbp80p. Alternatively, Hmt1p may have cellular functions in addition to its role as a methyltransferase that are required in strains lacking *CBP80*.

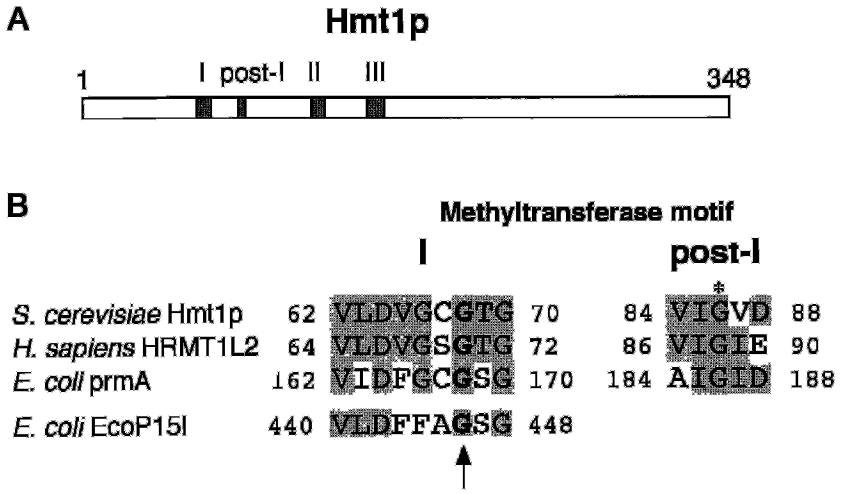

FIG. 1. **Methyltransferase motifs in Hmt1p.** *A*, the primary structure of Hmt1p is shown schematically. The methyltransferase motifs are *shaded*. *B*, alignment of motifs I and post-I from various methyltransferases: Hmt1p (GenBank™ accession number S45890), the most closely related human arginine methyltransferase (HRMT1L2; accession number Y10807), *Escherichia coli* ribosomal protein L11 methyltransferase (prmA; accession number S67010), and *E. coli* DNA methyltransferase EcoP15I (accession number X06287). The highly conserved penultimate glycine of motif I is shown in *boldface*, and the post-I motif glycine mutated in the *hmt1–1* allele is marked with an *asterisk*. Note that resequencing of the HRMT1L2 cDNA revealed that the last residue in the post-I motif was a glutamate rather than a valine. *H. sapiens*, *Homo sapiens*.

We have now tested directly whether the methyltransferase activity is crucial for its biological function. In addition we have discovered a new mutation that affects the ability of Hmt1p to methylate certain substrates and have defined its effect *in vivo*.

MATERIALS AND METHODS

Yeast Strains and Media—The yeast strains used in this study are listed in Table I. All strains were grown and genetic manipulations performed as described previously (33). The plasmids used here are listed in Table II. Oligonucleotides used in plasmid construction and sequencing were synthesized at Integrated DNA Technologies, Inc. and are shown in Table III. All sequences that were amplified by PCR were verified by automatic sequencing at the Dana-Farber Cancer Institute Molecular Biology Core Facility.

Construction of G68 Mutant hmt1 Alleles—HMT1 sequences were amplified by PCR from pPS1307 with oligonucleotides 5′HMT1a and either AM2 or AM3 to introduce mutations that would result in 1) an Arg or Ala change at amino acid 68, respectively, and 2) the loss of a *Kpn*I site. The PCR products were digested with *Age*I and *Nco*I, and pPS1307 was partially digested with *Nco*I, then digested with *Age*I. The 7-kilobase pair fragment of pPS1307 was ligated to each 526-base pair PCR product, resulting in plasmids pPS1750 (G68R) and pPS1751 (G68A). The 2.7-kilobase pair *Xba*I fragments from pPS1750 and pPS1751 were ligated into the *Spe*I site of either pRS315 or pRS305, resulting in plasmids pPS1752 and pPS1753 or pPS1760 and pPS1761, respectively. The integration plasmids pPS1760 and pPS1761 were linearized with *Nco*I, transformed into PSY865 (Δ*hmt1*::*HIS3*) or PSY1812 (Δ*hmt1*::*HIS3 npl3–27*),[2] and selected on plates lacking leucine. Integrated alleles were confirmed by Southern blot analysis or PCR, and expression and activity of mutant Hmt1 proteins were tested by immunoblotting.

Bacterial Expression of His-Hmt1 Proteins—His-Hmt1p expression plasmids were constructed as follows. *HMT1* sequences were amplified by PCR from plasmids pPS1750 (G68R), pPS1751 (G68A), and pPS1759 (E18V) using primers HMT5′NDE and HMT3′BAM. Resulting fragments were digested with *Nde*I and *Bam*HI and ligated into the *Nde*I and *Bam*HI sites of pET-15b. Amplified sequences were verified by automatic sequencing. Purification of recombinant His-Hmt1 proteins, His-Hrp1p, and Npl3-myc was as described in Shen *et al.* (9). A polyclonal rabbit antiserum was raised against purified His-Hmt1p at Covance Research Products, Inc.

Immunoblot Analysis—Strains were grown overnight in the appropriate medium and then diluted and grown at the appropriate temperature. The cells were at mid-log phase (about 10^7 cells/ml) when harvested. For expression studies, yeast cell pellets were frozen before lysis in radioimmune precipitation buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 8.0) supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 2.5 μg/ml each leupeptin, chymostatin, antipain, pepstatin A, and aprotinin) using a Fast-Prep instrument (Bio 101) set at 6.5 for 30 s. Insoluble material was pelleted by centrifugation at 4 °C for 10 min, and the protein concentration of the supernatant was determined by Bio-Rad assay. Indicated amounts of total protein were resolved by SDS, 10% PAGE (34) and immunoblotted essentially as described previously (35). Blots were incubated overnight in phosphate-buffered saline with 0.25% Tween (PBST), 2.5% milk powder and different dilutions of primary antibody (anti-Hmt1p serum, 1:5000; anti-Npl3p serum, 1:1000 (24); 1E4 antibody 1:500, provided by M. Swanson (Gainesville, FL) (36)). After washing in PBST, blots were incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody solution (1:5000 in PBST with milk; Jackson Immunoresearch Laboratories) and washed, and proteins were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech).

In Vitro Methyltransferase Activity Assays—Methyltransferase activity assays were performed in a 15-μl reaction containing 20 mM MOPS, pH 7.2, 400 mM KCl, 2 mM EDTA, 30 μM SAM (Sigma), 3.5 μCi of [methyl-^{3}H]SAM (NEN Life Science Products, 80 Ci/mmol) in addition to enzyme and substrate proteins. In the case of the G68 mutants, 2 μg of enzyme and 2 μg of substrate were used to test methylation of His-Hrp1p. To test the methylation of Npl3-myc by the G68 mutants, 0.2 μg each of enzyme and substrate were used. The reactions were incubated at 30 °C for 30 min.

For the methylation of His-Hrp1p and Npl3p-myc by wild-type Hmt1p and the E18V mutant Hmt1p, reactions were performed in the reaction mix described above, but with 0.7 μM substrate and with increasing amounts of enzyme ranging from 0.07 μM to 7.0 μM. Methylation of the peptide FGGRGGF, which was synthesized by Charles E. Dahl (Biopolymers Facility, Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School), was carried out similarly, but with lower enzyme:substrate ratios. The final concentration for the peptide was 100 μM, with enzyme concentrations ranging from 0.1 μM to 10 μM. Reactions were incubated at 14 °C or 30 °C for 30 min. All reactions were terminated by the addition of SDS-PAGE sample buffer and boiling. Samples were resolved by SDS-10% PAGE (15% for peptide) followed by Coomassie staining and fluorography (Entensify; NEN Life Science Products). Autoradiographs were exposed for 4–16 h at −80 °C.

Immunofluorescence Microscopy—Localization of Npl3p by immunofluorescence microscopy was performed essentially as described previously (37). Wild-type and *npl3–27* strains were grown to mid-log phase at 25 °C and then shifted to 37 °C for 30 min. Cells were fixed in formaldehyde for 1 h at 37 °C before preparation for microscopy. A 1:1000 dilution of anti-Npl3p and a 1:100 dilution of Texas red-conjugated anti-rabbit antibody were used to detect Npl3p.

Generation of Conditional hmt1 Alleles—HMT1 sequences were amplified from pPS1307 by PCR using a variety of conditions to optimize the degree of mutagenesis. All 100-μl reactions contained 1 μl of *Taq*

[2] H. Krebber, unpublished data.

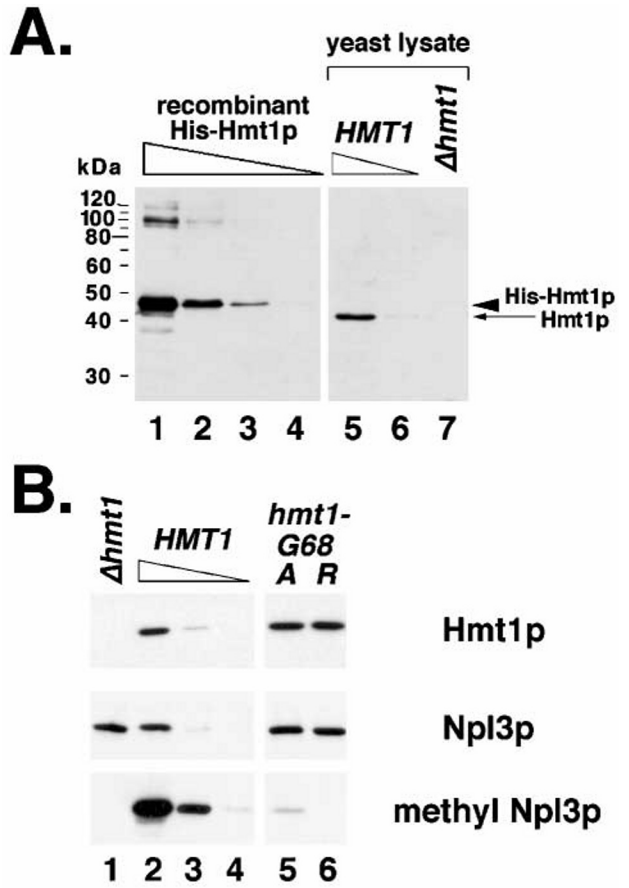

FIG. 2. *A*, anti-Hmt1p serum recognizes only Hmt1p. A polyclonal serum was raised in rabbits against recombinant, histidine-tagged Hmt1p. The original antigen and yeast lysates were resolved by SDS-PAGE, and immunoblot analysis was used to assess the specificity of the antiserum. *Lanes 1–4* contain a dilution series of His-Hmt1p (40, 8, 1.6, 0.32 ng). *Lanes 5* and *6* contain wild-type yeast lysate (10 and 1 μg total protein; PSY867), and *lane 7* contains 10 μg of total lysate from Δ*hmt1* cells (PSY865). *B*, *in vivo* methylation of Npl3p by wild-type and mutant Hmt1 proteins. Mutant *hmt1* alleles with G68 changed to either an alanine (A) or an arginine (R) were integrated into a Δ*hmt1* strain (PSY865) such that only the mutant Hmt1p was expressed. Cells were grown at 25 °C to mid-log phase and lysed, and total protein was resolved on SDS-PAGE gels. Samples were analyzed by immunoblotting with anti-Hmt1p and anti-Npl3p antisera and the monoclonal antibody 1E4, which specifically recognizes methylated Npl3p. All immunoblots compare protein levels in the mutant strains with those in the Δ*hmt1* strain and in a wild-type (*HMT1*) strain (5-fold dilution series). *Lanes 1*, *2*, *5*, *6* contain 5 μg of total protein, *lane 3* contains 1 μg, and *lane 4* contains 0.2 μg of total protein for anti-Hmt1p and anti-methyl Npl3p. Half as much total lysate was used for the anti-Npl3p blot.

polymerase (Amplitaq, Perkin-Elmer), 20 nmol of pPS1307, 50 pmol each of primer 5′HMT1a and 3′HMT1a, 7 mM $MgCl_2$, 1× Perkin-Elmer PCR buffer. Mutagenesis was increased by using 1 mM dTTP, 1 mM dCTP, 0.2 mM dATP, and 0.2 mM dGTP rather than 0.5 mM each dNTP or by also adding 0.5 mM $MnCl_2$ (38).

PCR fragments were purified by elution from Qiaquick columns (Qiagen) and co-transformed into PSY1191 with pPS1305 that had been digested with *Nco*I and *Msc*I. Strains that contained gap-repaired plasmids were selected at 25 °C on plates lacking leucine and then transferred to 5-FOA plates lacking leucine to select for strains that had lost pPS1307. Candidate conditional alleles were identified by transferring cells to YEPD plates at 13 °C, 25 °C, and 37 °C and selecting strains that grew at 25 °C but not at one of the two possible restrictive temperatures. Plasmids were rescued from these strains and retransformed into PSY1191 to test plasmid linkage of the conditional phenotype.

Amplification of *HMT1* with *Taq* polymerase in the absence of further mutagenic conditions resulted in 2–7% lethality. After screening of 26,500 colonies from these transformations, no candidates were identified. Screening of 18,000 colonies from PCR reactions containing altered dNTP concentrations (11–17% lethality) also resulted in no candidate conditional alleles. However, when PCR conditions were altered by changing dNTP concentrations and adding manganese chloride (60% lethality), cold-sensitive alleles were found after screening 20,000 colonies. The *hmt1* alleles that caused conditional growth after retransformation were sequenced from plasmids pPS1757 (*hmt1–20*) and pPS1758 (*hmt1–46*) with oligonucleotides 5′HMT1a, AM4, AM7, and AM8.

To separate N-terminal and C-terminal mutations in the conditional *hmt1* alleles, 0.5-kilobase pair *Nco*I-*Aat*II fragments were exchanged between plasmid pPS1305 and plasmids pPS1757 and pPS1758. Two mutations, one leading to an E18V substitution and the other introducing a silent *Hin*dIII site 5′ to the E18 codon, were introduced into pPS1305 by QuikChange mutagenesis (Stratagene) using gel-purified oligonucleotides AM9 and AM10, following the manufacturer's protocol. The resulting plasmid, which contained the *hmt1-E18V* allele, was pPS1759.

Plasmids for integration of *hmt1–20*, *hmt1–46*, and *hmt1-E18V* alleles were constructed by ligating the 3.5-kb *Bam*HI-*Hpa*I fragment from plasmids pPS1757, pPS1758, and pPS1759 into pRS305 that had been digested with *Bam*HI and *Hpa*I. Integration was performed, and integrated alleles were confirmed as for G68 mutant alleles. Synthetic lethality of integrated alleles was tested by mating with PSY1191 (Δ*hmt1* Δ*cbp80*) or PSY773 (*npl3–1*) and subsequent tetrad analysis.

RESULTS

Mutations in the SAM Binding Motif Disrupt Activity of Hmt1p—To test the importance of methyltransferase activity of Hmt1p *in vivo*, we wished to design point mutations in Hmt1p that would disrupt its catalytic activity (Fig. 1). Sequence alignments of methyltransferases have revealed sequence motifs shared by a diverse set of proteins (17), and the location of these motifs in Hmt1p is shown schematically (Fig. 1*A*). Although residues involved in substrate binding vary among methyltransferases that target nucleic acids, proteins, and small molecules, motifs I and post-I (Fig. 1*B*) are involved in binding to the cofactor SAM (3, 18, 39, 40).

A mutation in the EcoP15I DNA methyltransferase, chang-

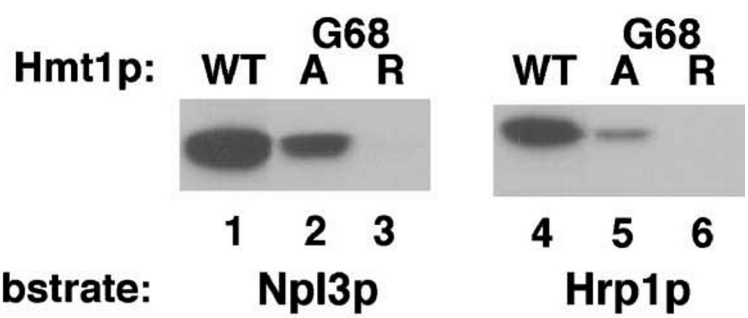

FIG. 3. **Hmt1p G68 mutants have decreased methylation activity *in vitro*.** Recombinant wild-type (*WT*), G68A, and G68R mutant proteins were analyzed for their abilities to methylate both the Hrp1p and Npl3p substrate proteins. *Lanes 1–3* show wild-type, G68A, and G68R, respectively, methylating His-Hrp1p (2 μg each enzyme and substrate), and *lanes 4–6* show them methylating Npl3p-myc (0.2 μg each of enzyme and substrate). Methylation reactions containing 30 μM SAM and 3.5 μCi of [methyl-^{3}H]SAM were incubated for 30 min at 30 °C before SDS-PAGE.

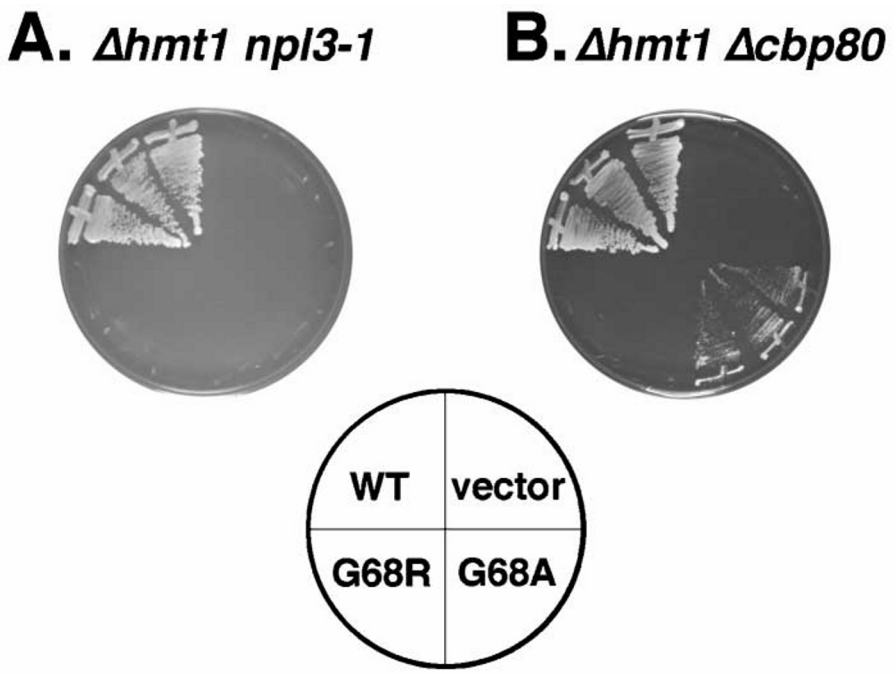

FIG. 4. **Mutations in G68 are synthetically lethal with *npl3–1* and *Δcbp80*.** Synthetic lethal strains Δ*hmt1 npl3–1* (PSY866; *A*) and Δ*hmt1* Δ*cbp80* (PSY1191; *B*) bearing a *URA3 HMT1 CEN* plasmid were transformed with *LEU2 CEN* plasmids that express either no Hmt1p (vector), wild-type (*WT*) Hmt1p, or mutant Hmt1 proteins (G68A or G68R). Cells were transferred to 5-FOA plates and tested for their ability to grow at 25 °C (3–4 days).

ing the highly conserved penultimate glycine in motif I (*bold*, Fig. 1) to a serine or an arginine, reduced the *in vitro* methyltransferase activity more than 400-fold (41). We therefore chose to mutate the equivalent residue in Hmt1p, G68. This residue was mutated to either an arginine or an alanine, as a more conservative change, and the mutant proteins were tested for methyltransferase activity and biological function.

To determine expression levels of wild-type and mutant Hmt1 proteins, an antiserum was raised in rabbits immunized with recombinant, histidine-tagged Hmt1p. Fig. 2*A* demonstrates that the antiserum recognizes both the original antigen (*lanes 1–4*), which migrates as a 45-kDa protein, and endogenous yeast Hmt1p (*lanes 5* and *6*), which migrates as a 40-kDa protein. The absence of a band in lysate from cells lacking *HMT1* (Δ*hmt1*) and the lack of other molecular weight bands indicates the high specificity of the antiserum. Comparison of the His-Hmt1p and wild-type lysate dilution series allowed an approximate calculation of the amount of Hmt1p in the cell, which was $\sim 4 \times 10^5$ molecules/cell.

To test the *in vivo* expression and activity of mutant Hmt1 proteins, mutated alleles were integrated into the genome of Δ*hmt1* cells such that only mutant forms were expressed. Both mutant alleles expressed Hmt1p to the same level as the wild-type strain (*top panel*, Fig. 2*B*). In addition, the anti-Npl3p immunoblot shows that equivalent amounts of Npl3p were found in the lysates (*middle panel*, Fig. 2*B*).

Swanson and co-workers (36) raised a monoclonal antibody, 1E4, against purified yeast Npl3p. The ability of this antibody to recognize Npl3p in yeast lysates but not recombinant bacterially expressed Npl3p led to a series of experiments showing that this antibody specifically recognizes methylated Npl3p, although its exact epitope has not been elucidated (20). We used 1E4 to determine the activity of the mutant Hmt1 proteins *in vivo*. The 1E4 immunoblot (*bottom panel*, Fig. 2*B*) reveals that the methylation of Npl3p in strains expressing mutant Hmt1 proteins is significantly reduced compared with that in the wild-type strain at 25 °C. As expected, Hmt1-G68A, with its less disruptive amino acid change, retains some activity (down >5- but <25-fold), whereas Hmt1p with arginine at position 68 has no detectable activity (>25-fold less than wild type).

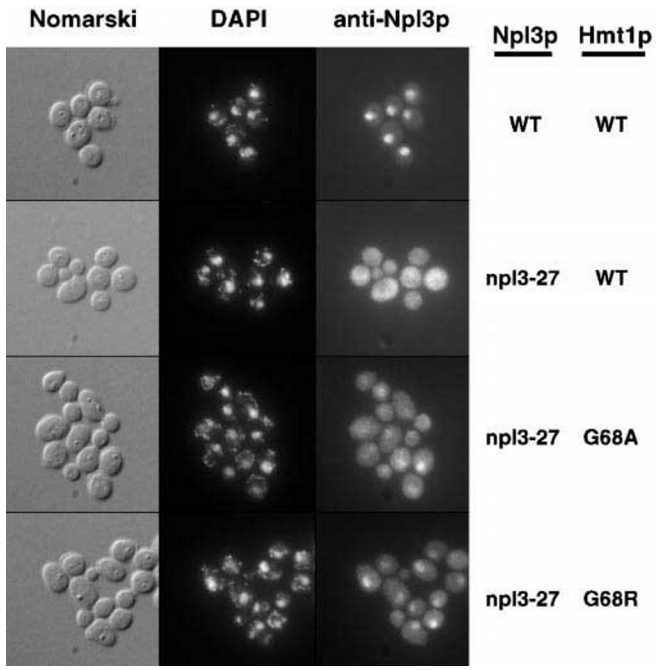

FIG. 5. **Methyltransferase activity of Hmt1p affects localization of Npl3p.** Yeast strains PSY867 (wild type (*WT*)), 1031 (npl3–27), 1826 (npl3–27, G68A), and 1825 (npl3–27, G68R) were grown to mid-log phase at 25 °C and then shifted to 37 °C for 30 min. After a 1-h fixation in formaldehyde at 37 °C, cells were prepared for immunofluorescence microscopy. Npl3p was detected with the anti-Npl3p polyclonal antiserum and a Texas red-conjugated anti-rabbit antibody. Nuclei were visualized by staining with 4,6-diamidino-2-phenylindole (*DAPI*).

The loss of *in vitro* methyltransferase activity of recombinant G68 mutant Hmt1 proteins correlates with their reduced *in vivo* activities (Fig. 3). Histidine-tagged wild-type and mutant Hmt1 proteins were expressed in bacteria, purified, and tested for methylation activity. Equal amounts of His-Hmt1p and substrate were incubated with [methyl-^{3}H]SAM to test if mutant proteins were able to methylate either His-Hrp1p or Npl3-myc. Hmt1-G68A (*lanes 2* and *5*) was less active than wild-type Hmt1p (*lanes 1* and *4*) when either His-Hrp1p or Npl3-myc was

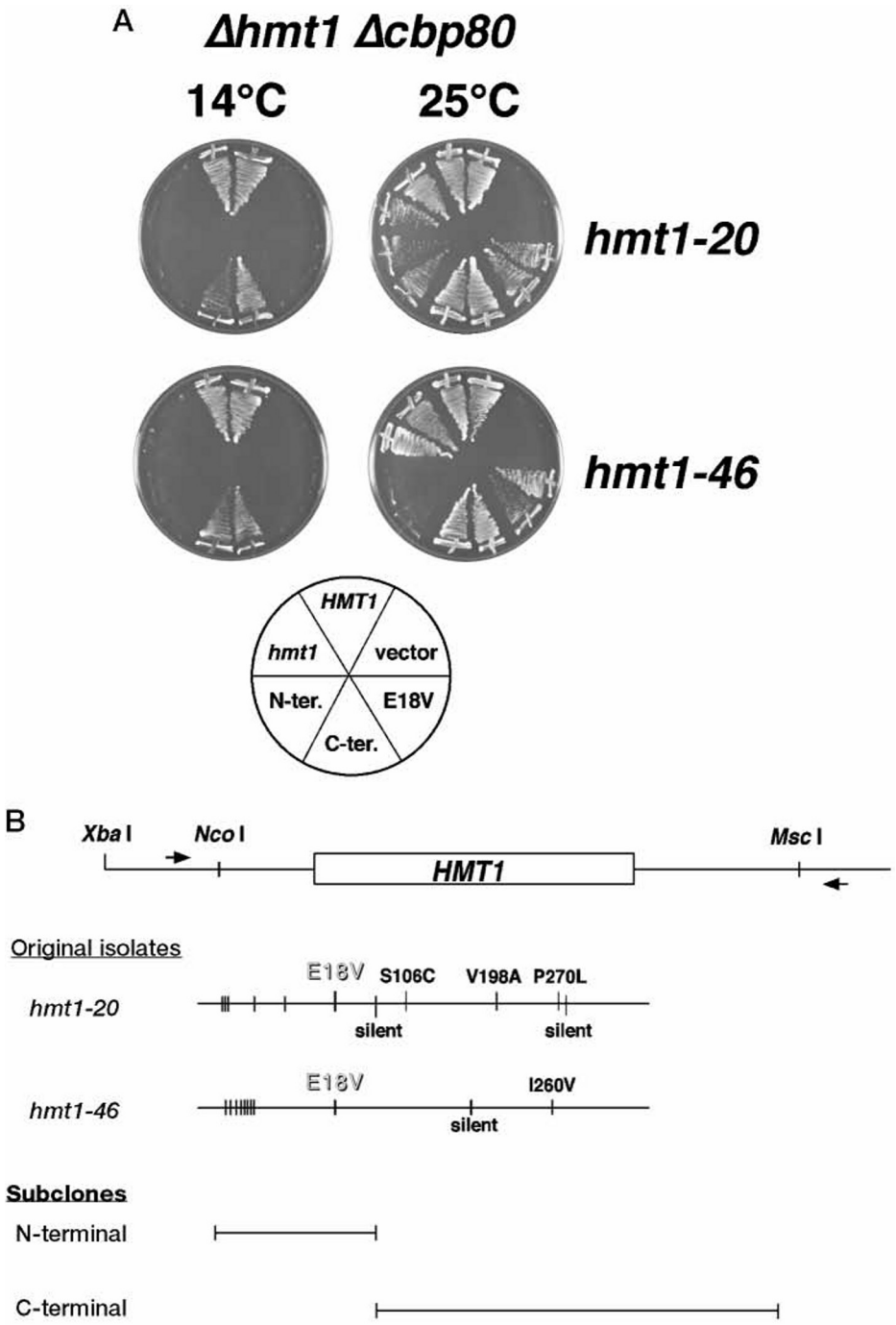

FIG. 6. **Cold-sensitive *hmt1* alleles generated in Δ*hmt1* Δ*cbp80* background.** *A*, PCR mutagenesis of *HMT1* and screening of mutant alleles in PSY1191 (Δ*hmt1* Δ*cbp80* + *HMT1 URA3 CEN*) revealed two cold-sensitive alleles of *hmt1*. PSY1191 was transformed with *LEU CEN* plasmids bearing wild-type *HMT1*, the original mutant alleles (*hmt1–20* and *hmt1–46*; *hmt1*), separated N-terminal and C-terminal mutations (*N-ter.* and *C-ter.*), or the single point mutant allele *hmt1-E18V*. Transformants were transferred to FOA plates lacking leucine and grown at 14 °C (3 weeks) or 25 °C (3–4 days). *B*, the location of mutations in the *hmt1* alleles and the division between N-terminal and C-terminal mutations used in *A* are shown. Restriction sites and oligonucleotides (*arrows*) used in mutagenesis are also shown. Note that both original alleles contain one mutation in common, the E18V mutation.

used as a substrate (Fig. 3). Hmt1-G68R, however, failed to methylate either substrate (*lanes 3* and *6*).

SAM-binding Site Mutations Disrupt Hmt1p Function in Vivo—To study the *in vivo* effect of these mutations that inhibit methyltransferase activity, we took advantage of strain backgrounds that require *HMT1*. Plasmids that express the mutant proteins were transformed into Δ*hmt1 npl3–1* and Δ*hmt1* Δ*cbp80* strains bearing an *HMT1 URA3* plasmid to test for their ability to suppress the synthetic lethality. The growth of strains that had lost the *URA3* plasmid bearing the wild-type *HMT1* was monitored on FOA plates at 25 °C (Fig. 4). Neither mutant *hmt1* was able to suppress the Δ*hmt1 npl3–1* synthetic lethality after 3 days of growth (Fig. 4*A*), and *hmt1-G68A* only partially suppressed the Δ*hmt1* Δ*cbp80* synthetic lethality after 4 days of growth (Fig. 4*B*). Thus, methyltransferase activity correlates with growth in the Δ*cbp80* background, whereas complete wild-type methyltransferase activity appears to be necessary in the *npl3–1* background. Therefore, the methyltransferase activity of Hmt1p is important for its function *in vivo*.

To test whether methyltransferase activity of Hmt1p is important for Npl3p export from the nucleus, we used the *npl3–27* allele, which encodes a mutant Npl3 protein that is imported slowly into the nucleus (37). In the presence of *HMT1*, wild-type Npl3p is predominantly nuclear at 37 °C, whereas npl3–27p is found throughout the cell (Fig. 5 (21)). In the presence of G68 mutant *hmt1* alleles, however, the steady state localization of npl3–27p becomes more nuclear. The severity of this phenotype correlates with loss of methyltransferase activity. Nuclear npl3–27p is found in <50% of hmt1-G68A cells, whereas npl3–27p is concentrated in the nucleus of 60–80% of hmt1-G68R cells. Thus methyltransferase activity of Hmt1p

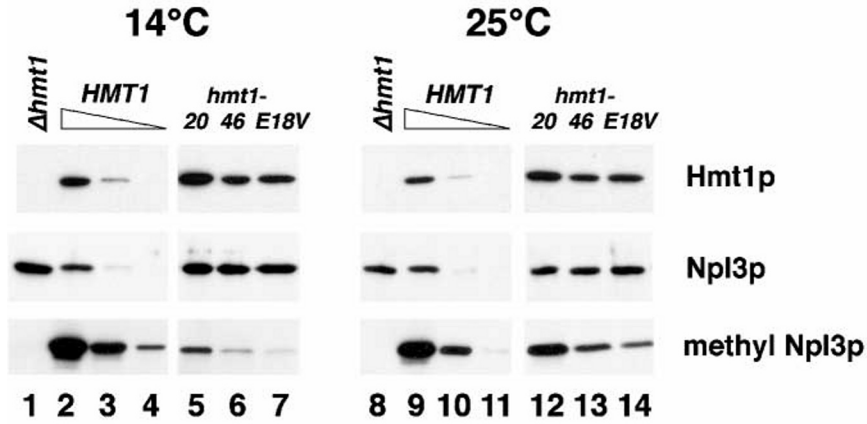

FIG. 7. ***In vivo* methylation of Npl3p by cold-sensitive Hmt1p.** Cold-sensitive *hmt1* alleles were integrated into the genome as in Fig. 2*C*. Cells were grown and lysed, and total protein was used for immunoblot analysis of the expression and activity of the mutant proteins. Lysate from Δ*hmt1* strains and a 5-fold dilution series of wild-type *HMT1* lysate (same as Fig. 2) are shown for comparison. *Lanes 1*, *2*, *5–9*, *12–14* contain 5 μg of total protein, *lanes 3* and *10* contain 1 μg, and *lanes 4* and *11* contain 0.2 μg of total protein for anti-Hmt1p and anti-methyl-Npl3p. Half as much lysate was used for anti-Npl3p blots.

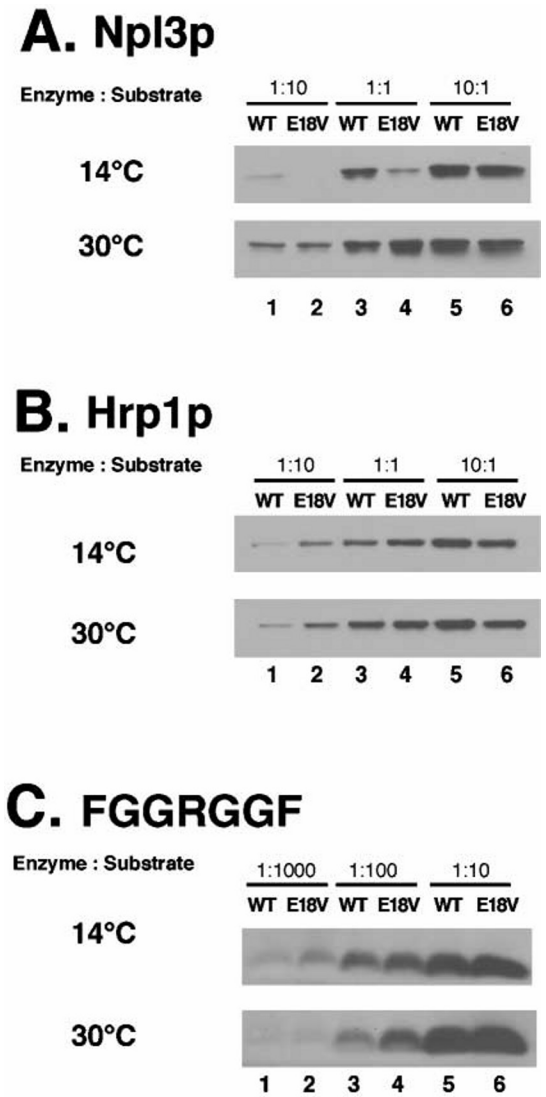

FIG. 8. ***In vitro* activity of wild-type and E18V Hmt1p.** *A*, recombinant Npl3-myc (0.7 μM) was incubated in the presence of [methyl-^{3}H]SAM and increasing amounts of wild-type (*WT*, *lanes 1*, *3*, *5*) or E18V (*lanes 2*, *4*, *6*) mutant His-Hmt1p (*lanes 1* and *2*, 0.07 μM; *lanes 3* and *4*, 0.7 μM; *lanes 5* and *6*, 7 μM enzyme) at 14 °C or 30 °C for 30 min. Proteins were resolved by SDS-PAGE and visualized by fluorography. *B*, recombinant His-Hrp1p was tested for methylation by wild-type and mutant His-Hmt1 proteins exactly as described in *A*. *C*, a synthetic peptide with a single site for methylation (100 μM) was incubated in the presence of increasing amounts of wild-type (*lanes 1*, *3*, *5*) or E18V (*lanes 2*, *4*, *6*) His-Hmt1p (*lanes 1* and *2*, 0.1 μM; *lanes 3* and *4*, 1 μM; *lanes 5* and *6*, 10 μM enzyme). Methylation of the peptide was detected as in *A*.

facilitates export of Npl3p.

Generation of Cold-sensitive Alleles of Hmt1p—To investigate further the *in vivo* function of *HMT1*, we again took advantage of its being essential in Δ*cbp80* strains to generate conditional alleles of *hmt1*. Error-prone PCR was used to amplify and to introduce random mutations into *HMT1* sequences. The entire *HMT1*-coding region and some 5′- and 3′-noncoding sequences were excised from a *LEU2 HMT1 CEN* plasmid. The gapped plasmid and PCR product were co-transformed into a Δ*hmt1* Δ*cpb80* strain that contained a *URA3 HMT1 CEN* plasmid (PSY1191). After *in vivo* gap repair (42), the *URA3* plasmid was eliminated by transferring Leu$^+$ colonies to FOA. *LEU2* plasmids bearing temperature-sensitive or cold-sensitive mutant *hmt1* alleles were then identified by plating on rich medium at different temperatures.

Two plasmids, pPS1762 and 1763, still showed conditional suppression of synthetic lethality when retransformed into the Δ*hmt1* Δ*cpb80* strain. The *hmt1* alleles in these plasmids were named *hmt1–20* and *hmt1*-46. Δ*hmt1* Δ*cpb80* strains bearing these alleles can grow on FOA plates at 25 °C but not at 14 °C, demonstrating the cold sensitivity of these alleles (compare *hmt1*, upper left section and wild-type *HMT1*, top section, Fig. 6*A*).

Given that the conditions used for mutagenesis were predicted to result in multiple mutations, the N- and C-terminal mutations were subcloned separately into the wild-type *HMT1* plasmid pPS1305. The cold sensitivity of each allele mapped to the N terminus (Fig. 6*A*, compare lower left and bottom sections). Sequencing of the 5′-noncoding region, the open reading frame and a portion of the 3′-noncoding region revealed that each allele did contain multiple mutations (Fig. 6*B*). Remarkably, the two alleles shared one mutation in common, A to T at nucleotide 53 of the open reading frame. This mutation resulted in the substitution of a valine for glutamate 18 of Hmt1p (E18V). Introduction of this point mutation into *HMT1* and expression of the resultant *hmt1-E18V* allele in PSY1191 demonstrated that cold sensitivity is mapped to this residue (Fig. 6*A*, lower right section).

The E18V Mutation in Hmt1p Reduces Methylation of Npl3p—Although E18 does not lie within predicted methyltransferase motifs of Hmt1p, mutating this residue might affect methyltransferase activity of the protein. To test this possibility *in vivo*, the original mutant alleles and *hmt1-E18V* were integrated into the genome of a Δ*hmt1* strain (PSY865), and lysates were immunoblotted for expression and activity of the mutant proteins (Fig. 7). The activity of all three mutant alleles, as detected by the relative abundance of methyl-Npl3p, is reduced at 14 °C compared with 25 °C, from slightly less than wild type to more than 20-fold less than wild type (Fig. 7, *bottom panel*). Thus cold-sensitive growth correlates with cold-

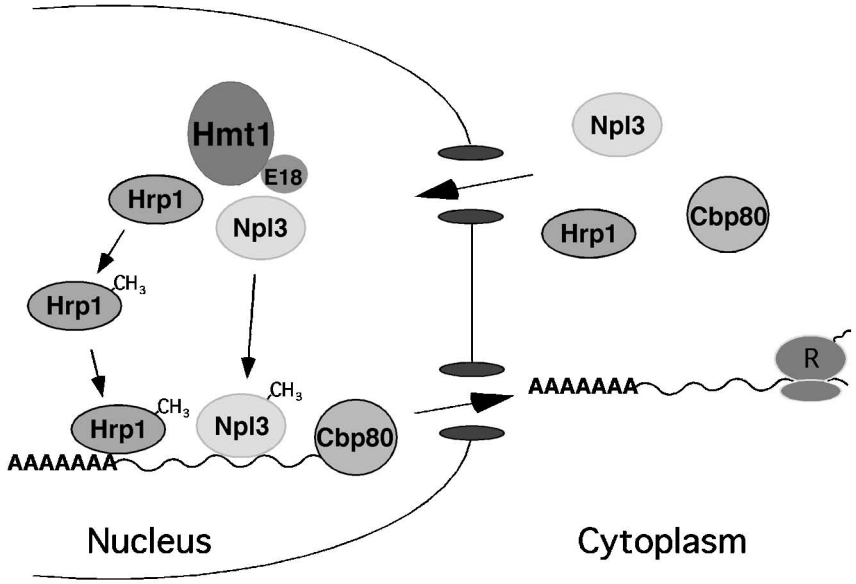

FIG. 9. **Model: Hmt1p and nuclear transport.** Proteins involved in nuclear transport that interact with Hmt1p are shown schematically. Hrp1p and Npl3p are Hmt1p substrates, and the cap-binding protein gene *CBP80* interacts genetically with *HMT1*. These proteins are thought to play a role in packaging mRNA for export into the cytoplasm for subsequent translation (the ribosome is indicated by 60 S and 40 S subunits).

sensitive methyltransferase activity.

Histidine-tagged E18V Hmt1p was expressed in bacteria, purified, and tested for methylation of recombinant Npl3p-myc and His-Hrp1p. Although methylation of Npl3-myc by recombinant E18V Hmt1p was equivalent to that by wild-type Hmt1p at 30 °C, [methyl-^{3}H]Npl3-myc was significantly reduced for E18V reactions incubated at 14 °C (Fig. 8*A*). To test whether the cold sensitivity of methyltransferase activity was substrate-specific, methylation of His-Hrp1p, a synthetic peptide substrate, and GST-Hrb1p were tested at two temperatures (Fig. 8, *B* and *C*, and data not shown). A higher concentration of the peptide substrate was used to allow detection of the tritiated peptide within a responsive range of enzyme-to-substrate ratios. Notably, hmt1-E18V appeared to methylate these substrates as well as, if not better than, wild-type Hmt1p at both temperatures, suggesting that the hmt1-E18V defect is specific for Npl3p (Fig. 8, *B* and *C*, and data not shown).

To test whether the *hmt1-E18V* allele was synthetically lethal with *npl3–1*, a *hmt1-E18V* strain (PSY1686) was crossed to a *npl3–1* strain (PSY773; Ref. 43). After sporulation of the diploid, 20 tetrads were dissected: 6 parental ditypes, 3 nonparental ditypes, and 11 tetratypes. Only two spores of nonparental ditypes and three spores of the tetratypes were viable, and in each case the inviable spore was predicted to be *hmt1-E18V npl3–1* (Leu$^+$, temperature-sensitive phenotype). Thus the cold-sensitive *hmt1* allele was synthetically lethal with *npl3–1*, as expected if methylation of npl3–1p is important for strain viability.

DISCUSSION

The primary yeast protein arginine methyltransferase, Hmt1p, has been shown to be essential for viability of strains with mutations in the RNA-binding proteins Npl3p and Cbp80 (4, 9). In this report, the introduction of point mutations into the co-factor binding site of Hmt1p, one of which abolishes its enzymatic activity, has revealed that its methyltransferase activity is crucial for the survival of these strains. Random mutagenesis of *HMT1* led to the identification of a cold-sensitive allele, *hmt1-E18V*. The N-terminal E18V substitution affected *in vitro* methylation of Npl3p but not of other substrates.

The cofactor-binding site of methyltransferases comprises both specific residues for SAM coordination and a loop that lies between a β-sheet and an α-helix (18, 40). The methyltransferase motif I contains the loop residues, and the third amino acid in the loop, corresponding to G68 in Hmt1p, is structurally constrained (40). Given the sizes of the amino acids substituted for glycine at this residue, arginine would be expected to perturb SAM binding by Hmt1p to a greater extent than alanine would. Correspondingly, no methyltransferase activity of Hmt1-G68R was detected *in vivo* or *in vitro*, whereas the activity of Hmt1-G68A was detectable but significantly reduced. The lower activity of Hmt1-G68A protein was sufficient to partially suppress the Δ*hmt1* Δ*cbp80* synthetic lethality but not that of Δ*hmt1 npl3–1*.

Upon sequencing of the original *hmt1–1* allele, which was found to be synthetically lethal with *npl3–1* (4), we found a mutation that would result in a glycine-to-aspartate change at residue 86 (*asterisk*, Fig. 1*B*; data not shown). The location of this mutation in the post-I methyltransferase motif, which is also thought to be involved in SAM binding (3, 39), suggests that the lethality of the *hmt1–1 npl3–1* strain may also be due to defective cofactor binding and a concomitant reduction in methyltransferase activity.

We were interested in determining whether there are any regions of Hmt1p other than those implicated in SAM binding that are important for its biological function. Such regions might, for example, be involved in Hmt1p-substrate interactions. The change of a glutamate to a valine at residue 18 of Hmt1p caused cold sensitivity of a Δ*cbp80* strain; in a *npl3–1* strain, the *hmt1-E18V* allele was lethal. The E18V substitution resulted in an enzyme with lowered methyltransferase activity toward Npl3p, but not other substrates, at low temperature. This *in vitro* substrate specificity and the location of residue 18 N-terminal to the defined methyltransferase motifs suggest that this residue might be involved in substrate binding or positioning rather than catalysis. It is not yet clear why a mutation that affects Npl3p methylation causes cold sensitivity in the Δ*cbp80* background, but both *NPL3* and *CBP80* have been implicated in RNA export.

The majority of the RNA in the cell is thought to be associated with proteins throughout its lifetime. For example, cap-binding proteins Cbp80p and Cbp20p bind nascent mRNAs co-transcriptionally and are joined by splicing and polyadenylation factors (*e.g.* Hrp1p) and other nuclear RNA-binding proteins, such as Npl3p (44) (Fig. 9). These proteins are likely to escort the RNAs through the nucleus and nuclear pores to the cytoplasm and to be exchanged for cytoplasmic proteins before

translation. In Δ*hmt1* cells, in which Npl3p export is severely slowed, Cbp80 may expedite the export of mRNAs and allow survival (9). Conversely, in the absence of Cbp80, RNA export may require the efficient methylation of Npl3p, which is inhibited at low temperature in *hmt1-E18V* cells. Alternatively, the E18V mutation may disrupt methylation of another substrate that is required at low temperature in the absence of *CBP80*. Future studies should help to define the interactions among these genes implicated in RNA binding and nuclear transport.

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