In vivo analysis of nucleolar proteins modified by the yeast arginine methyltransferase Hmt1/Rmt1p

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ABSTRACT

In this report, we have investigated the impact of arginine methylation on the Gar1, Nop1, and Nsr1 nucleolar proteins in *Saccharomyces cerevisiae*. Although previous reports have established that protein arginine methylation is important for nucleocytoplasmic shuttling, they have focused on the examination of heterogeneous nuclear ribonucleoproteins (hnRNPs). We have extended this analysis to several nucleolar proteins that represent a distinct functional class of arginine-methylated proteins. We first developed an in vivo assay to identify proteins methylated by the Hmt1 arginine methyltransferase. This assay is based on the fact that the Hmt1 enzyme utilizes S-Adenosyl-L-methionine as the methyl donor for protein arginine methylation. Following SDS polyacrylamide electrophoresis, 11 distinct proteins were identified as substrates for the Hmt1 methyltransferase. Hmt1p overexpression did not increase the methylation level on these proteins, suggesting they are fully methylated under the conditions examined. Three of the radiolabeled proteins were confirmed to be Gar1p, Nop1p, and Nsr1p. To monitor the cellular localization of these proteins, functional GFP fusion proteins were generated and found to be localized to the nucleolus. This localization was independent of arginine methylation. Furthermore, all three proteins examined did not export to the cytoplasm. In contrast, arginine methylation is required for the export of the nuclear RNA-binding proteins Npl3p, Hrp1p, and Nab2p. The observation that three nucleolar proteins are modified by Hmt1p but are not exported from the nucleolus implies an alternate role for arginine methylation.

Keywords: Nop1, Gar1, Nsr1, arginine methylation, posttranslational modification

INTRODUCTION

Posttranslational protein modification allows the cell to create novel amino acids that serve to expand its polypeptide diversity and function. Although the methylation of protein at arginine residues was first discovered over 30 years ago (Paik and Kim 1968), only more recent experiments have begun to elucidate the physiological importance of this modification. These experiments have been aided by the identification and cloning of arginine methyltransferases from both higher and lower eukaryotic species (Lin et al. 1996; Scott et al. 1998; Tang et al. 1998; Chen et al. 1999; Pollack et al. 1999; Frankel et al. 2002). 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. Although the impact of arginine methylation on protein function is not yet clear, it has been implicated in a variety of cellular processes ranging from signaling (Altschuler et al. 1999; Bedford et al. 2000; Tang et al. 2000) and transcriptional activation (Chen et al. 1999; Mowen et al. 2001) to nucleocytoplasmic transport (Shen et al. 1998; Yun and Fu 2000).

Two distinct types of protein arginine methyltransferase activities were initially identified in mammalian cell lysates (Ghosh et al. 1988). The two methyltransferases differ in that the Type I enzymes catalyze the formation of asymmetric ω -N^G, N^G-dimethylarginine residues whereas Type II enzymes form symmetric ω -N^G, N^G-dimethylarginine residues. Mammalian Type I substrates are predominately RNA-binding proteins that include hnRNPA1 and A2 (Kim et al. 1997; Nichols et al. 2000), nucleolin (Lischwe et al. 1985b), poly(A)-binding protein II (Smith et al. 1999), and fibrillarin (Lischwe et al. 1985a). The majority of Type I arginine methylation occurs within a domain referred to as an RGG box. This domain is made up of repeated Gly-Gly dipeptides interspersed with arginines and aromatic residues (Beyer et al. 1977). Myelin basic protein (Baldwin and

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Carnegie 1971), coilin (Hebert et al. 2002), the Sm ribonucleoproteins B/B', D1 and D3 (Brahms et al. 2000, 2001), and the Sm-like protein Lsm4 (Brahms et al. 2001) are the only known substrates for the Type II protein arginine methyltransferases.

The first arginine methyltransferase gene, *HMT1* (heterogeneous nuclear ribonucleoprotein methyltransferase) was discovered in the yeast *Saccharomyces cerevisiae* on the basis of its interaction with an abundant yeast mRNA-binding protein termed Npl3p (Henry et al. 1996). The same gene, alternatively called *RMT1*, was also found in a comprehensive search of the yeast genome for proteins containing methyltransferase domains (Gary et al. 1996). *HMT1* is not required for normal cell viability, but is synergistically lethal in at least two genetic backgrounds related to RNA maturation: in strains lacking the 80-kD cap-binding protein gene and strains harboring the temperature-sensitive *npl3-1* allele (Henry and Silver 1996; Shen et al. 2000). Furthermore, although sequence comparison does not identify a clear *HMT1* homolog in the yeast genome, the fact that a yeast strain disrupted for *HMT1* retains 15% arginine methyltransferase activity leaves open the possibility of a second yeast methyltransferase gene (Gary et al. 1996).

The first two human methyltransferase cDNAs isolated, HRMT1L1/PRMT2 and HRMT1L2/PRMT1, were identified by virtue of their sequence similarity to the yeast arginine methyltransferase (Lin et al. 1996; Scott et al. 1998; Scorilas et al. 2000). Since these initial reports, four additional human arginine methyltransferases (PRMT3–6) have been uncovered utilizing a variety of screens designed to identify proteins involved in different cellular processes (Tang et al. 1998; Chen et al. 1999; Pollack et al. 1999; Frankel et al. 2002). Of these, the only symmetrical arginine methyltransferase is JBP1/PRMT5 (Pollack et al. 1999; Rho et al. 2001). The finding that human HRMT1L2/PRMT2 is a functional homolog of yeast *HMT1* indicates that the cellular mechanisms involving arginine methylation are conserved throughout eukaryotes (Scott et al. 1998).

Studies in *S. cerevisiae* to elucidate the importance of Hmt1p methylation have focused on identifying substrate proteins and examining the impact of arginine methylation on their function. Substrate proteins analyzed thus far have included the nuclear RNA-binding proteins Npl3, Hrp1/Nab4, Nab2, and Hrb1 (Bossie et al. 1992; Wilson et al. 1994; Henry et al. 1996; Shen et al. 1998). The Npl3 protein has been implicated in silencing (Loo et al. 1995), pre-rRNA processing (Russell and Tollervey 1992; Singleton et al. 1995), and nucleocytoplasmic protein transport (Bossie et al. 1992), and may act as a carrier for poly(A)+ RNA export from the nucleus (Lee et al. 1996). The Hrp1 and Nab2 proteins are both required for efficient mRNA polyadenylation (Kessler et al. 1997; Minvielle-Sebastia et al. 1998), whereas, Hrp1 plays an additional role in the nonsense-mediated-decay pathway (Gonzalez et al. 2000). In addition, all of these proteins share the ability to shuttle between

the nucleus and the cytoplasm. The first evidence for the importance of HMT1 in cellular processes was the finding that its function is required for the efficient export of the Npl3, Hrp1, and Nab2 proteins from the nucleus (Shen et al. 1998; Green et al. 2002).

To date, arginine methylation studies in *S. cerevisiae* have been primarily restricted to examining arginine-methylated proteins in the nucleus that play roles in pre-mRNA processing and nuclear export. However, a search of the *S. cerevisiae* genome for candidate methylation substrates suggests that not all arginine-methylated proteins fall into this broad functional class. A number of potential substrates for the Hmt1 methyltransferase are nucleolar proteins implicated in rRNA maturation. These proteins include Gar1p, Nop1p, and Nsr1p. Gar1p, which contains 18 RGG repeats, is required for the pseudouridylation of rRNAs (Bousquet-Antonelli et al. 1997). Nop1 contains 17 RGG repeats and is the yeast homolog to mammalian fibrillarin (Schimmang et al. 1989), whereas Nsr1, the yeast homolog to mammalian Nucleolin, contains 5 RGG repeats and has been linked to 18S rRNA processing (Kondo and Inouye 1992; Lee et al. 1992). Although all these nucleolar proteins are likely substrates, no definitive evidence has been presented that these proteins are methylated by the Hmt1 methyltransferase.

To gain a more complete picture of the physiological role of arginine methylation, we examined these potential nucleolar substrate proteins in greater detail. To facilitate our analysis, we first developed an assay to permit the easy identification of arginine-methylated proteins in vivo. With this assay, we demonstrate that all three nucleolar proteins examined in this study are indeed modified by the Hmt1 enzyme. However, unlike the arginine-methylated nuclear proteins previously examined, they do not shuttle between the nucleus and cytoplasm. The observation that these nucleolar proteins are methylated by Hmt1 but are not exported from the nucleus implies that arginine methylation plays an alternate role for these three proteins.

RESULTS

The nucleolar proteins, Gar1p, Nop1p, and Nsr1p, are methylated in vitro

The yeast Hmt1/Rmt1 protein has previously been shown to have methyltransferase activity in vitro, methylating a range of heterogeneous nuclear ribonucleoproteins containing RGG repeats (Gary et al. 1996; Henry and Silver 1996; Shen et al. 1998; Green et al. 2002). However, RGG-containing proteins localized to the nucleolus have not been directly tested as substrates. These potential methyl-accepting substrates include Gar1p, Nop1p, and Nsr1p (Schimmang et al. 1989; Kondo and Inouye 1992; Lee et al. 1992; Bousquet-Antonelli et al. 1997). To determine whether these nucleolar proteins were in vitro substrates for the Hmt1 enzyme, each

protein was fused to either glutathione S-transferase (GST) or a 6-histidine tag and expressed in *Escherichia coli*. The absence of arginine methyltransferase activity in *E. coli* (Liu and Dreyfuss 1995) allows the use of this bacterium as a source of unmodified yeast protein substrates for in vitro methylation. *E. coli* extracts containing the recombinant nucleolar proteins were incubated with the radiolabeled methyl donor, S-Adenosyl-L-[methyl- ^3H]methionine (^3H -SAM) in the presence (Fig. 1A, lanes 2,4,6) or absence (Fig. 1A,B, lanes 3,5,7) of purified GST-Hmt1p enzyme. The expression and stability of each fusion protein was verified by Western blot analysis of the methylation reactions using either anti-GST or anti-Express antibodies (Fig. 1B, lanes 1–7). Proteins were resolved by SDS-PAGE, and labeling was detected by fluorography.

FIGURE 1. Purified Hmt1p methylates Gar1p, Nop1p, and Nsr1p in vitro. (A) Gar1p, Nop1, and Nsr1 are methylated in vitro. *E. coli* lysates from cells expressing recombinant methyl-acceptor proteins, either GST-Gar1p (lanes 2 and 3), GST-Nsr1p (lanes 3 and 4), or His(6)-Nop1p (lanes 6 and 7), were incubated with S-Adenosyl-L-[methyl- ^3H]methionine ([methyl- ^3H]SAM) in the presence (lanes 2, 4, and 6) or absence (lanes 3, 5, and 7) of GST-Hmt1p purified from *E. coli*. As a control, a lysate containing GST alone was incubated in a reaction mixture containing both [methyl- ^3H]SAM and GST-Hmt1p (lane 1). Following incubation, the mixtures were subjected to SDS-PAGE and labeling was detected by fluorography. Sizes are indicated on the left in kilodaltons. (B) Yeast recombinant methyl-acceptor proteins are expressed full length in *E. coli*. Methyl-accepting proteins present in each of the above methylation reactions were resolved by SDS-PAGE and analyzed by blotting with either monoclonal anti-GST or anti-Xpress antibody. The GST-Hmt1p protein bands (lanes 1, 2, and 4) detected by the anti-GST antibody are indicated with an arrow. Sizes are indicated on the left in kilodaltons.

As shown in Figure 1A, only the recombinant yeast nucleolar fusion proteins present in the *E. coli* cell extract are radiolabeled (Fig. 1A, lanes 2,4,6). The observed methylation is not due to the presence of either the endogenous *E. coli* proteins or GST present in the extracts, as cells expressing this tag alone are not labeled (Fig. 1A, lane 1). These results demonstrate that the Gar1, Nop1, and Nsr1 nucleolar proteins are substrates for the Hmt1 enzyme in vitro.

Gar1p, Nop1p, and Nsr1p are methylated by Hmt1p in vivo

To demonstrate that Hmt1p is required for the methylation of Gar1p, Nop1p, and Nsr1p in living cells, an in vivo arginine methylation assay was developed. The assay is based on the ability of the methyl donor SAM to be efficiently transported across the plasma membrane of yeast (Rouillon et al. 1999) and directly utilized for Hmt1-dependent arginine methylation. To allow detection of methylated proteins, the transferable methyl group on SAM is tritium labeled. As described in Materials and Methods, both wild-type and $\Delta hmt1$ mutant yeast cells were labeled with ^3H -SAM, cell extracts were prepared, and proteins were resolved by gel electrophoresis. Methylated proteins were detected by fluorography, and those modified by the Hmt1 methyltransferase were identified as radiolabeled bands present in the wild-type extract but not in the $\Delta hmt1$ extract. The radiolabeling is due to methylation, not translational incorporation, as the proteins were still labeled in the presence of the translational inhibitor cycloheximide (Fig. 2, lanes 2,4).

Eleven radiolabeled bands detected in the wild-type extract were not observed in the $\Delta hmt1$ extract (Fig. 2). These proteins migrated at the following molecular weights: 25.5, 28, 36, 38, 45, 58, 62, 71, 75, 77 and 160 kD. Assuming that each band represents a single polypeptide, the fact that these proteins were only labeled in the wild-type strain indicates that their methylation is Hmt1 dependent. Furthermore, the absence of any detectable label at the corresponding positions in the $\Delta hmt1$ strain indicates that Hmt1p is the predominant, if not sole, methyltransferase that modifies these substrates.

Predictably, labeling in the presence of the translational inhibitors cycloheximide and chloramphenicol reduces the sensitivity of the assay approximately fourfold (Fig. 2, cf. lanes 1,2 and 3,4). However, it also eliminates cotranslational label incorporation, which results in the labeling of at least two additional proteins that migrate at 32 and 46 kD (Fig. 2, lanes 1,3). Importantly, the fact that the number and relative intensity of Hmt1-dependent bands is unchanged in the presence of cycloheximide indicates that the drug does not alter Hmt1p methylation in vivo. Thus, depending on the application, the assay can be performed with or without cycloheximide addition.

FIGURE 2. The methylation of 11 proteins is Hmt1-dependent in vivo. Wild-type (lanes 3 and 4) and $\Delta hmt1$ cells (lanes 1 and 2) were collected at mid-log phase (15 mL), then resuspended in 1 mL synthetic-defined medium and incubated for 10 min at 30°C. For lanes 2 and 4, cells were resuspended in the presence of cycloheximide and chloramphenicol. Labeling was initiated by adding 50 μ Ci of [methyl- 3 H]SAM and the cells were incubated at 30°C for an additional 90 min. The cells were then lysed and soluble proteins were resolved by SDS-PAGE. Proteins were visualized by Coomassie Blue staining and labeling was detected by fluorography. The 11 bands radiolabeled only in the wild-type extract are denoted with arrows on the right. Protein molecular weights are indicated in kilodaltons in the middle.

We next sought to match the Gar1, Nop1, and Nsr1 proteins to the corresponding radiolabeled bands present in our in vivo methylation assay. Many polypeptides containing RGG domains migrate anomalously “slowly” on a gel; thus, we utilized the observed molecular weight values of these proteins on SDS gels for our comparisons rather than the calculated weights. It has been previously reported that although the Gar1, Nop1, and Nsr1 nucleolar proteins have calculated molecular weights of 21.5, 34.5, and 44.5 kD, they migrate aberrantly on a SDS-polyacrylamide gel at 24.5, 38, and 67 kD, respectively (Schimmang et al. 1989; Henriquez et al. 1990; Lee et al. 1991; Girard et al. 1992; Lubben et al. 1995). In addition, on some gels, we observed the mobility of the 36-kD methyl-accepting substrate protein to shift closer to the 38-kD band. Based on observed molecular weight values, we speculated that the radiolabeled proteins migrating at approximately 25.5, 38, and 62 kD on our gels represented the Gar1, Nop1, and Nsr1 proteins, respectively (Fig. 2, WT lanes).

Arginine methylation of the yeast hnRNP, Npl3p, has previously been shown to be Hmt1 dependent in vivo

(Henry and Silver 1996). Based upon its reported observed molecular weight, we reasoned that the radiolabeled band migrating at 58 kD represented Npl3p (Fig. 2, WT lanes).

To confirm our protein assignments, we repeated the in vivo methylation assay using cells over expressing Gar1p, Nop1p, or Nsr1p. As a control for a substrate known to be methylated in vivo, we also overexpressed the yeast hnRNP protein Npl3. We reasoned that increased protein expression would result in increased labeling of the corresponding gel band. To allow regulated overexpression, each gene was placed under control of the GAL1 promoter. Transcription from this promoter is induced in the presence of galactose and repressed in the presence of glucose.

Western blot analysis of yeast extracts prepared from cells following 1 h incubation in galactose confirmed the induction of each protein (Fig. 3A). As expected, overexpression of Gar1p, Nop1, Nsr1p, and Npl3p resulted in increased label incorporation of the 25.5-, 38-, 67-, and 58-kD methyl-accepting bands, respectively (Fig. 3B, lanes 3–6). In each case, the intensity of only a single band increased. Thus, our results suggest that the radiolabeled proteins at 25.5, 38, 58, and 62 kD represent the Gar1, Nop1, Npl3, and Nsr1 proteins, respectively. Furthermore, the in vivo methylation of these proteins is Hmt1 dependent, as they are not labeled in a strain lacking the arginine methyltransferase.

The lack of Hmt1 enzyme activity does not alter the nucleolar localization of Nop1p and Nsr1p

The steady-state location of Gar1p, Nop1p, and Nsr1p is predominantly nucleolar as determined by indirect immunofluorescence (Fig. 4, panels C and I; Henriquez et al. 1990; Lee et al. 1991; Girard et al. 1992). We next tested whether the absence of arginine methylation would alter the localization of these nucleolar proteins. The subcellular localization of Nop1p and Nsr1p was determined by indirect immunofluorescence utilizing anti-Nop1 and anti-Nsr1 antibodies. In both wild-type and $\Delta hmt1$ cells, the Nop1 and Nsr1 proteins are located in the nucleolus (Fig. 4, panels C, F, I, and L). These results demonstrate that arginine methyltransferase activity is not required for the steady-state nucleolar localization of Nop1p and Nsr1p.

To determine the intracellular localization of Gar1p, the green fluorescent protein (GFP) was fused to Gar1p, and the cellular localization was visualized in both wild-type and $\Delta hmt1$ strains by directly monitoring the GFP signal. The Gar1-GFP fusion protein localized to the nucleolus in both wild-type and $\Delta hmt1$ strains (Fig. 5A, panels A,E). This nucleolar accumulation is not due to the presence of the GFP, as GFP alone localizes to the cytoplasm (data not shown). This result is consistent with previous work indicating that the glycine/arginine-rich domains of Gar1p are not required for its steady-state accumulation in the nucleolus (Girard et al. 1992).

FIGURE 3. The methylation of Gar1p, Nop1p, and Nsr1p is Hmt1-dependent in vivo. (A) Galactose induction increases Gar1, Nop1, Nsr1, and Npl3p protein levels. Wild-type cells harboring plasmids encoding either Gar1, Nop1, Nsr1, and Npl3 under control of GAL1 promoter were grown in either glucose or raffinose. Protein expression was induced by adding galactose for 1 h immediately prior to collection. The cells were concentrated, labeled with [*methyl*-³H]SAM, lysed, and soluble proteins were resolved by SDS-PAGE and analyzed by immunoblotting with their corresponding antibodies. (B) Induction of methyl-accepting proteins results in an increase in their labeling. Radiolabeled extracts from A were resolved by SDS-PAGE. Proteins were visualized by Coomassie Blue staining, and labeling was detected by fluorography. The migration of the Nsr1, Npl3, Nop1, and Gar1 proteins are indicated by arrows to the left. Gal-induced proteins are designated with an asterisk on the gel.

To confirm our indirect immunofluorescence results in living cells, we also generated GFP-Nop1 and GFP-Nsr1 fusion proteins. In agreement with our previous results, both fusion proteins remained nucleolar in a $\Delta hmt1$ strain (Fig. 5A, panels B,C,F,G). As previously reported (Lee et al. 1996), the steady-state localization of the nuclear control protein, Npl3, remains nuclear (Fig. 5A, panels D,H). In sum, these results indicate that the nucleolar localization of Gar1p, Nop1p, and Nsr1p is Hmt1 independent.

The ability of the nucleolar GFP fusion proteins to functionally replace the equivalent endogenous proteins was tested by transforming each plasmid construct into the corresponding null strains. As shown in Figure 5B, all GFP fusion proteins rescue the loss of the corresponding wild-type genes, indicating they are functional. In each case, the nucleolar-GFP fusion gene was constructed to allow expression from its own promoter. Thus, each fusion protein is functional when expressed at normal cellular levels. Furthermore, the fusion proteins were shown to be expressed full length as determined by immunoblot analysis using anti-GFP antibody (Fig. 5C). Interestingly, the level of Nop1 is significantly higher than Gar1, Nsr1, or Npl3.

Gar1, Nop1, and Nsr1 proteins do not shuttle

Previous studies have shown that three yeast hnRNPs, Npl3p, Hrp1p, and Nab2p, are nucleocytoplasmic shuttling proteins and that their export to the cytoplasm is arginine methylation-dependent (Shen et al. 1998; Green et al. 2002). To test whether Gar1p, Nop1p, and Nsr1p were also shuttling proteins, we performed a nuclear export assay utilizing a thermosensitive nucleoporin mutant *nup49-313* strain (Lee et al. 1996). At the restrictive temperature, import into the nucleus is blocked, whereas export out of the nucleus is unaffected in this mutant (Doye et al. 1994). In our experiment, plasmids encoding GFP fusions of either the Gar1, Nop1, Nsr1, or Npl3 protein under control of the GAL1 promoter were transformed into a *nup49-313* *Ts*⁻ mutant strain. GFP-fusion protein synthesis was induced by galactose, and then repressed by glucose followed by a temperature shift. We then monitored the GFP signal to localize the fusion proteins. If the GFP-nucleolar fusion proteins shuttle, they should accumulate in the cytoplasm of the *nup49-313* cells after a shift to 36°C. Interestingly, whereas the nuclear Npl3 control protein was cytoplasmic after a temperature shift to

36°C (Fig. 6, panels N and P), the Gar1, Nop1, and Nsr1 proteins remained in the nucleolus (Fig. 6, panels B,D,F,H,J,L), demonstrating that these three nucleolar proteins do not shuttle.

GFP fusion proteins are also methylated in vivo

Although our results suggest that Gar1p, Nop1p, and Nsr1p are methylated by the Hmt1 enzyme and their respective GFP fusion proteins do not shuttle, it is still possible that they do not shuttle simply because the GFP domain blocks methylation of the substrate protein. To eliminate this possibility, we performed the in vivo methylation assay with cells expressing each fusion protein to verify their methylation state. Because we utilized null mutant strains transformed with plasmids encoding the corresponding GFP fusion proteins, we predicted that the matching wild-type protein band would be replaced with a slower migrating fusion protein band.

As expected, a new methyl-accepting protein band was detected in cells expressing GFP-Nop1p (Fig. 7, lane 3),

FIGURE 4. Nucleolar localization of Nop1p and Nsr1p does not require arginine methyltransferase activity. Wild-type (A,B,C,G,H,I) and $\Delta hmt1$ (D,E,F,J,K,L) cells fixed for indirect immunofluorescence were stained with anti-Nop1 (C,F) or anti-Nsr1 (I,L) antibodies and visualized with Alexa Fluor 488-labeled anti-mouse antibody. Cells were viewed by Nomarski optics (A,D,G,J) and nuclei were stained with DAPI (B,E,H,K).

GFP-Nsr1p (Fig. 7, lane 6), and GFP-Npl3p (Fig. 7, lane 4). The observed molecular weights of these proteins closely approximate the predicted size of the Nop1, Nsr1, and Npl3 GFP fusion proteins. Concurrently, the predicted Nop1p, Npl3p, and Nsr1p bands (Fig. 7, lanes 3, 4, and 6) were absent in the corresponding deletion strains.

The analysis of $\Delta gar1$ cells expressing Gar1-GFP was complicated by the fact that both tagged and untagged Gar1p comigrates with the 50-kD and 25.5-kD proteins labeled in the absence of Hmt1 on our in vivo methylation gels (Fig. 7, lanes 2,5). Immunoblot analysis of wild-type cells expressing both Gar1p and Gar1p-GFP confirms that the untagged and tagged proteins migrate at 25.5 and 50 kD, respectively (Fig. 3A, top panel; Fig. 5C, lane 4). The fact that the labeling intensity of the 25.5-kD band is significantly stronger in a wild-type strain than in the $\Delta hmt1$ strain suggests that the band represents two methyl acceptor proteins, one Hmt1 dependent and the other independent. Furthermore, because the intensity of the 25.5-kD band in the $\Delta gar1$ strain drops to a level equal to the $\Delta hmt1$ strain, we predict that the HMT1 dependent protein is likely Gar1p. Unfortunately, because the 50-kD protein is much stronger, this type of comparison cannot be extended to the Gar1-GFP fusion protein. Together, these experimental results indicate that the GFP fusion proteins utilized to detect nucleocytoplasmic shuttling are substrates for arginine methylation.

Gar1p, Nop1p, and Nsr1p are fully methylated in vivo

The inability of Gar1p, Nop1, and Nsr1 to shuttle could conceivably be due to partial methylation of these substrates. To determine whether additional arginine residues could be modified, we examined whether elevated levels of Hmt1p could increase methylation. Figure 8, lanes 1 and 3, shows that when Hmt1p expression is increased during labeling by inducing transcription from the *GAL1* promoter, the in vivo methylation of the Gar1, Nop1, and Nsr1 proteins remains unchanged. Western blot analysis showed that Hmt1p levels were increased at least 20-fold following galactose induction (data not shown), and these levels have no phenotypically detectable impact on cell growth. Under these conditions, the level of Npl3 methylation as well as the remaining methyl-accepting substrates remained unchanged. This finding suggests that under the conditions

FIGURE 5. The nucleolar GFP fusion proteins function as wild type. (A) Localization of the Gar1, Nop1, Nsr1, and Npl3 fusion proteins is independent of arginine methylation. Cells expressing the GFP fusion proteins in wild-type (upper panels) and $\Delta hmt1$ (lower panels) background were viewed by direct fluorescence microscopy. (B) The fusion proteins rescue the loss of the corresponding wild-type genes. Plasmids encoding the GFP fusion proteins were transformed into the corresponding null mutant as indicated by the plate schematic. Transformants were restreaked at 30°C on SC medium supplemented with glucose. (C) GFP fusion proteins are expressed full length. Protein extracts from yeast cells expressing GFP-Nop1 (lane 2), GFP-Npl3 (lane 3), Gar1-GFP (lane 4), or GFP-Nsr1 (lane 5) were analyzed by Western blot. As a control, extract was also prepared from a wild-type strain not expressing a GFP fusion protein (lane 1). The fusion proteins were detected with a monoclonal anti-GFP antibody. Sizes are indicated on the left in kilodaltons.

FIGURE 6. Gar1, Nop1, and Nsr1 proteins do not shuttle. *nup49-313 Ts⁻* mutant cells carrying reporter plasmids expressing either Gar1-GFP (A–D), GFP-Nop1 (E–H), GFP-Nsr1 (I–L), or GFP-Npl3 (M–P) were subjected to the export assay. Cells were incubated for 5 h at 25°C (A,B,E,F,I,J,M,N) or 36°C (C,D,G,H,K,L,O,P). Living cells were photographed by Nomarski optics (A,C,E,G,I,K,M,O) or the GFP signal (B,D,F,H,J,L,N,P).

tested, the arginine-methylated proteins detected in our assay are fully modified by the Hmt1 enzyme in vivo.

DISCUSSION

In this report, we have investigated the impact of arginine methylation on the Gar1, Nop1, and Nsr1 nucleolar proteins. We first show that these RGG-containing proteins are modified in vitro by the yeast arginine methyltransferase, Hmt1/Rmt1. To monitor the methylation of these substrates within the cellular environment, we developed a system to detect Hmt1-dependent methylation in vivo. Using this assay, we have confirmed that the Gar1, Nop1, and Nsr1 nucleolar proteins are indeed methylated by Hmt1p within the cell. Previous studies have demonstrated that the nuclear shuttling proteins Npl3, Hrp1/Nab4, and Nab2 depend on arginine methylation to exit the nucleus (Shen et al. 1998; Green et al. 2002). In contrast, we have found that although Gar1p, Nop1p, and Nsr1p are arginine methylated, their cellular function does not include the ability to exit the nucleus. Furthermore, arginine methylation does not alter the localization or stability of these proteins. Taken together, these data suggest that the effect of arginine methylation on substrate function is protein specific.

A biochemical approach was used to provide direct evidence that Hmt1p modifies the Gar1, Nop1, and Nsr1 nucleolar proteins. In a previous report, the Gar1 and Nop1 proteins were inferred to be methyl-accepting substrates (Frankel and Clarke 1999). This tentative identification was

based on the observation that the molecular weights and isoelectric points of Gar1p and Nop1p were similar to proteins modified by recombinant Hmt1p in extracts prepared from Hmt1-deficient cells. In this report, we directly demonstrate that recombinant Hmt1p specifically modifies recombinant Gar1p and Nop1p, and Nsr1p in vitro, using SAM as the methyl donor. In the GST-Gar1p and GST-Nsr1p reactions, we were able to standardize enzyme and substrate levels based on Western blot analysis to permit an estimation of relative in vitro methylation levels. Not surprisingly, Gar1, which contains 18 RGG repeats, was methylated at a significantly higher level than Nsr1, which contains only 5 RGG repeats.

Our assay system monitors the predominant arginine-methylated proteins in vivo. The assay is based on the fact that ^3H -SAM is transported across the plasma membrane of *S. cerevisiae* and directly utilized as the methyl donor to

FIGURE 7. GFP fusion proteins are methylated in vivo. Fifteen milliliters of wild-type (lane 1), $\Delta hmt1$ (lane 2), and null mutant cells harboring corresponding GFP fusion proteins (lanes 3–6) were collected at mid-log phase and resuspended in 1 mL synthetic-defined medium containing cycloheximide and chloramphenicol. Following a 10-min incubation at 30°C, labeling was initiated by adding 50 μCi of [$\text{methyl-}^3\text{H}$]SAM and incubation was continued for 90 min. The cells were lysed, and soluble proteins were resolved by SDS-PAGE. Proteins were visualized by Coomassie Blue staining, and labeling was detected by fluorography. Sizes are indicated on the left in kilodaltons. The migration of the GFP fusion proteins are indicated with asterisk on the gel.

FIGURE 8. Hmt1p-dependent substrate proteins are fully methylated in vivo. Wild-type (lane 1), $\Delta hmt1$ (lane 2), and wild-type cells transformed with a plasmid carrying HMT1 under control of the *GAL1* promoter (lane 3) were grown to mid-log phase in 15 mL synthetic-defined medium containing raffinose. Induction and labeling were initiated by adding 2 mL of 20% galactose and 50 μ Ci of [*methyl*- 3 H]SAM. Following a 90-min incubation at 30°C, proteins were visualized by Coomassie Blue staining, and labeling was detected by fluorography. Sizes are indicated on the left in kilodaltons.

transfer a radiolabeled methyl group to substrate proteins (Rouillon et al. 1999). Growing yeast cells are labeled with 3 H-SAM and those proteins modified by the Hmt1 methyltransferase are detected in cell lysates as radiolabeled bands that are present on SDS gels in wild-type but not in $\Delta hmt1$ cells. Our laboratory and others have previously utilized L-[*methyl*- 3 H]methionine as an indirect label for in vivo arginine methylation (Liu and Dreyfuss 1995; Green et al. 2002). However, because this label can be directly incorporated into protein during translation, its use required the addition of translational inhibitors, resulting in reduced methyl incorporation. Furthermore, the potential secondary effects of this inhibitor on arginine methylation had not been fully explored. When labeling with 3 H-SAM directly, labeling can be extended and sensitivity increased. In this study, we have demonstrated that the labeling of methylarginines is reduced, but otherwise unaffected by the addition of the translational inhibitors cyclohexamide and chloramphenicol. For our application, a benefit of this addition is that cotranslational labeling is also inhibited. By this method, we reproducibly detect 11 arginine methylated proteins in *S. cerevisiae*.

The strategy utilized to confirm the identity of the meth-

ylated Nop1, Gar1, Nsr1, and Npl3 proteins on our gel system can easily be extended to identify the seven remaining methyl-acceptor substrate bands. Possible candidates in the yeast database are those RGG-containing proteins whose apparent molecular weights on SDS gels match the methylated species visualized in our assay. Based on these criteria, likely potential methyl-acceptor substrates include the nonessential proteins Gbp2, Hrb1, Psp2, Sbp1, Ski2, and Tif4361, as well as the essential proteins Dbp2, Ded1, Hrp1/Nab4, and Rps2. Strains lacking these genes are all available as part of the *Saccharomyces* Genome Deletion Project (Giaever et al. 2002) and would be amenable to analysis in this assay system. Proteins not required for cell viability could be tested directly by assay of the corresponding haploid deletion strain observing loss of a radiolabeled band. Those proteins required for cell viability could be tested by overproduction of the protein in either a wild-type or deletion strain. Prior to this study, the Npl3, Hrp1/Nab4, Hrb1, and Nab2 nuclear proteins have been shown to be arginine methylated in vitro (Henry and Silver 1996; Shen et al. 1998; Green et al. 2002). However, only Npl3p and Nab2p have been shown to be in vivo substrates of Hmt1p (Henry and Silver 1996; Green et al. 2002).

A search of the *Saccharomyces* genome database (genome-www.stanford.edu) for proteins containing arginine residues in a glycine-rich context yields more than the 11 proteins detected in our assay. In fact, the number of potential candidates increases as the stringency of the search is reduced. How can this excess of potential substrates be reconciled with the limited number of proteins labeled in our in vivo methylation assay? One possibility is that not all proteins containing RGG repeats are methylated in vivo. Although previous studies have roughly defined a recognition consensus sequence consisting of RGG repeats, the consensus is not precise enough to predict all the proteins that receive arginine methylation (Birney et al. 1993; Najbauer et al. 1993; Kim et al. 1997). In fact, we know that even within the context of a single protein not all RGG sequences are modified. For example, only three of four RGG residues within a terminal tryptic peptide of human hnRNP A1 were found to be modified (Kim et al. 1997). Another possibility is that the tritium-based assay lacks the sensitivity to detect all the potential methyl-acceptor proteins. Labeling could fall below detection levels if the methyl-accepting substrate was present in low amounts or contained few methylation sites. This appears to be the case for the yeast Hrp1 protein. Although the Hrp1 protein contains four potential sites for arginine methylation within its RGG domains and has previously been shown to be methylated in vitro (Shen et al. 1998), it was not detected on our standard in vivo methylation gels. However, we found Hrp1p could be detected by increasing Hrp1 protein levels (unpubl. results). This was accomplished by either Gal overexpression during labeling or by concentrating the protein following labeling by immunoprecipitation. Thus, the

sum of these results suggests that the assay may only detect abundant arginine methylated proteins.

Arginine methylation represents but a single class of protein methylation that occurs within the cell (Aletta et al. 1998). Enzymes catalyzing these reactions utilize S-adenosylmethionine and can modify a variety of nucleophilic oxygen, nitrogen, and sulfur atoms on the polypeptide chain. These processes result in methyl esters, methyl amines, methyl amides, and other derivatives on the side chains of 9 of the 20 common amino acids, as well as on several additional residues when present at the amino or carboxyl terminus (Clarke 1993). Given the plethora of protein methyltransferases that utilize SAM as a methyl donor, how do you reconcile the fact that in our system a significant percentage of the methylated proteins detected are HMT1 dependent? One explanation is that those methylated proteins low in abundance or modified at only a few residues would not be detected in our assay. A second explanation is that not all potential protein methylation occurs during our experimental conditions. Finally, as shown by our results, some methylation is cotranslational and is inhibited when labeling is performed in the presence of cyclohexamide.

It has been an open question as to whether Hmt1p is the sole asymmetrical arginine dimethyltransferase in *S. cerevisiae*. An early experiment suggested the presence of a second enzyme, as a *HMT1* disruption strain retained a 15% level of asymmetric dimethylarginine residues compared to wild-type cells (Gary et al. 1996). However, no clear *HMT1* homologs are present in the yeast genome database and, to date, no additional protein with asymmetric dimethylarginine activity has been identified in yeast. The results of our in vivo methylation assay would suggest that for those methyl-accepting substrates only modified in the wild-type strain, Hmt1p is the sole enzyme responsible for their modification. However, it is also possible that some proteins modified in both the wild-type and Δ *HMT1* cells are substrates for a second arginine methyltransferase.

Methylation of the Npl3, Hrp1, and Nab2 shuttling hnRNP proteins facilitates their export from the nucleus, thus implicating this modification in the export of RGG-motif proteins (Shen et al. 1998; Green et al. 2002). In contrast, the characterization of another hnRNP protein, Hrb1, whose export was not affected by methylation, implied that arginine methylation could play an alternate role (Shen et al. 1998). However, this second interpretation was tempered by the possibility that although Hrb1p was methylated in vitro, it may not be a bona fide substrate in vivo. In this study, we have expanded the list of known substrates for the Hmt1 methyltransferase to include the nucleolar proteins Gar1p, Nop1p, and Nsr1p. The idea that arginine methylation affects alternate cellular processes is supported by the results of this study in which three arginine-methylated nucleolar proteins do not shuttle.

The finding that Nsr1p does not shuttle in yeast is interesting in light of the fact that the orthologous protein,

nucleolin, shuttles in mammalian cells (Borer et al. 1989). Given that these two proteins are not true homologs, it is reasonable to expect that these proteins could differ in their ability to shuttle. Interestingly, another possible interpretation of this result is that nucleolin shuttling in mammalian cells is independent of arginine methylation. To our knowledge the impact of arginine methylation on nucleolin shuttling has not been tested.

Given that arginine methylation does not impact the localization dynamics of Gar1, Nop1, and Nsr1, future experiments will need to focus on alternate roles for this modification. Likely functions to be modulated include those shared by these three nucleolar proteins: protein-protein interactions, RNA binding, and snoRNA processing.

MATERIALS AND METHODS

Strains and Media

The following yeast strains were used in this study: W303a (*MATa ura3-1 leu2-3,112 ade21 his3-11,15 trp11*); BY4741 (*MATa his3D1 leu2D0 met15D0 ura3D*) (*Saccharomyces* Deletion Project; SDP); #23710 (*MATa/MAT α his3D1/his3D1 leu2D0/leu2D0 lys2D0/LYS2 ura3D0/ura3D0 met15D0/MET15 Δ nop1::KanMX/NOP*) (SDP); MHY629 (*MAT α His3D1 leu2D0 ura3D0 lys2D0 met15D0 Δ nop1::KanMX* plus pMHY193); #3171 (*MATa his3D1 leu2D0 met15D0 ura3D0 Δ hmt1::KanMX*) (SDP); #14789 (*MAT α his3D1 leu2D0 lys2D0 ura3D0 Δ nsr1::KanMX*) (SDP); nup49-313 (*MAT α Δ nup49::TRP1 ura3 leu2 his3 ade2 ade3* plus pUN100-nup49^{ts}-LEU2) (Doye et al. 1994); BMA64 (*MATa/MAT α ura3-1/ura3-1 ade2-1/ade2-1 trp1 Δ leu2-3,112/leu2-3,112 his3-11,15/his3-11,115 can1-100/can1-10*) (Baudin-Baillieu et al. 1997); MHY285 (*MAT α ade2 ade8 leu2 his3 ura3 Δ hmt1::HIS3 Δ nup49::TRP1* plus nup49-313-LEU2); MHY447 (*MATa Δ hmt1::HIS3 his3 leu2 lys1 ura3 ade2 ade8*) and MHY675 (*MATa ura3-1 leu2 ade2 ade3 Δ npl3::HIS3* plus pMHY216). The remaining strains are plasmid transformants. Strains MHY418 (pPS811), MHY479 (pMHY134), MHY481 (pMHY135), and MHY536 (pMHY174) are nup49-313 transformants. Strains MHY419 (pPS811), MHY478 (pMHY134), MHY480 (pMHY135), and MHY541 (pMHY174) are MHY285 transformants. Strains MHY805 (pMHY194), MHY806 (pMHY193), MHY807 (pMHY207), and MHY829 (pMHY216) are #3171 transformants. Strains MHY769 (pMHY229), MHY770 (pMHY230), MHY771 (pMHY231), MHY813 (pPS426), and MHY822 (pMHY260) are BY4741 transformants. Strain MHY800 (pMHY194) is a transformant of #14789.

Strain MHY686, Δ *gar1::TRP1*, was constructed as follows: A PCR strategy (Baudin-Baillieu et al. 1997) was used to produce a DNA fragment in which *TRP1* is flanked by the 5' and 3' sequences of *GARI*. This fragment was amplified from pUC19-TRP1 with the hybrid primers 5' TGAR1 and 3' TGAR1. Following amplification, the reaction mixture was directly transformed into strain BMA64 to disrupt one copy of the genomic *GARI*. Transformants were confirmed by PCR to have the appropriate disrupted as well as wild-type *GARI* genes. To create the nup49-313 Δ *hmt1* double mutant (MHY285), nup49-313 was mated to strain MHY447, a strain created exactly as described for PSY865 (Henry

and Silver 1996). Standard yeast genetic methods and selective growth media were as described previously (Henry et al. 1996).

Plasmid constructs

All plasmids and primers used in this study are summarized in Table 1. A series of plasmids was constructed to facilitate the analysis of each nucleolar protein. Each set consists of a yeast centromere plasmid harboring (1) the wild-type allele, (2) the same allele fused to the GFP gene under control of its own promoter or (3) that of *GAL1*, and (4) an *E. coli* expression vector encoding the wild-type allele fused to either GST or His(6).

The *GAR1* set of plasmids was constructed as follows. The *GAR1* gene was PCR amplified from yeast genomic DNA using primers Gar1 5'/Gar1 3' and the *EcoRI/SalI* digested product was inserted into pRS316 to yield pMHY122. To generate *P_{GAL1} GAR1-GFP* (pMHY134), the *GAR1* ORF was amplified from pMHY122 with primers 5' Gar-Spe /3' Gar-Xho (to simultaneously introduce a *SpeI* site 2 bp upstream of the start codon and replace the stop codon with a *XhoI* site) and the *SpeI/XhoI*-digested product was then inserted into the *XbaI/XhoI* sites of pMHY68. To construct *P_{GAR1} GAR1-GFP* (pMHY207), a *PshA1-SalI* fragment encoding the *GAR1* C terminus was excised from pMHY122 and replaced with a *PshA1-NsiI* fragment encoding the *GAR1-GFP* C terminus from pMHY134. The *SalI* and *NsiI* sites were blunted to allow ligation. To construct *P_{GAL1} GAR1* (pMHY229), *GAR1* was PCR amplified from pMHY122 with primers Bam-GAR1/GAR1 3'-HindIII and then the *BamHI/HindIII*-digested product was inserted into pPS311. To generate *GST-GAR1* (pMHY176) the *GAR1* ORF was PCR amplified from pMHY134 with primers Bam-GAR1/3' Gar1-EcoRI and the *BamHI/EcoRI*-digested product was inserted into pGEX-4T-1 (Amersham Biosciences).

The *NOP1* set of plasmids was constructed as follows. The *NOP1* gene was PCR amplified from genomic DNA using primers Nop1 5'/Nop1 3' and the *EcoRI/SacI*-digested product was inserted into pRS316 to yield pMHY123. To generate *P_{GAL1} GFP-NOP1* (pMHY135), the *NOP1* gene was amplified from pMHY123 using primers Bam-NOP1/NOP1-HindIII (to introduce a *BamHI* site immediately upstream of the *NOP1* start codon and a *HindIII* site downstream from its stop codon) and inserting the *BamHI/HindIII*-digested product into pCGF-1A. As a first step to generate *P_{NOP1} GFP-NOP1* (pMHY193), an *NheI* site was introduced immediately after the ATG codon (pMHY185) by inserting two PCR products (from pMHY123) encoding flanking sequences at a common *NheI* site into the *EcoRI/HindIII* sites of pRS316. The primer combinations used were Nop-Nhe #1/NOP1-HindIII and Nop-Nhe #2/pRS316 MCS. DNA encoding GFP and the N terminus of Nop1p was PCR amplified from pMHY135 with primers GFP-NheI/Nop1 600 and the *NheI/BglII*-digested product was swapped into the *NheI/BglII* sites of pMHY185 to yield pMHY193. To construct *P_{GAL1} NOP1* (pMHY230), *NOP1* was PCR amplified from pMHY123 with primers Bam-NOP1/NOP1-HindIII and the *BamHI/HindIII*-digested product encoding the entire ORF of *NOP1* was inserted into pPS311. This same product was inserted into pRSET-B to generate a *His(6)-NOP1* fusion gene.

The *NSR1* series of plasmids was constructed as follows. A fragment encoding the *NSR1* gene was PCR amplified from genomic DNA using primers Nsr1 5' #2/Nsr1 3' #2. The *SpeI/HindIII*-digested product was then inserted into pRS316 to generate pMHY139. To generate *P_{GAL1} GFP-NSR1* (pMHY174), the *NSR1*

gene was amplified from pMHY139 (with primers 5' NSR1 BamHI and 3' NSR1-HindIII) and the *BamHI/HindIII*-digested PCR product was inserted into pCGF-1A. To construct *P_{NSR1} GFP-NSR1* (pMHY194), an *NheI* site was first introduced at the GFP start codon by inserting two PCR products encoding flanking sequences at a common *NheI* site into the *SpeI/HindIII* sites of pRS315 (pMHY186). The Nsr1-Nhe #1/pRS316 MCS and Nsr1-Nhe #2/Nsr-SpeI primer combinations were used. A fragment encoding GFP and the N terminus of Nsr1p was amplified from pMHY174 with primers GFP-NheI and Nsr1-800. The resulting *NheI/BglII*-digested PCR product was swapped into plasmid pMHY186 to yield pMHY194. To construct *P_{GAL1} NSR1* (pMHY231), *NSR1* was amplified from pMHY139 (with primers 5' NSR1 BamHI and 3' Nsr1 HindIII) and the *BamHI/HindIII*-digested product was inserted into pPS311. To generate a *GST-NSR1* fusion (pMHY178), the *NSR1* ORF was amplified from pMHY174 (with primers 5' NSR1 BamHI and 3' Nsr1 SmaI) and inserting the *BamHI/SmaI*-digested product into pGEX-4T-1 (Amersham Biosciences).

A *P_{NPL3} GFP-NPL3* plasmid was constructed as follows. The unique pRS316 vector *KpnI* site was eliminated by *KpnI* digestion followed by blunt end formation and religation to yield pMHY199. A *NheI* site was then introduced immediately after the ATG codon of the *NPL3* ORF by ligating two PCR products (from pMHY3) encoding flanking sequences at a common *NheI* site and inserting them into the *SacI/SmaI* sites of pRS316 to yield pMHY203. The primer combinations used were Npl3-Nhe #1/Npl3-ScaI and Npl3-Nhe #2/Npl3-Sac. A fragment encoding GFP and the N terminus of Npl3p was amplified from pPS811 with primers GFP-NheI and NPL3-KpnI. The resulting PCR product was digested with *NheI/KpnI* and swapped into plasmid pMHY203 to yield pMHY216.

In vitro methylation reaction

Recombinant GST-Hmt1p (0.2 µg) expressed and purified from *E. coli* (Henry and Silver 1996) was incubated with 3 µL ³H-SAM (specific activity of 84 mCi/mmol; Amersham Biosciences) and *E. coli* whole-cell extracts in a 15-µL volume of methylation assay buffer (50 mM Na-MOPS buffer at pH 7.2, 2 mM EDTA, 0.3 M NaCl, 0.1 µg each of the following protease inhibitors per milliliter: aprotinin, chymostatin, leupeptin, and pepstatin) for 40 min at 30°C. For GST-Gar1 and GST-Nsr1, the amount of extract added to each reaction was adjusted to give equal amounts of recombinant fusion protein, 30 µg and 5 µg, respectively. For His(6)-Nop1, a whole-cell lysate was first precipitated with 55% ammonium sulfate and 20 µg extract was added to each reaction. Terminated reactions were loaded onto a 13% SDS polyacrylamide gel. Following electrophoresis, the gel was fluorographed, dried, and exposed to preflashed HyperFilm (Amersham Biosciences) at -80°C for 12–24 h.

In vivo methylation assay

Cells were grown to 1 × 10⁷ cells per milliliter at 30°C in synthetic-defined media supplemented with either leucine, uracil if plasmid selection was required, or both, and 2% glucose. Cells (1.5 × 10⁸) were collected by centrifugation, washed once in sterile H₂O, and resuspended in 950 µL of the same media containing 40.0 µL 250

TABLE 1. Plasmids and primers used in this study

Plasmid/primer	Description	Source
pRS315	yeast centromere shuttle vector <i>LEU2</i>	Sikorski & Hieter 1989
pRS316	yeast centromere shuttle vector <i>URA3</i>	Sikorski & Hieter 1989
pUC19-TRP1	<i>E. coli</i> cloning vector carrying <i>TRP1</i>	Baudin-Baillieu et al. 1997
pGEX4T-1	<i>E. coli</i> GST expression vector	Amersham Biosciences
pRSET B	<i>E. coli</i> His(6) tag expression vector	Invitrogen, Inc.
pPS311	yeast centromere <i>GAL1</i> promoter expression vector <i>LEU2</i>	Flach et al. 1994
pPS426	yeast centromere galactose inducible expression <i>LEU2</i> vector containing <i>NPL3</i>	Flach et al. 1994
pCGF-1A	yeast high-copy galactose inducible expression vector for fusion to the carboxyl terminus of GFP	Kahana and Silver 1996
pGAL-HMT1	0.6-kb <i>Bam</i> HI- <i>Xba</i> I <i>HMT1</i> fragment in pPS311	Henry and Silver 1996
pMHY3	3.8-kb chromosomal fragment containing <i>NPL3</i> in Ycp50	Henry et al. 1996
pMHY68	yeast high-copy <i>GAL1</i> promoter expression vector carrying FGF-GFP	P. Henry and M. Henry (unpubl. plasmid)
pMHY122	1.9-kb <i>Eco</i> RI- <i>Sal</i> I chromosomal fragment containing <i>GAR1</i> in pRS316	This study
pMHY123	2.3-kb <i>Eco</i> RI- <i>Sal</i> I chromosomal fragment containing <i>NOP1</i> in pRS316	This study
pMHY134	620-bp <i>Spe</i> I- <i>Xho</i> I fragment containing <i>GAR1</i> ORF replacing FGF in pMHY68	This study
pMHY135	1.6-kb <i>Bam</i> HI- <i>Hind</i> III fragment containing <i>NOP1</i> in pCGF-1A	This study
pMHY139	2.3-kb <i>Spe</i> I- <i>Hind</i> III chromosomal fragment containing <i>NSR1</i> in pRS316	This study
pMHY174	1.6-kb <i>Bam</i> HI- <i>Hind</i> III fragment containing <i>NSR1</i> in pCGF-1A	This study
pMHY176	1-kb <i>Bam</i> HI- <i>Eco</i> RI fragment containing <i>GAR1</i> in pGEX4T-1	This study
pMHY178	1.6-kb <i>Sma</i> I- <i>Bam</i> HI fragment containing <i>NSR1</i> in pGEX4T-1	This study
pMHY180	1.6-kb <i>Bgl</i> II- <i>Hind</i> III fragment containing <i>NOP1</i> in pRSET B	This study
pMHY185	pMHY123 with <i>Nhe</i> I site at <i>NOP1</i> start codon	This study
pMHY186	pRS315 carrying <i>NSR1</i> with <i>Nhe</i> I site at start codon	This study
pMHY193	<i>GFP-NOP1</i> under control of the <i>NOP1</i> promoter in pRS316	This study
pMHY194	<i>GFP-NSR1</i> under control of the <i>NSR1</i> promoter in pRS315	This study
pMHY203	pMHY3 with <i>Nhe</i> I site at <i>NPL3</i> start codon	This study
pMHY207	<i>GAR1-GFP</i> under control of the <i>GAR1</i> promoter in pRS316	This study
pMHY216	<i>GFP-NPL3</i> under control of the <i>NPL3</i> promoter in Ycp50	This study
pMHY229	1.1-kb <i>Bam</i> HI- <i>Hind</i> III <i>GAR1</i> fragment in pPS311	This study
pMHY230	1.5-kb <i>Bam</i> HI- <i>Hind</i> III <i>NOP1</i> fragment in pPS311	This study
pMHY231	1.5-kb <i>Bam</i> HI- <i>Hind</i> III <i>NSR1</i> fragment in pPS311	This study
Gar1 5'	GCATCAAGCTCAGGATCCGT	This study
Gar1 3'	GTCGGTCATGGTAGACCAAC	This study
Nop1 5'	GAGCTAGCACTGTGCCATCT	This study
Nop1 3'	GTCTGTCTGGGTACAACCTC	This study
5' Gar-Spe	TTCCTTCCACTAGTTAATGAGTTTCAGAGGAGGT	This study
3' Gar-Xho	CCTTCTTCTCGAGATCTTCTACCTCCTCTGAAACC	This study
Bam-GAR1	AAGTAGGAAGGATCCATGAGTTTCAGAGGAGGTAATAGAGGTG	This study
GAR1 3'-HindIII	CGCAAGCTTGGTTCGACAAGTTATCACC	This study
Nop1 5'	GAGCTAGCACTGTGCCATCT	This study
Nop1 3'	GTCTGTCTGGGTACAACCTC	This study
Bam-NOP1	ACCCACCAAGGATCCATGTCATTGAGACCAGGTAGCAGAG	This study
NOP1-HindIII	AACCACCAAGCTTCCAATCAACGCAACACACGCTC	This study
pRS316 MCS	GTTGTGTGGAATTGTGAGCG	This study
Nop-Nhe #2	CAGAGCTAGCCATTACTGTTTATGTTGATTGAG	This study
Nop-Nhe #1	AGGAATGGCTAGCAGACCAGGTAGCAGAGGTG	This study
GFP-NheI	GGAATGGCTAGCAAAGGAGAAGAACTCTTCACTGG	This study
Nop1 600	AATTCTCTGCCTGGTCTGTG	This study
Nsr1 5' #2	CCTCATACGCAGAGACAGTG	This study
Nsr1 3' #2	CTGATAACACCTTAATGTGAGTA	This study
5' NSR1 BamHI	AAGTAGGAAGGATCCATGGTCAAGACTACTAAAGTAAA	This study
3' NSR1 HindIII	GCACAACGCCCAAGCTTTTA	This study
Nsr-SpeI	AAGGACTAGTATTTAAACCCCATCACGTTGGATTG	This study
Nsr1-Nhe #2	GTTGGCTAGCCATCTATTTTATCTGCTCC	This study
Nsr1-Nhe #1	AGGATGGCTAGCACTTACTAAAGTAAAGGTAACAAG	This study
Nsr1 800	GTGTCAGATGGTTCAGATGGG	This study
Npl3-Sac	CGCTCCACAAATCCACGTC	This study
Npl3-Nhe #2	GTTGGCTAGCCATTATCCTTATGGTTTATAGC	This study

(continued)

TABLE 1. Continued

Plasmid/primer	Description	Source
Npl3-Nhe #1	AGGATGGCTAGCTCTGAAGCTCAAGAACTCACG	This study
Npl3-ScaI	CACAGATTTAACAGGCCATAATAGTAC	This study
NPL3-KpnI	CTTCTGATTGGTGGAGGATTG	This study
5' TGAR1	CCAAAAGATCACCTGAGGATAATTTTAAACAAAAGAA	This study
3' TGAR1	GATAAAGGATGGTTTTTTTTTTTATTTTTTCTAGCTAG	This study
3'Gar1 EcoRI	CGGAATTCGTCGACAAGTTATCACCTAC	This study
3'Nsr1 SmaI	TCCCCGGGGCACAACGCCCAAGCTTTTA	This study

mM potassium phthalate buffer (pH 5.0). When required, translational inhibitors were added to the resuspended cells at the following amounts: 15 μ g each of cycloheximide and chloramphenicol. Cells were immediately transferred to 12 $\times$ 75 mm culture tubes and incubated for 10 min at 30°C on a rolling drum. Labeling was initiated by the addition of 50.0 μ Ci of 3 H-SAM (specific activity of 84 mCi/mmol; Amersham Biosciences) and incubation was continued for 90 min at 30°C. Cells were then pelleted, washed once in lysis buffer (25 mM Tris-HCl, 1 mM sodium EDTA, 1 mM sodium EGTA at pH 7.5), and resuspended in 100 μ L of lysis buffer supplemented with protease inhibitors (10 mg/mL phenylmethylsulfonyl fluoride and 2.5 μ g/mL [final] each of aprotinin, leupeptin, chymostatin, and pepstatin) and 1 M DTT. Cells were lysed and protein extracts clarified by a 10-min centrifugation at 13,000 r.p.m. at 4°C. Proteins were then resolved by SDS-PAGE and analyzed via Coomassie staining, fluorography, and immunoblot analysis.

Immunoblot analysis

Total cell extracts were prepared (Bossie et al. 1992) from cells grown in either YEPD or SC medium. Proteins were resolved by SDS-PAGE, transferred, and immunoblotted as described previously (Bossie et al. 1992). Rabbit polyclonal anti-Gar1p and affinity purified anti-Npl3p (Bossie et al. 1992) were used at 1:200 and 1:1000, respectively. Mouse monoclonal anti-GFP (Clontech), anti-GST B-14 (Santa Cruz Biotechnology), anti-Nop1 A66 (Henriquez et al. 1990), and anti-Nsr1 5B5 antibodies were used at concentrations of 1:1000, 1:5000, 1:10,000 and 1:5000, respectively. The blots were then incubated with horseradish peroxidase (HRP)-conjugated anti-mouse antibodies at a concentration of 1:5000 and detected with ECL Western Blotting Reagents (Amersham Biosciences).

Export assay

The nuclear export assay was performed exactly as described (Lee et al. 1996). Briefly, cells containing various reporter plasmids are induced for 2 h in medium containing galactose to express the reporter followed by repression for 2 h in medium containing glucose. Cells are then incubated for 5 h at either 25°C or 36°C and examined for GFP fluorescence.

Indirect immunofluorescence

Indirect immunofluorescence microscopy was performed as previously described (Sadler et al. 1989). Mouse monoclonal anti-

Nop1p A66 (Henriquez et al. 1990) was used at 1:2000, and mouse monoclonal 5B5 anti-Nsr1p was used at 1:2000.

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