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Bms1p, a novel GTP-binding protein, and the related Tsr1p are required for distinct steps of 40S ribosome biogenesis in yeast

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ABSTRACT

Bms1p and Tsr1p define a novel family of proteins required for synthesis of 40S ribosomal subunits in *Saccharomyces cerevisiae*. Both are essential and localize to the nucleolus. Tsr1p shares two extended regions of similarity with Bms1p, but the two proteins function at different steps in 40S ribosome maturation. Inactivation of Bms1p blocks at an early step, leading to disappearance of 20S and 18S rRNA precursors. Also, slight accumulation of an aberrant 23S product and significant 35S accumulation are observed, indicating that pre-rRNA processing at sites A_0, A_1, and A_2 is inhibited. In contrast, depletion of Tsr1p results in accumulation of 20S rRNA. Because processing of 20S to 18S rRNA occurs in the cytoplasm, this suggests that Tsr1p is required for assembly of a transport- or maturation-competent particle or is specifically required for transport of 43S pre-ribosomal particles, but not 60S ribosome precursors, from the nucleus to the cytosol. Finally, Bms1p is a GTP-binding protein, the first found to function in ribosome assembly or rRNA processing.

Keywords: 18S rRNA; GTP-binding; nucleolus; P-loop; rRNA processing

INTRODUCTION

In eukaryotic cells, ribosome formation occurs mainly in a specialized region of the nucleus called the nucleolus. Here, ribosomal RNA (rRNA) is transcribed, modified, processed, and assembled with 78 ribosomal proteins (r proteins) to produce the large and small ribosomal subunits (reviewed in Woolford & Warner, 1991). In *Saccharomyces cerevisiae*, the 18S, 5.8S, and 25S rRNAs are transcribed as part of a 35S precursor molecule, which is heavily methylated, modified by conversion of uridines to pseudouridines, and cleaved in a series of processing steps to release the mature rRNAs (Kressler et al., 1999; Venema & Tollervey, 1999). 18S rRNA is produced from the 35S precursor by cleavage at sites A_0, A_1, and A_2 to generate a 20S intermediate, followed by transport of the 20S rRNA to the cytosol as part of a pre-ribosomal particle, where it is processed to the mature 18S rRNA (Udem & Warner, 1973).

Correct formation of mature 18S rRNA requires many *cis*-acting signals and a growing number of *trans*-acting factors. These include exo- and endonucleases, snoRNAs and their associated proteins, RNA helicases, and other proteins with poorly defined functions (reviewed in Kressler et al., 1999; Venema & Tollervey, 1999). The most numerous class of accessory factors are snoRNAs, which guide methylation and pseudouridylation enzymes to specific sites (Kiss-Laszlo et al., 1996; Ni et al., 1997). Almost all snoRNAs are dispensable for ribosome production, except for the U3, U14, snR10, and snR30 snoRNAs, which are required for early steps in processing that lead to 18S rRNA (Tollervey, 1987; Morrissey & Tollervey, 1993; Tollervey & Kiss, 1997). Of these, U3 is the best characterized snoRNA, and several proteins have been identified that associate with U3 (see Kressler et al., 1999; Venema & Tollervey, 1999; and references therein). U3 is required for two distinct steps in 18S processing; one which involves base pairing to the 5′ end of the 35S precursor and is necessary for cleavage at sites A_0, A_1, and A_2,

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and another which involves base pairing to the 18S rRNA and is required for A_1 and A_2 cleavage (Sharma & Tollervey, 1999). In addition, several putative, ATP-dependent RNA helicases of the DEAD/H box family are required for 18S rRNA formation; these presumably function in formation of rRNA structure and/or assembly of proteins onto the rRNA (de la Cruz et al., 1999). Other than ATP-dependent helicases, no other nucleotide-binding proteins are known to play a role in rRNA processing. Finally, nucleo/cytoplasmic transport factors are required for export of 20S rRNA-containing pre-ribosomes to the cytosol, although no transport factors specific for the small ribosomal subunit have as yet been identified (Moy & Silver, 1999; Stage-Zimmermann et al., 2000).

Although the pre-rRNA cleavage and modification steps of ribosome synthesis are relatively well understood, assembly of the r proteins with the rRNA remains mysterious. This is largely due to the tightly interconnected nature of assembly and processing, such that defects in assembly cause inhibition of rRNA processing (Tollervey et al., 1993; Baudin-Baillieu et al., 1997). In addition, feedback inhibition mechanisms block rRNA processing if downstream events do not occur properly (Lafontaine et al., 1998; Allmang et al., 2000).

In this report, we describe the identification of two novel related, essential nucleolar proteins, Bms1p and Tsr1p. These proteins share significant sequence similarity and represent a new family of factors required for ribosome biogenesis. Depletion of either protein leads to a defect in production of 18S rRNA and 40S ribosomal subunits; however, the two proteins do not perform identical roles in 40S synthesis. Bms1p is required early in small ribosome subunit assembly or rRNA processing, whereas Tsr1p (Twenty S rRNA accumulation) acts later in 40S synthesis and may be required for maturation of 43S pre-ribosomal subunits or for their transport to the cytosol. Interestingly, Bms1p contains a nucleotide-binding motif and specifically binds GTP, the first such protein found to be required for ribosome biogenesis.

RESULTS

Bms1p is a novel, essential protein conserved throughout eukaryotes

We initially identified *BMS1* in a synthetic lethal screen for mutants sensitive to reduced levels of 14-3-3 proteins (D. Gelperin, L. Horton, J. Hensold, and S.K. Lemmon, in prep.: see Discussion). The mutant isolated, designated *bms1-1*, is temperature sensitive for growth, and this phenotype was used to clone the gene. A previously uncharacterized open reading frame (ORF; YPL217c) was able to complement the temperature sensitivity of *bms1-1*. Integration and segregation analysis confirmed that YPL217c is allelic to *BMS1*. To determine the phenotype of a *bms1* null mutation, the entire coding sequence was replaced by *HIS3* in a wild-type diploid strain (SL3768) and haploid progeny were examined by tetrad analysis. No His^+ spores were recovered, indicating that *BMS1* is an essential gene.

The sequence of *BMS1* predicts a hydrophilic protein of 1183 amino acids (Fig. 1). Bms1p contains three tandem bipartite nuclear localization sequences (NLS) at its carboxyl terminus. In addition, near the amino terminus is a P-loop nucleotide binding motif (GXXXXGK[S/T]; Saraste et al., 1990), which is predicted to function in binding of ATP or GTP. The region surrounding this motif has homology to bacterial IF-2 proteins, suggesting that it may bind to GTP (Fig. 1D; Bourne et al., 1991). In agreement with this, Sanchez and Sali (1998) identified this region as being similar to the GTPase EF-Tu using a structural modeling method.

Comparison of Bms1p to current databases revealed two classes of sequences with similarity to Bms1p in a wide range of eukaryotes, including humans (Fig. 1). Members of the first class are similar in size to Bms1p and contain a GTP-binding motif and at least one NLS. These are 30–42% identical to Bms1p with up to 60% identity in the amino and carboxyl terminal regions (Fig. 1B,C). Members of the second class are usually ~800 amino acids in length, compared to nearly 1,200 amino acids for Bms1p family proteins. Typically, members of the second group show 30–40% identity to one another, and 20–25% identity to the corresponding Bms1p family member from the same species. Class 2 sequences do not contain any recognizable nucleotide-binding motif, although several have recognizable bipartite NLS. Members of this class, exemplified by the uncharacterized *S. cerevisiae* ORF YDL060w (which we now call *TSR1*; see below), have a smaller central acidic/variable domain with regions of sequence identity to Bms1p concentrated at the amino and carboxyl-termini (Fig. 1). Most species in the databases appear to contain one representative member of each class, a Bms1p and a Tsr1p type, and most likely represent the respective functional counterparts of these yeast proteins.

Bms1p binds GTP

Because the P-loop motif in Bms1p is predicted to function as part of a nucleotide-binding site, possibly GTP specific, we tested whether the P-loop is required for Bms1p's function by mutating the P-loop lysine at position 82 to alanine. This mutation is predicted to abolish nucleotide binding as well as nucleotide hydrolysis, by analogy to the effects of similar mutations made in other P-loop-containing proteins (Deyrup et al., 1998). Bms1p-K82A could not serve as the sole source of Bms1p (Fig. 2A), even though the protein was relatively stable when expressed in a *BMS1* strain (Fig. 3B). This demonstrates that this amino acid is critical for Bms1p function.

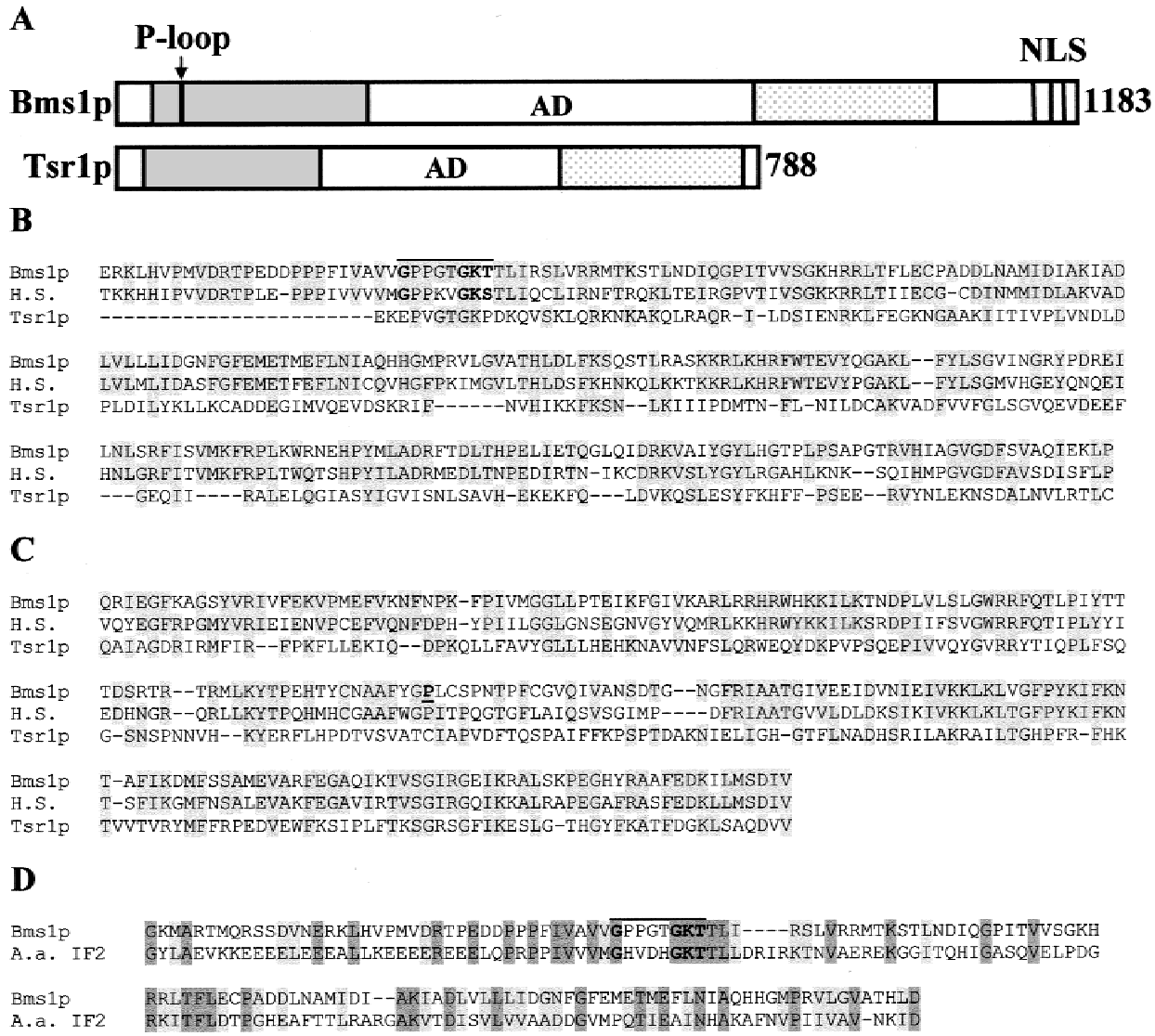

FIGURE 1. Bms1p and Tsr1p share two conserved regions. **A:** Diagram of Bms1p and Tsr1p highlighting the major N-terminal (gray) and C-terminal (stippled) regions of shared homology flanking a central acidic domain (AD) (26–28% Asp/Glu). Locations of Bms1p P loop and NLS are indicated. **B:** Alignment of amino-terminal region of Bms1p (51–305) with *Homo sapiens* KIAA0187 (65–314) and *S. cerevisiae* Tsr1p (36–243). Identical amino acids are shaded; P loop motif is indicated with bold/overline. **C:** Alignment of C-terminal regions of Bms1p (778–1001), KIAA0187 (875–1096) and Tsr1p (550–769). P888, which is mutated to leucine in *bms1-1*, is indicated in bold/underline. This proline is universally conserved in all Bms1p-class homologs. **D:** Bms1p P loop region is similar to bacterial IF-2. Alignment of residues 37–175 of Bms1p to 276–419 of *Aquifex aeolicus* IF-2, which are 27% identical and 48% similar. The P loop motif is indicated by an overline. Identities are shaded with dark gray; similarities are shaded with light gray. Sequences were aligned using Clustal W (Thompson et al., 1994).

To test whether K82A is part of a functional nucleotide-binding fold, Bms1p and Bms1p-K82A were purified as GST fusions from yeast, incubated with ^{32}P-ATP and ^{32}P-GTP, and exposed to UV light for crosslinking. The results indicate that ^{32}P-GTP, but not ^{32}P-ATP, crosslinked to GST-Bms1p (Fig. 2B). Furthermore, the P-loop mutant, GST-Bms1p-K82A, showed no crosslinking to ATP and only faint interaction with GTP, despite being present at fourfold higher amounts than GST-Bms1p in the reactions, as judged by immunoblotting (Fig. 2B). These data demonstrate that Bms1p is a bona fide GTP binding protein.

Bms1p is a nucleolar protein required for 40S ribosomal subunit biogenesis

To investigate the function of Bms1p, its cellular localization was examined by indirect immunofluorescence. A strain (SL4012) was constructed in which Bms1p was tagged with two copies of the hemagglutinin epitope (HA) and the gene was expressed from its own promoter on a low copy plasmid as the sole source of the protein. Indirect immunofluorescence revealed strong staining of a caplike structure within the nucleus, adjacent to the main mass of DNA (Fig. 2C). This

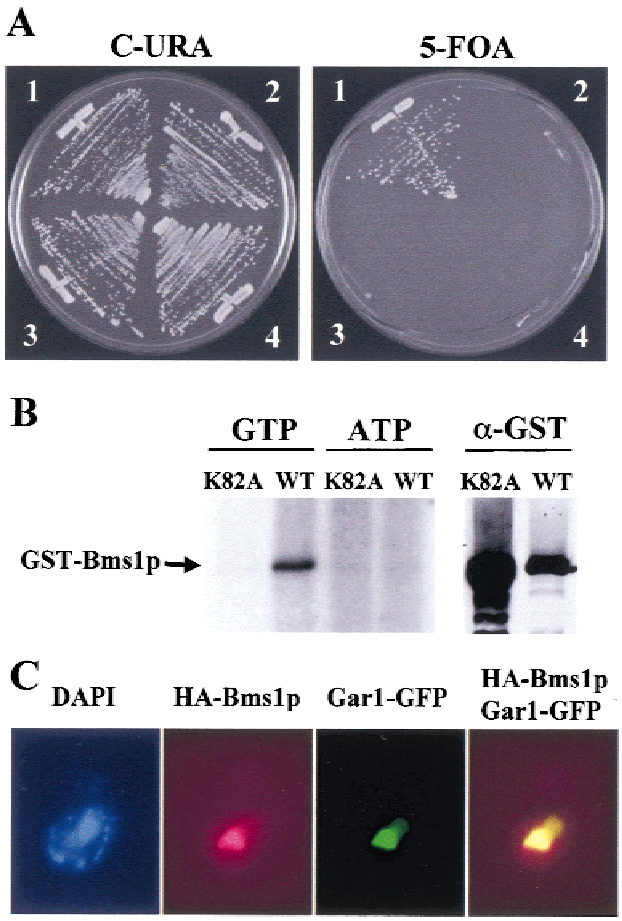

FIGURE 2. Bms1p binds GTP and is localized to the nucleolus. **A:** Bms1p-ΔNLS and Bms1p-K82A (ΔP loop) are nonfunctional. A *bms1-*Δ strain with a *BMS1, URA3* plasmid (SL3875) was transformed with *CEN*, *LEU2* plasmids containing (1) *HA-BMS1* (pDG110); (2) empty vector (pRS315); (3) *HA-bms1-*Δ*NLS* (pDG114); and (4) *HA-bms1-K82A* (pDG116). Transformants were grown at 30 °C for 3 days on C-URA or 5-FOA plates to select against the *BMS1*, *URA3* plasmid. *HA-bms1-*Δ*NLS* and *bms1-K82A* were also unable to support growth of *bms1-*Δ spores germinating on rich medium (data not shown). **B:** Bms1p binds to GTP but not to ATP. GST-Bms1p (WT) and GST-Bms1p-K82A (K82A) were expressed in strain BJ2168 transformed with pDG98 and pDG115, respectively. GST purification and crosslinking to α-^{32}P GTP (GTP) or α-^{32}P ATP (ATP) were carried out as described in Materials and Methods. An equal aliquot for each glutathione-purified sample was analyzed by immunoblotting with anti-GST antibodies (α-GST). **C:** HA-Bms1p and Gar1-GFP were visualized by fluorescence microscopy in a *bms1-*Δ*/bms1-*Δ strain carrying HA-*BMS1* and *GAR1*-GFP plasmids (SL4012+pZUT3). HA-Bms1p localization with anti-HA antibodies is false-colored in red, Gar1p-GFP is in green, and DNA stained with DAPI is in blue.

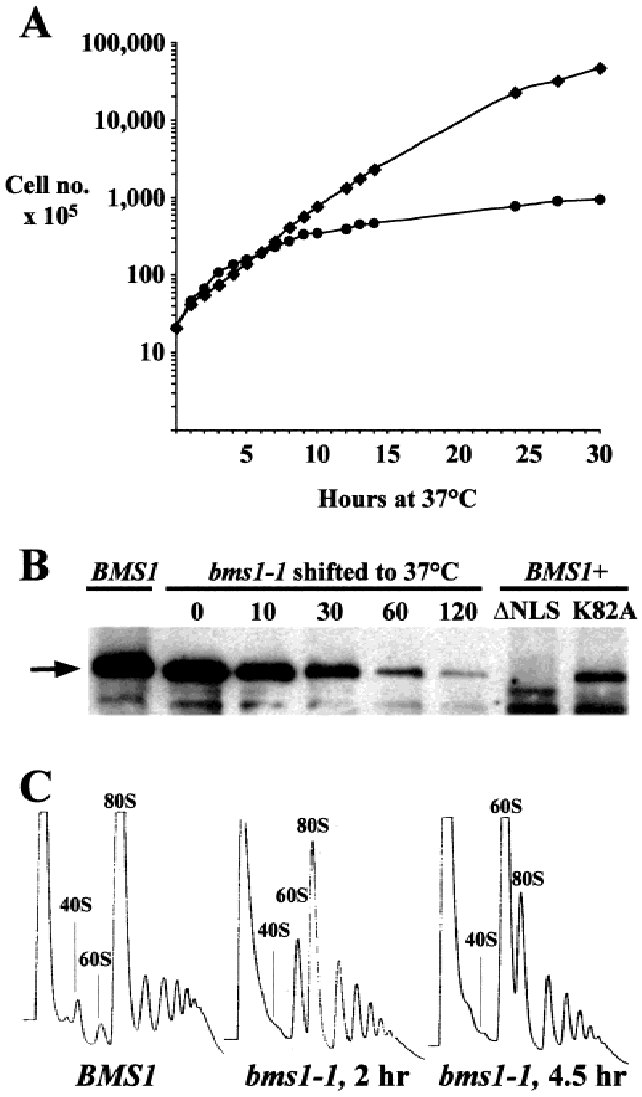

FIGURE 3. *bms1-1* cells are depleted of 40S ribosomes after a shift to 37 °C. **A:** Growth curves of isogenic *BMS1* (SL3492, -♦-) and *bms1-1* (SL3491, -▪-) cells after a shift to 37 °C. **B:** Immunoblot of extracts from cells expressing the indicated HA-tagged alleles of *BMS1*. Strains are: SL4012 (*BMS1*) or SL4013 (*bms1-1*) shifted to 37 °C for the times indicated; SL3875 (*BMS1*) + pDG116 (*bms1-*Δ*NLS*) or pDG114 (*bms1-K82A*). **C:** Polysome profiles from isogenic *BMS1* (SL3492) and *bms1-1* (SL3491) cells shifted to 37 °C for 2 or 4.5 h. Positions of 40S and 60S free subunits, as well as 80S monomers are indicated.

structure is strongly reminiscent of the nucleolus. This was confirmed by coexpressing HA-Bms1p and GFP-tagged Gar1p, a snoRNP protein that localizes to the nucleolus (Trumtel et al., 2000). The predominant staining with anti-HA antibodies overlapped with the major GFP fluorescence, although there was also faint staining of HA-Bms1p in the cytosol (Fig. 2C). Our observations are in agreement with those of Rout et al. (2000), who, in the course of examining a large number of proteins that copurify with nuclear pore complexes, reported that Bms1p localizes to the nucleolus and nucleus, with small amounts in the cytoplasm.

To determine whether nuclear localization of Bms1p is important for its function, stop codons were inserted immediately upstream of the sequence encoding the first *BMS1* NLS (pDG116), removing 56 C-terminal amino acids, including all three NLS. *HA-bms1-*Δ*NLS* was unable to complement *bms1-*Δ at either 25 °C (not shown) or 30 °C (Fig. 2A). Immunoblotting indicated that HA-Bms1p-ΔNLS was expressed and migrated at the predicted size when pDG116 was transformed into a wild-type *BMS1* strain (Fig. 3B). Furthermore, immu-

nolocalization of HA-Bms1p-ΔNLS revealed diffuse cytoplasmic staining (not shown). These data suggest that the ability of Bms1p to localize to the nucleus is important for it to perform its essential function.

Because the nucleolus is the site of intense biosynthetic activity during the formation of ribosomes, we investigated whether *bms1-1* mutants are defective in ribosome production by generating polysome profiles on sucrose gradients. Growth analysis showed that a strain carrying the *bms1-1* mutation (P888L) stopped dividing after about 9 h at 37 °C (Fig. 3A). However, Bms1p-P888L levels declined rapidly after shift to the nonpermissive temperature (Fig. 3B), indicating that the mutant protein is unstable. Free 40S ribosomal subunits also declined rapidly after shifting to 37 °C, and this was paralleled by an increase in free 60S subunits unable to enter polysomes (Fig. 3C).

Decreased ribosomal subunit production is often caused by defects in assembly of pre-ribosomal particles and/or rRNA processing that lead to reduced production of mature ribosomal RNA (Kressler et al., 1999; Venema & Tollervey, 1999; and references therein). 40S ribosomal subunits contain a single rRNA species, the 18S rRNA, that is transcribed as part of a 35S precursor. The 35S precursor contains the 18S, 5.8S, and 25S rRNA products separated by internal spacers and bounded on each end by external spacers. These spacer sequences are removed by specific processing events during the course of ribosome maturation (Fig. 4). To produce an 18S rRNA, the 35S rRNA is first cleaved at site A_0 to yield a 33S precursor, which is then cleaved at sites A_1 to yield 32S and A_2 to yield a 20S rRNA (Kressler et al., 1999; Venema & Tollervey, 1999). The 20S rRNA is exported to the cytosol as part of a 43S pre-ribosomal particle, where it is cleaved at site D to yield 18S rRNA as part of a mature 40S ribosomal subunit (Udem & Warner, 1973). Following cleavage of the 33S precursor at sites A_1 and A_2, the remaining $27SA_2$ precursor is processed to the mature 25S and 5.8S rRNA components of the 60S ribosomal subunit in one of two ways. The majority of $27SA_2$ is cleaved at site A_3 ($27SA_3$) and rapidly exonucleolytically digested back to site $B1_S$ (27SB) to form the mature 5′ end of 5.8S rRNA (Fig. 4). A minor amount of $27SA_2$ is processed directly at site $B1_L$ to yield a slightly longer, alternative mature 5′ end of 5.8S rRNA (not shown). The 3′ end of 5.8S rRNA and the 5′ end of 25S rRNA are formed by cleavage at sites C_1 and C_2, followed by exonucleolytic digestion from C_2 to site E to generate the mature 3′ end of 5.8S rRNA. The 3′ end of mature 25S rRNA is then generated by processing at site B_2.

To assess the kinetics of rRNA processing in cells lacking Bms1p function, isogenic *bms1-1* and *BMS1* strains (SL3491 and SL3492) were shifted to 37 °C and subjected to pulse-chase labeling with ^{3}H-methyl methionine to label methylated RNAs (Fig. 5). The wild-type strain rapidly processed the 35S precursor to 27SA,

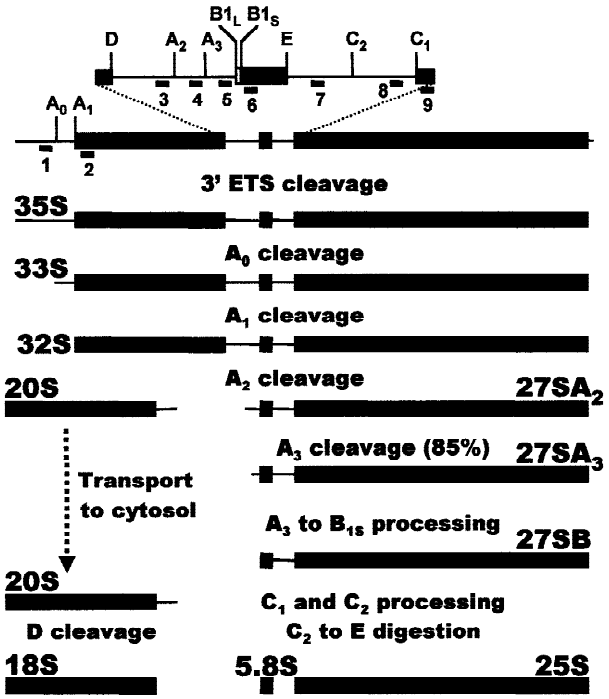

FIGURE 4. Pre-rRNA processing in *S. cerevisiae*. The 35S precursor contains 18S, 5.8S, and 25S rRNAs separated by two internal spacer sequences (ITS1 and ITS2) and two external spacer sequences (5′ ETS and 3′ ETS; see text for details). A second pathway leading to the production of $5.8S_L$ is not shown. In this minor pathway (15% of total 5.8S produced) the $27SA_2$ precursor is cleaved directly at site $B1_L$, leading to a slightly longer 5′ end of 5.8S rRNA. Subsequent events generating the 3′ end of 5.8S rRNA and the 5′ end of 25S rRNA occur as diagramed. Positions of probes used for northern blots are indicated.

27SB, and 20S intermediates, which were then cleaved to mature 25S and 18S rRNA. In contrast, in the *bms1-1* strain, processing of 35S, 32S, and 27SA was slightly delayed and levels of 20S precursor and 18S rRNA were nearly undetectable, whereas 25S rRNA was produced with nearly wild-type kinetics. No other aberrant intermediates accumulated, indicating that the 18S rRNA precursors were unstable. Similar results were seen when cells were labeled with ^{3}H-uracil (not shown). There was no difference in the extent of labeling of pre-rRNA in *BMS1* or *bms1-1* with ^{3}H-methyl methionine or ^{3}H-uracil, indicating that *bms1-1* does not cause a general defect in rRNA methylation.

Processing of rRNA in the *bms1-1* mutant was examined in more detail by northern blotting of total RNA using probes specific for different regions of the 35S rRNA precursor (see Fig. 4 for locations of probes used). Loss of Bms1p function caused a progressive decrease in steady-state levels of 18S rRNA, whereas 25S, 5.8S, and 5S levels remained unchanged over 12 h at 37 °C (Fig. 6A and data not shown). Hybridization with probe 3 (D/A_2), which is directed to the 5′ end of ITS1, showed rapid disappearance of 20S and 32S precursors, paralleled by significant accumulation of

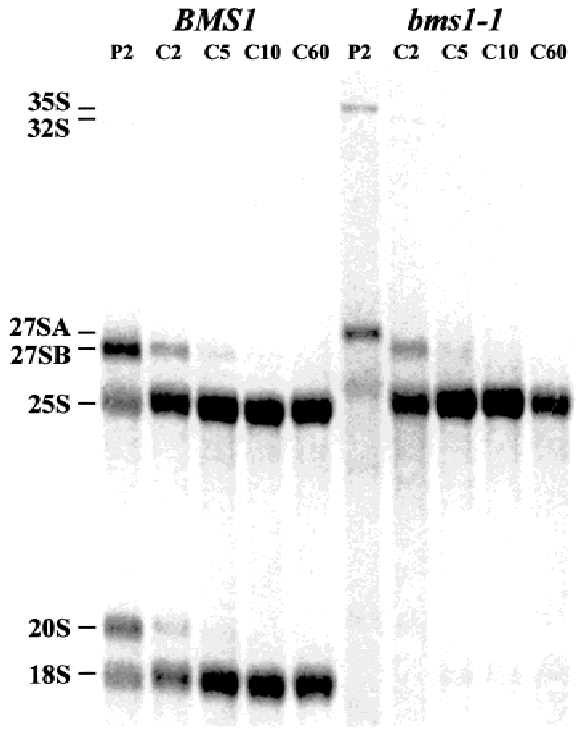

FIGURE 5. Loss of Bms1p function blocks 18S rRNA formation. *BMS1* (SL3492) and *bms1-1* (SL3491) cells were shifted to 37°C for 2 h, pulsed for 2 min (P2) with ^{3}H-methyl-methionine and chased for 2, 5, 10, and 60 min (C2, C5, C10, C60) by addition of excess unlabeled methionine. Samples were then processed as described in Materials and Methods.

35S precursor and appearance of a small amount of an aberrant 23S intermediate (Fig. 6C). The 23S RNA arises due to direct cleavage of 35S precursor at site A_3 in the absence of processing at sites A_0, A_1, and A_2. The 23S species extends from the 5′ end of the transcript to site A_3, as it was labeled with the 5′ETS (Fig. 6B) and A_2/A_3 (Fig. 6D) probes, but not the A_3/B probe (Fig. 6E). Direct cleavage of 35S rRNA at site A_3 also produces the $27SA_3$ rRNA intermediate, which is rapidly trimmed to 27SB and further processed to form the 5.8S and 25S rRNA species. Accordingly, whereas the 27SA products were depleted in the *bms1-1* strain (Fig. 6D,C,F), the 27SB, 25S, and 5.8S rRNAs were present at normal levels (Fig. 6F,A and data not shown).

The defects in pre-rRNA processing were further analyzed by primer extension (Fig. 7). There was a rapid increase in products extending from the 18S region to the beginning of the transcript (+1), which was paralleled by a dramatic decline in products ending at the A_2 cleavage site, consistent with the 35S and 23S RNA accumulation. Products ending at site A_0 were depleted more slowly, suggesting that there was some residual cleavage at A_0 and generation of 33S pre-rRNA, a product that is not readily detected in northern blot analysis. However, processing at A_0 was not revealed by any obvious accumulation of 22S RNA (extending from A_0 to A_3) in northern blots or pulse-chase analysis (Figs. 5 and 6), suggesting that this cleavage is minor and/or any 22S product generated is rapidly degraded in *bms1-1* cells shifted to the nonpermissive temperature. Primer extension stops at sites A_3, B_{1L}, and B_{1S} remained relatively constant during the time course, as expected from the northern analysis, thus confirming normal accumulation of 5.8S and 25S rRNAs.

Taken together, the results of pulse-chase, northern, and primer-extension analysis indicate that inactivation of Bms1p leads to a defect in rRNA processing at sites A_0, A_1, and A_2, possibly with a less severe effect on A_0 cleavage, whereas processing steps leading to production of 25S and 5.8S rRNAs are unaffected. Furthermore, 22S and 23S products expected to result from the cleavage defects may be unstable.

The Bms1p-related protein, Tsr1p (YDL060w), is also involved in 40S ribosome biogenesis

The similarity of sequences within the amino- and carboxyl-terminal regions of Tsr1p and Bms1p (Fig. 1) prompted us to begin to investigate the function of this related protein. Notably, Tsr1p lacks a P-loop and an obvious NLS, which are essential for Bms1p's function. Disruption of *TSR1* in a diploid (SL3998) led to inviable *tsr1*-Δ spore progeny, which could be rescued to wild-type growth by the addition of *TSR1* on a low-copy plasmid (pYCG060; data not shown). These results agree with those of Casalone et al. (1999), who disrupted the gene as part of an ongoing genomic functional analysis project and also found it to be essential. In addition, we found that overexpression of *BMS1* was unable to suppress *tsr1*-Δ lethality and *TSR1* overexpression could not suppress the lethality of *bms1*-Δ or the temperature sensitivity of a *bms1-1* strain (data not shown). These data indicate that despite their sequence similarity, *BMS1* and *TSR1* are not functionally equivalent.

To localize Tsr1p, the coding sequence for GFP was fused to the 3′ end of the ORF and expressed from a CEN, *URA3* plasmid (pJB1). This fusion protein is functional, as pJB1 is able to complement the *tsr1*-Δ lethality. Examination of a diploid *tsr1*-Δ/*tsr1*-Δ strain carrying pJB1 (SL4125) by fluorescence microscopy showed a strong GFP signal in the nucleolus (Fig. 8), suggesting that Tsr1p might also be involved in ribosome biogenesis.

To examine the effect of loss of Tsr1p, the genomic copy of *TSR1* was placed under control of the repressible *GAL1* promoter. Simultaneously, the coding sequence for a triple HA tag was added to the 5′ end of the ORF to allow detection of the protein. This strain, SL4078, grew well on galactose medium, but growth plateaued approximately 15 h after transcriptional repression in glucose medium (Fig. 9A); protein levels declined to undetectable levels by 9–12 h after shift to

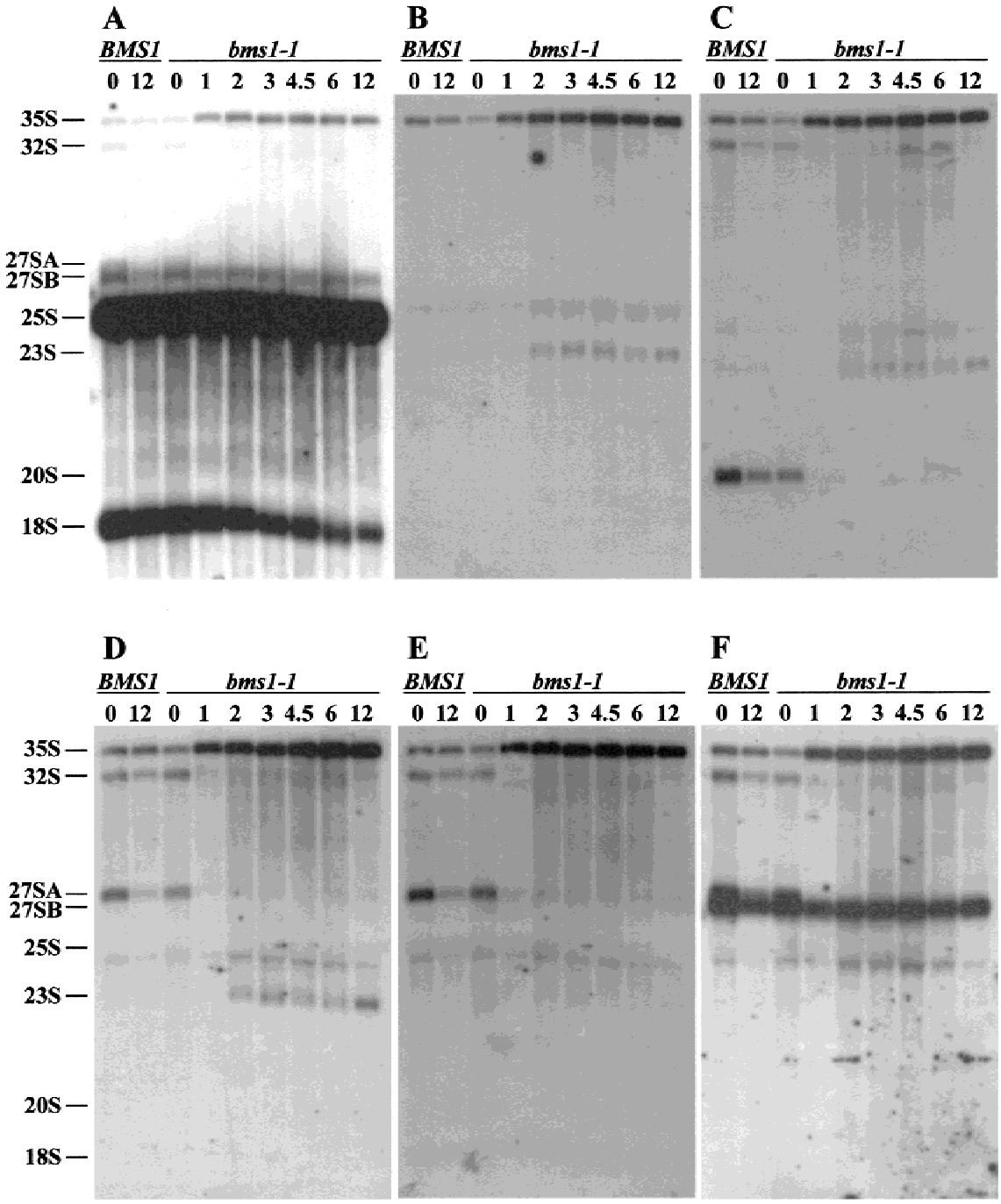

FIGURE 6. Analysis of steady-state rRNA from *BMS1* and *bms1-1* strains. *BMS1* (SL3492) and *bms1-1* (SL3491) were shifted to 37 °C for the times indicated before total RNA was isolated and separated for northern blotting. Probes used (see Fig. 4) were (**A**) 18S (probe 2) and 25S (probe 9); (**B**) 5′ ETS (probe 1); (**C**) D/A_2 (probe 3); (**D**) A_2/A_3 (probe 4); (**E**) A_3/B (probe 5); and (**F**) E/C_2 (probe 7). Positions of rRNA products and intermediates are indicated.

glucose (Fig. 9B). To determine whether Tsr1p depletion causes a ribosome biogenesis defect, polysome profiles were generated from cells grown on galactose or shifted to glucose medium (Fig. 9C). As early as 12 h after shift to glucose, a dramatic loss of free 40S subunits was observed, together with a concomitant increase in free 60S subunits and loss of 80S ribosomes. This defect was even more extreme after 24 h of Tsr1p depletion. Thus, like Bms1p, Tsr1p is required for 40S ribosomal subunit production.

To determine whether depletion of Tsr1p affects rRNA processing, *GAL:TSR1* and isogenic *TSR1* strains were shifted from galactose to glucose medium for 24 h, and then subjected to pulse-chase labeling analysis with ^{3}H-methyl methionine (Fig. 10). Depletion of Tsr1p slowed processing of 35S slightly and strongly reduced

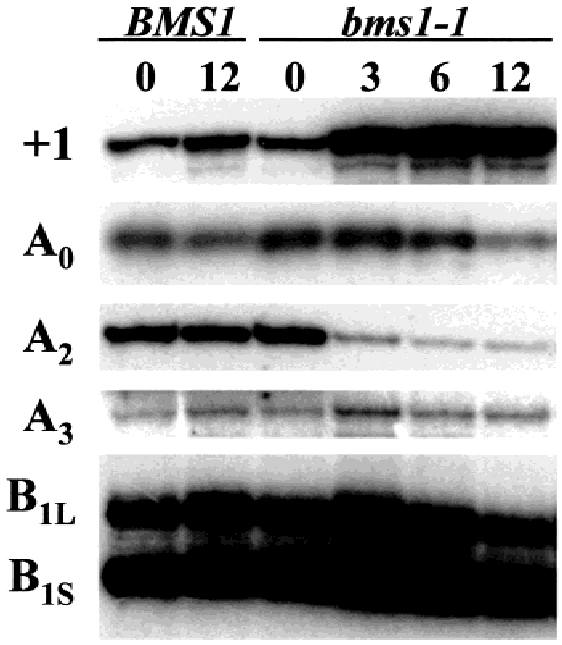

FIGURE 7. Primer extension analysis of *bms1-1* rRNA after a shift to 37°C. Primer extensions were performed on RNA isolated from *BMS1* (SL3492) and *bms1-1* (SL3491) strains shifted to 37°C for the indicated times in hours. Sites of extension stops due to rRNA processing are indicated. Primer 7 (E/C2) was used to examine sites A_2, A_3, B_{1L}, and B_{1S}. Primer 2 (18S) was used to identify products ending at the 5′ end of the transcript (+1) and cleavages at sites A_0 and A_1. Bands at A_3 represent a sixfold longer exposure than A_2, B_{1S}, and B_{1L}. The A_1 cleavage is obscured by preexisting 18S rRNA and is not shown.

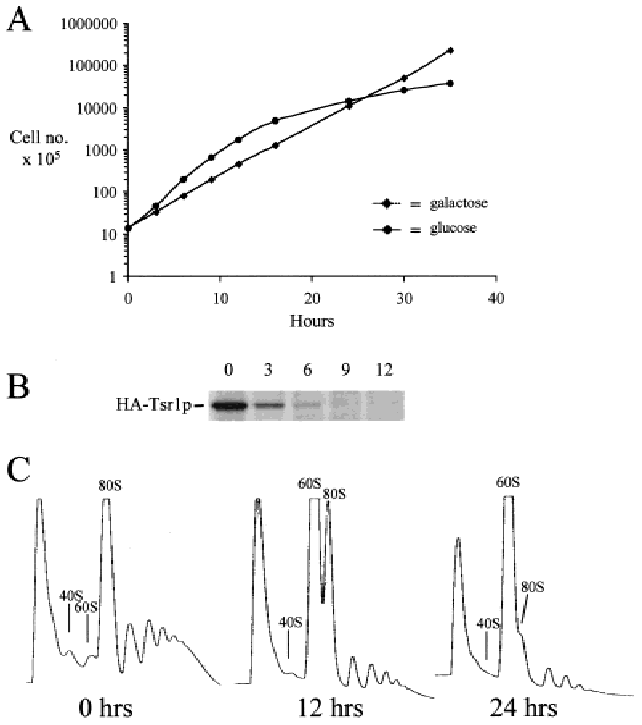

FIGURE 9. Depletion of Tsr1p leads to loss of 40S ribosomal subunits. **A:** SL4078 (*GAL1:HA-TSR1*), grown in YEP-GAL at 30°C, was shifted to glucose (YEPD, -•-) or retained in YEP-GAL (-◆-) and cell numbers were monitored by OD_{600} at indicated times after shift. **B:** HA-Tsr1p is depleted after shift to glucose. *GAL1:HA-TSR1* cells from **A** were harvested at the indicated times (in hours) after a shift to glucose, lysed, and processed for immunoblotting. **C:** Polysome profiles of extracts from *GAL1:HA-TSR1* cells shifted from galactose to glucose medium for the indicated times.

the rate of processing 20S to 18S rRNA. The amount of 20S and 18S rRNA produced was also diminished. Production of 25S rRNA was unaffected, similar to the *bms1-1* mutant. Further analysis by northern blotting showed that, as expected, steady-state levels of 18S rRNA progressively declined whereas 25S levels remained constant over the course of Tsr1p depletion (Fig. 11A). Hybridization with a probe directed to the 5′ end of ITS2 showed that depletion of Tsr1p causes accumulation of 35S precursor, similar to *bms1-1*. However, in contrast to *bms1-1*, 27SA and 32S intermediates persisted for some time (Fig. 11D), even after the complete disappearance of the Tsr1p protein (see Fig. 9).

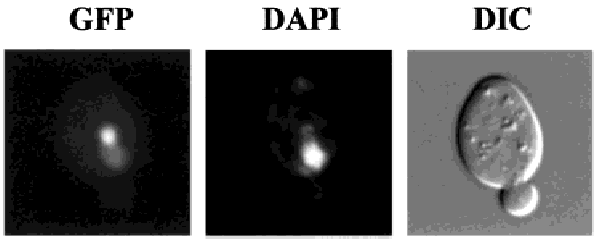

FIGURE 8. Tsr1p-GFP localizes to the nucleolus. Living diploid cells expressing Tsr1p-GFP (SL4125) were stained with DAPI and visualized by epifluorescence.

Also, very little, if any, 23S intermediate accumulated. Instead, Tsr1p-deficient yeast strongly accumulated a 20S rRNA species (Fig. 11C), unlike Bms1p-deficient cells in which this intermediate was not produced or was unstable (see Fig. 6C). This is the normal 20S precursor extending from site A_1 to A_2, as it was seen only with a probe hybridizing to the 5′ end of ITS1 (Fig. 11C) and not with a probe hybridizing to the 3′ end of ITS1 (Fig. 11D), or with any other probes tested. These results indicate that Tsr1p is important for a later step in 40S ribosome assembly. Based on these results, we now refer to YDL060w as *TSR1*, for "Twenty S rRNA accumulation."

DISCUSSION

In this report, we describe the identification of two related novel, essential proteins, Bms1p and Tsr1p, that are each independently required for 40S ribosomal subunit biogenesis. Both are crucial for the formation of 18S rRNA but, despite their similarity, the two proteins affect different steps of the 18S processing pathway. Bms1p is a GTP-binding protein that is required for an early step of 18S rRNA production. Moreover, GTP bind-

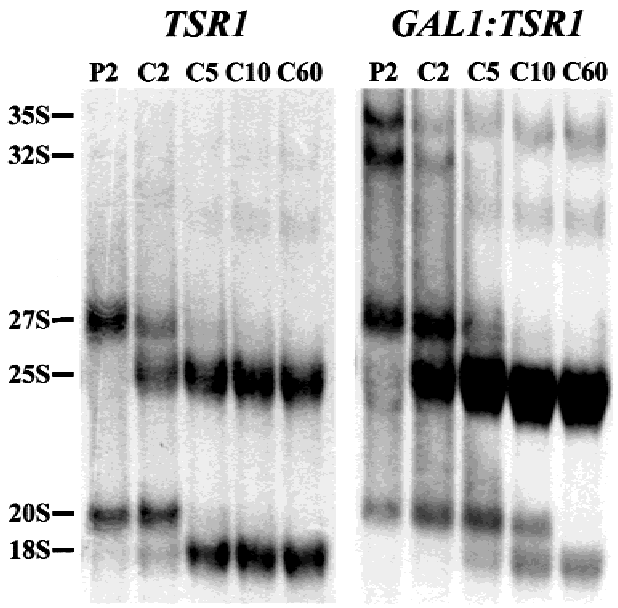

FIGURE 10. Depletion of Tsr1p leads to delayed 18S rRNA processing. *TSR1* (SL1462) and *GAL1:HA-TSR1* (SL4078) cells were shifted to glucose medium for 24 h before being pulsed for 2 min (P2) with ^{3}H-methyl-methionine and chased for 2, 5, 10, and 60 minutes (C2, C5, C10, C60) by addition of excess unlabeled methionine. RNA was processed as described in Materials and Methods.

ing is critical for Bms1p's essential role in the cell. In contrast, Tsr1p is not predicted to bind GTP and functions at a later step of 40S ribosome production, possibly in assembly and/or export of 43S pre-ribosomal subunits to the cytosol.

Relationship of Bms1p to 14-3-3 proteins

BMS1 was isolated as a mutation that is sensitive to reduced levels of 14-3-3 proteins (D. Gelperin, L. Horton, J. Hensold, and S.K. Lemmon, in prep.). 14-3-3 proteins regulate a variety of signaling pathways by binding to target proteins and altering their localizations and/or activities (reviewed in Fu et al., 2000). Bms1p contains a candidate 14-3-3 binding motif that is conserved among Bms1p-like sequences from different species. However, two-hybrid analysis and GST pull-downs have failed to detect any physical interaction between Bms1p and 14-3-3, and mutation of the 14-3-3 binding motif in Bms1p had no detectable effect on Bms1p's function (D. Gelperin, unpubl. observations). Also, we found that *S. cerevisiae* cells lacking 14-3-3 function have a normal pattern of ribosome and polysome accumulation (D. Gelperin and L. Horton, unpubl. observations), suggesting that 14-3-3 does not regulate ribosome synthesis. Thus, the explanation for the *BMS1*/14-3-3 genetic interaction may be more indirect. It is possible that 14-3-3 affects ribosome biogenesis through regulation of growth, stress, or nutrient sensing pathways. Consistent with this, 14-3-3 proteins act as downstream components of the TOR signaling pathway that regulates ribosome synthesis and translation in response to nutrient availability (Bertram et al., 1998; Beck & Hall, 1999; Powers & Walter, 1999). Because inactivation of the TOR pathway results in decreased ribosome synthesis and inhibition of translation initiation (Powers & Walter, 1999), 14-3-3 mutants may be hypersensitive to mutations causing decreased ribosome synthesis. However, it is unlikely that Bms1p itself is regulated directly by TOR, because *bms1-1* strains are not hypersensitive to the TOR-specific inhibitor rapamycin (D. Gelperin, L. Horton, J. Hensold, and S.K. Lemmon, in prep.). Therefore, further studies will be required to understand 14-3-3′s role in Bms1p-related processes.

Bms1p functions at an early step in 40S ribosome biogenesis

Bms1p is required for processing of 35S pre-rRNA at sites A_0, A_1, and A_2, as demonstrated by rapid accumulation of unprocessed 35S precursor and a small amount of an aberrant 23S intermediate product upon inactivation of Bms1p. The 23S product results from cleavage of 35S pre-rRNA at site A_3 in the absence of prior cleavage at sites A_0, A_1, and A_2. Furthermore, pulse-chase analysis shows that production of 20S and 18S rRNA is nearly completely blocked in a *bms1-1* strain at the nonpermissive temperature. Overall, our results indicate that Bms1p plays a role early in 40S ribosome formation. Furthermore, because no early cleavage products and very little 23S RNA accumulate upon Bms1p inactivation, this early function for Bms1p may be important for rRNA precursor stability.

In a companion report, Filipowicz and colleagues (Wegierski et al., 2001, this issue) describe their identification of Bms1p as a protein that binds to Rcl1p. Rcl1p is an essential protein involved in 40S ribosome biogenesis that affects rRNA processing at sites A_1 and A_2, with less effect on processing at site A_0 (Billy et al., 2000). U3 snoRNP has two major functions: one affecting cleavages at A_0, A_1, and A_2, and another affecting primarily A_1 and A_2 processing (Sharma & Tollervey, 1999). The *rcl1* phenotype resembles that observed upon inactivation of U3′s A_1/A_2 function, similar to mutants of 18S rRNA unable to base pair to U3 and to phenotypes seen upon depletion of U3-associated proteins Mpp10p and Dhr1p (Dunbar et al., 1997; Sharma & Tollervey, 1999; Colley et al., 2000). Indeed, Rcl1p specifically associates with U3 snoRNA, although it is not a structural component of the U3 mono-snoRNP (Billy et al., 2000). Taken together, these results suggest that Rcl1p may be involved in the A_1 and A_2-specific function of U3.

Though Bms1p and Rcl1p associate with each other, our results show that inactivation of Bms1p in the *bms1-1* mutant causes a distinct phenotype. Although primer extension experiments showed that some resid-

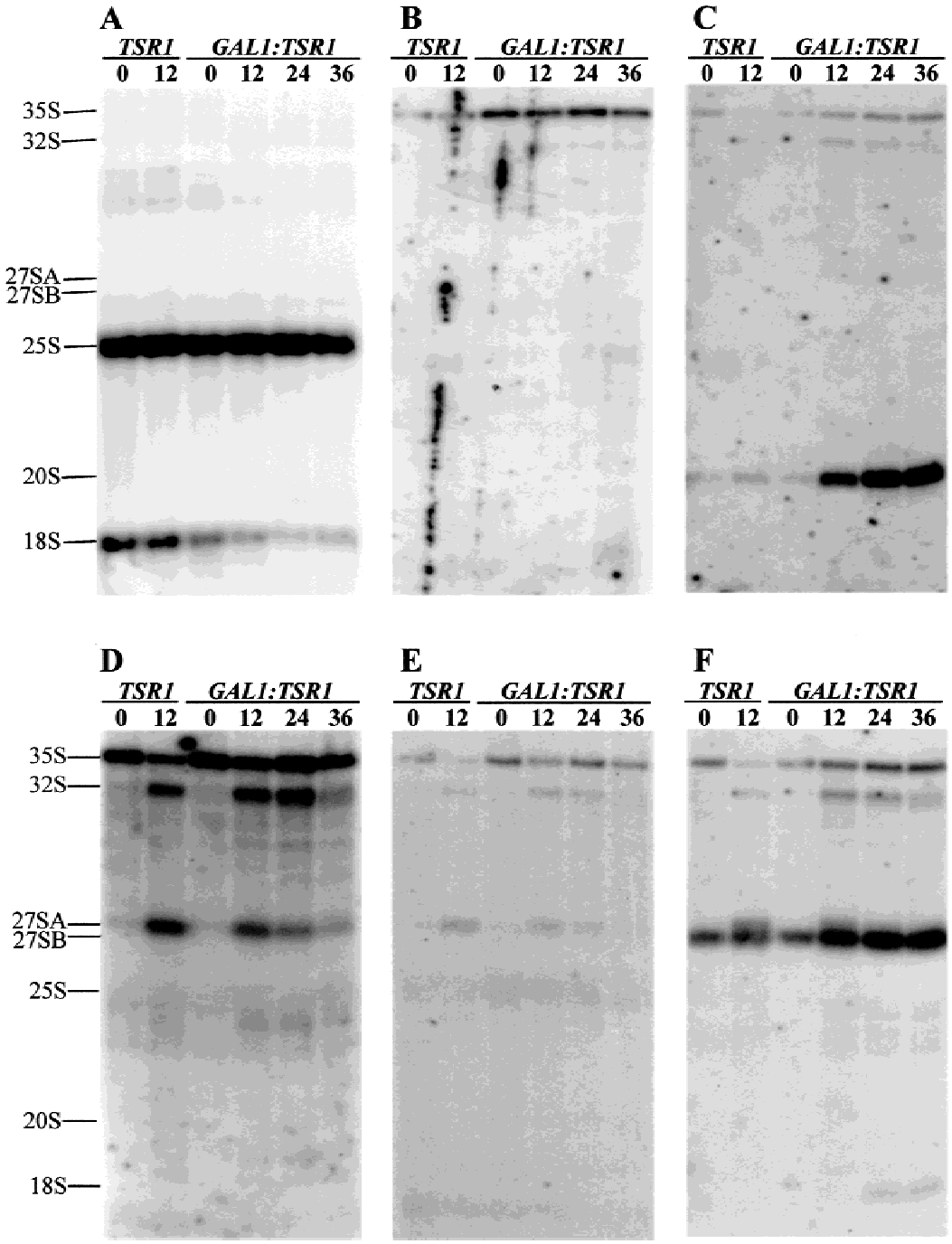

FIGURE 11. Analysis of steady-state rRNA after *GAL1:HA-TSR1* repression on glucose. Wild-type (SL1462) and *GAL1:HA-TSR1* (SL4078) were grown in YEP-GAL and shifted to YEPD for times indicated before total RNA was isolated and separated for northern blots. Probes used (see Fig. 4) were (**A**) 18S (probe 2) and 25S (probe 9); (**B**) 5′ ETS (probe 1); (**C**) D/A_2 (probe 3); (**D**) A_2/A_3 (probe 4); (**E**) A_3/B (probe 5); and (**F**) E/C_2 (probe 7). Positions of rRNA products and intermediates are indicated.

ual cleavage at A_0 occurred at early times after temperature shift in the *bms1-1* mutant, no 22S RNA (A_0-to-A_3 product) accumulated as assessed by pulse-chase or steady-state northern blot analysis. Thus, this cleavage was minor or the 22S RNA was very unstable. More dramatic in *bms1-1* was the rapid disappearance of the 20S intermediate, with only a minor accumulation of the aberrant 23S (+1 to A_3) RNA relative to accumulation of 35S pre-rRNA (Fig. 6). In contrast, both mutational inactivation and depletion of Rcl1p caused a less severe effect on processing at A_0 and resulted in significantly higher levels of aberrant 22S and 23S rRNA relative to 35S rRNA than is seen in the *bms1-1* mutant (Billy et al., 2000). Thus, Bms1p and Rcl1p associate

and may act early in 40S ribosome assembly, but they do not appear to have completely overlapping functions.

What is the role of Bms1p in 40S ribosomal subunit biogenesis? Most proteins with known roles in A_0, A_1, and A_2 processing can be generally grouped into (1) those affecting assembly of pre-ribosomal particles or processing complexes; (2) those involved in U3 snoRNP function; and (3) putative RNA helicases. Bms1p does not contain any of the signature sequence motifs that are hallmarks of RNA helicases (de la Cruz et al., 1999), so it is not likely to perform this function. It is possible that Bms1p regulates U3 snoRNP's association with the pre-ribosomal particle or stabilizes the activity of the U3 snoRNP. Finally, Bms1p may be required for assembly of r proteins or other nonribosomal factors, including Rcl1p, into the nascent pre-ribosomal particle. Consistent with this, Rcl1p's association with large pre-ribosomal complexes is dependent upon the presence of Bms1p (Wegierski et al., 2001).

Tsr1p functions in a late step of 40S ribosome maturation

Tsr1p is also required for 18S rRNA production and 40S ribosomal subunit synthesis and, like *bms1-1*, and many other mutants that cause a delay in rRNA processing, depletion of Tsr1p causes accumulation of 35S precursor RNA. However, despite its similarity to Bms1p, depletion of Tsr1p causes a dramatic accumulation of 20S rRNA. 20S precursor rRNA is a late intermediate that arises from 35S pre-rRNA after it has been cleaved at sites A_0, A_1, and A_2. 20S rRNA is then transported from the nucleus to the cytosol as part of a 43S pre-ribosomal particle, where it is cleaved at site D to yield 18S rRNA and a mature 40S ribosomal subunit (Udem & Warner, 1973). Thus, Tsr1p acts after processing steps leading to production of 20S rRNA, but before cytosolic maturation of 20S to 18S rRNA. Consistent with an activity late in 18S biosynthesis, depletion of Tsr1p results in a kinetic delay of 20S to 18S rRNA maturation, rather than the near complete loss of 20S and 18S rRNA products seen after inactivation of Bms1p (compare Figs. 5 and 10). This suggests that Tsr1p functions after the rRNA has been assembled into a relatively stable pre-ribosomal particle.

Accumulation of 20S rRNA after depletion of Tsr1p could be caused by defective production of 43S pre-ribosomal particles that are not competent to be transported to the cytosol or matured to the 40S ribosome. Alternatively, Tsr1p could be involved directly in transport from the nucleus. It seems unlikely that Tsr1p is directly involved in processing of 20S to 18S rRNA at site D in the cytosol, as Tsr1p's steady-state localization is in the nucleolus. However, we have not ruled out the possibility that Tsr1p is exported with 43S pre-ribosomes to the cytosol and is rapidly reimported into the nucleus.

To our knowledge, Tsr1p is the first identified non-ribosomal protein that acts at a late step in ribosome assembly or transport and is specific for the 40S subunit without affecting the 60S ribosome. A number of known proteins that function at late steps of 40S ribosome biosynthesis block production of both 40S and 60S subunits and appear to cause general blocks in nucleo/cytoplasmic transport (e.g., see Moy & Silver, 1999; Stage-Zimmermann et al., 2000). One other mutant, CLP-8, has been reported to have a similar phenotype; however, the mutant strain is genetically complex and the gene responsible for the 40S defect has not been cloned (Carter & Cannon, 1980; Carter et al., 1980). More interestingly, depletion of some small ribosomal subunit proteins, such as Rps31p/Ubi3p and Rps0, specifically affect late steps of 40S ribosome maturation and block 20S to 18S RNA conversion (Finley et al., 1989; Ford et al., 1999). Perhaps Tsr1p is required for assembly of these ribosomal proteins onto the pre-ribosome.

Role of Bms1p GTP binding

To our knowledge, Bms1p is the first GTP-binding protein shown to be required for ribosome biogenesis. Indeed, only one other putative GTP-binding protein (Nog1p) is known to localize to the nucleolus in *S. cerevisiae* (Park et al., 2001), but its function is unknown. All known proteins with the type of GTP-binding motif present in Bms1p also hydrolyze GTP (Bourne et al., 1991; Kjeldgaard et al., 1996). However, thus far we have only observed modest stimulation of GTPase activity by Bms1p in in vitro assays (D. Gelperin, unpubl. observations). This may indicate that Bms1p requires other factors, most likely in the context of the assembling ribosome, to regulate or activate hydrolysis of the bound nucleotide.

GTP-binding proteins function in a variety of important processes, including growth control (Ras), membrane trafficking (Rab, Arf), cytoskeletal organization (tubulin, Rho), and protein translation (EF-Tu, IF-2; reviewed in Kjeldgaard et al., 1996). Many act as molecular switches (e.g., Ras, Rho, Rab) or can promote movement or assembly (tubulin, EF-Tu), depending on whether they are bound to GDP or GTP. GTP binding is clearly required for Bms1p's function, but more work will be required to determine its exact role. A number of interesting possibilities exist. Bms1p may act as a checkpoint sensor to monitor events during ribosome biogenesis, for example, the completion of rRNA transcription or proper assembly of the pre-ribosomal particle, or to prevent processing until the earlier events are correctly completed. Alternatively, GTP binding could serve as an energy source for applying force, perhaps in assembling proteins onto rRNA or in properly positioning other *trans*-acting factors on the pre-ribosome.

Our work demonstrates that Bms1p and Tsr1p function in different steps of 40S ribosome biogenesis; nevertheless, because they share homology in two extended domains, they may have related biochemical activities or recognize a common binding partner/structure. Because Bms1p is required for an early event in 40S formation, whereas Tsr1p is required for a later event, this raises the possibility that Bms1p binds to a site on the pre-ribosome and must be released to allow binding of Tsr1p to the same site. Bms1p would be in a position to act as a checkpoint to regulate progression of pre-ribosome maturation to the later Tsr1p-dependent step, an event that could be regulated by Bms1p's GTPase activity.

In summary, we have identified two related proteins that are required for small ribosomal subunit biogenesis. Bms1p is the first known GTP-binding protein required for proper rRNA processing and functions at an early step of ribosome biogenesis. Tsr1p is required for a later step of 40S ribosome formation and is the first *trans*-acting factor identified that specifically affects 20S to 18S rRNA maturation without affecting 60S subunit formation.

MATERIALS AND METHODS

Yeast methods

Strains used in this study are listed in Table 1. Standard yeast genetic techniques were used (Guthrie & Fink, 1991). Yeast extract peptone (YEP) containing 2% dextrose (YEPD) or galactose (YEP-Gal) and synthetic selective dropout media were prepared as described (Nelson & Lemmon, 1993). When raffinose was used as the carbon source it was present at 2%. Growth curves were performed in synthetic glucose medium lacking uracil (C-URA; for *bms1-1* strains) or in YEPD and YEP-Gal (for *GAL1:HA-TSR1*). In both cases, cultures were diluted with fresh medium as needed to maintain log phase growth over the course of the experiment, and cell number was monitored by absorption at 600 nM. Yeast were transformed by the lithium acetate method (Gietz et al., 1992).

Cloning *BMS1*, *bms1-1*, and integrations at *BMS1* and *YDL060w*

BMS1 was cloned by complementation of the temperature-sensitive growth of a *bms1-1* mutant (SL3243) at 37°C following transformation with a YCp50 yeast genomic DNA library (Rose et al., 1987). Two rescuing plasmids containing overlapping genomic inserts were isolated and the ORF (YPL217c) responsible for rescue was identified by subcloning.

To generate a null allele of *BMS1* the complete ORF was replaced by *HIS3* using a PCR-based one-step gene replacement method (Baudin et al., 1993). The disruption PCR fragment was created with primers 5′-GCAAAGGCCTT TGCCGTGGCAGCCCCAGGTAAGATGGCGAGCTCTTGG CCTCCTCTAG-3′ and 5′-CCTCCTCATCTTACGTGGACG AGATTCATCGTCACCGCCCTCGTTCAGAATGACACG-3′ using pUC18-*HIS3* (Rothstein, 1991) as a template and was transformed into SL1528 to generate SL3768. Correct disruption was verified at both ends by colony PCR.

To confirm that YPL217c is allelic to the *bms1-1* temperature-sensitive mutation, the ORF was disrupted with *HIS3* in a *bms1-1*/*BMS1* diploid (SL3768) and segregation of *HIS3* and *bms1-1* temperature sensitivity were followed through tetrads. One class of *HIS3* integrants yielded a temperature-

TABLE 1. Strains used in this study.

Strain	Genotype	Source
BJ2168	*MAT*a *leu2 trp1 ura3-52 prb1-1122 pep4-3 prc1-407*	Elizabeth Jones
SL1462	*MAT*a *leu2 ura3-52 trp1 his3-Δ200 GAL2*	Gelperin et al., 1995
SL1528	*MAT*a/α *leu2/leu2 ura3-52/ura3-52 trp1/trp1 his3-200/his3-Δ200 GAL2/GAL2*	Gelperin et al., 1995
SL3243	*MAT*α *bms1-1 leu2 ura3-52 trp1 ade2 ade3 lys2*	This study
SL3491	*MAT*a/α *bms1-1/bms1-1 leu2/leu2 ura3-52/ ura3-52 trp1/trp1 his3-200/HIS3 ade2/ade2 ade3/ade3 lys2-801/LYS2* +pRS316	This study
SL3492	*MAT*a/α *bms1-1/bms1-1 leu2/leu2 ura3-52/ura3-52 trp1/trp1 his3-200/HIS3 ade2/ade2 ade3/ade3 lys2-801/LYS* +pDG75 (*CEN*, *URA3*, *BMS1*)	This study
SL3768	*MAT*a/α *bms1-Δ::HIS3/BMS1 leu2/leu2 ura3-52/ura3-52 trp1/trp1 his3-200/his3-Δ200 GAL2/GAL2*	This study
SL3875	*MAT*a/α *bms1-Δ::HIS3/bms1-Δ::HIS3 leu2/leu2 ura3-52/ura3-25 trp1/trp1 his3-200/his3-Δ200 GAL2/GAL2* +pDG75 (*CEN*, *URA3*, *BMS1*)	This study
SL3998	*MAT*a/α *tsr1-Δ::kanMx6/TSR1 leu2/leu2 ura3-52/ura3-52 trp1/trp1 his3-Δ200/his3-Δ200 GAL2/GAL2*	This study
SL4012	*MAT*a/α *bms1-Δ::HIS3/bms1-Δ::HIS3 leu2/leu2 ura3-52/ura3-52 trp1/trp1 his3-200/his3-Δ200 GAL2/GAL2* +pDG110 (*CEN*, *LEU2*, *HA-BMS1*)	This study
SL4013	*MAT*a/α *bms1-Δ::HIS3/bms1-Δ::HIS3 leu2/leu2 ura3-52/ura3-52 trp1/trp1 his3-200/his3-Δ200 GAL2/GAL2* +pJC10 (*CEN*, *URA3*, *HA-bms1-1*)	This study
SL4078	*MAT*a *HIS3Mx6:GAL1:3HA-TSR1 leu2 ura3-52 trp1 his3-Δ200 GAL2*	This study
SL4125	*MAT*a/α *tsr1-Δ::kanMx6/tsr1-Δ::kanMx6 leu2/leu2 ura3-52/ura3-52 trp1/trp1 his3-Δ200/his3-Δ200 GAL2/GAL2* +pJB1 (*CEN*, *URA3*, *TSR1-GFP:HIS3Mx6*)	This study

sensitive diploid that would not sporulate, presumably because the integration was in *BMS1*. In the second class, diploids sporulated, but no His^+ or temperature-sensitive His^- spores were recovered in 24 tetrads. This indicates that the disruption construct integrated in *bms1-1* and confirms YPL217c is allelic to *bms1-1*.

The *bms1-1* mutation was recovered from the genome by gap repair. pDG75, cut with *Afl*II and *Sna*BI to remove 1,692 bp of the ORF, was transformed into SL3243 yielding a strain that retained temperature sensitivity. The repaired plasmid (pJC1) was recovered, sequenced, and found have a single mutation causing a change in Bms1p proline-888 to leucine. This plasmid causes temperature-sensitive growth when present as the sole source of Bms1p on a low copy plasmid (data not shown).

TSR1 was disrupted in SL1528 by transformation with the kanMX4 deletion construct pYORC_YDL060w (Casalone et al., 1999; EUROSCARF, Frankfurt, Germany) digested with *Not*I. Transformants were selected on YEPD medium containing 200 μg/mL G418 (Gibco BRL), and proper integration generating a heterozygous diploid (SL3998) was confirmed by colony PCR. To place the genomic copy of *TSR1* under control of the repressible *GAL1* promoter, a PCR product with 45 bp of homology flanking *TSR1* on each end was made using primers 5′-AGAAGTTGATATGAGTTGTGATATTAAT CGCAGAAATTTATTTGTGAATTCGAGCTCGTTTAAAC-3′ and 5′-TTTGTGTCCGTTTTTTAATGATGACCTGTGTGA ATGACCTGCCATGCACTGAGCAGCGTAATCTG-3′ with pFA6a-His3Mx6-GAL:3HA (Longtine et al., 1998) as a template. The PCR product was integrated immediately upstream of and in frame with *TSR1* in the wild-type strain SL1462, selecting for growth on synthetic galactose medium lacking histidine. Proper integration was confirmed by PCR and by the production of an HA-tagged protein of the proper size.

Plasmid constructions

All PCR amplified regions and junctions in plasmids were sequenced to ensure that no changes were introduced. pRS plasmids were previously described (Sikorski & Hieter, 1989; Christianson et al., 1992). pDG75 (*CEN*, *URA3*, *BMS1*) and pDG77 (2μ, U*RA3*, *BMS1*) were created by ligating a 6,176 bp *Sal*I-*Sal*I fragment containing *BMS1* from a YCp50 genomic library plasmid into the *Xho*I sites of pRS316 and pRS426 respectively. pDG110 (*CEN*, *LEU2*, *HA-BMS1*) was created in multiple steps. First, a *Bam*HI site was introduced upstream of the initiating ATG by cloning a 731 bp PCR product generated with primers 5′-CAGGTACCAGGATCCCTT ATGGAGCAGTC-3′ and 5′-CATATAGGGATGTTCGTTCC TCC-3′ into the *Kpn*I (vector) and *Bsa*BI (insert) sites of pDG75 to generate pDG94. Next, the nutritional marker was changed from *URA3* to *LEU2* via homologous recombination, by cotransforming yeast with pDG94 linearized in *URA3* with *Nsi*I and a *LEU2*-containing *Aat*II-*Sac*I fragment of pRS305 to generate pDG94/*LEU2*. Finally, the *BMS1* promoter region was reconstituted and sequences encoding two hemagglutinin (HA) epitope tags were added to the 5′ end of *BMS1*. A PCR product with the HA coding sequence was amplified using pDG75 as a template and primers 5′-CTGGGATC CACTCGCGACCGATCCTGCATAGTCCGGGACGTCATAGG GATAGCCCGCATAGTCAGGAACATCGTATGGGTAAAAG ACCATCGTTTCAGATAACTCAGATGG-3′ and 5′-GCAGTG AGCTCAGTGTGATTCTGGCCTTTTAATTTC-3′ and cloned into the *Sac*I and *Bam*H1 sites of pDG94/*LEU2* to yield the final pDG110 plasmid.

pDG114 (*CEN, LEU2*, *HA-bms1-K82A*) was created using gap repair by cutting pDG110 with *Stu*I and *Sna*BI and cotransforming yeast with a PCR product coding for the K82A allele. This PCR product was made by the megaprimer method (Aiyar & Leis, 1993). The mutation was introduced in a half-product created with primers 5′-CCAACCCAAAGACAA GACC-3′ and 5′-CCTGGAACAGGCGCCACTACTTTGATT CG-3′, and primers 5′-CCATAGTGCAGATACTCG-3′ and 5′-CATATAGGGATGTTCGTTCCTCC-3′ were used for the other half-product. pDG116 (CEN, *LEU2*, *HA-bms1-ΔNLS:TRP1*) was made by integrating stop codons in all three reading frames immediately after codon 1125 of *BMS1*. A PCR product containing the stop codons followed by the selectable marker *TRP1* with 44 bp of flanking homology to *BMS1* on each side was made with primers 5′-GATCATTCATTAAA AAGTATTGACGATTTCGAAAGCCAAAGACAGCTGATTG ATTGAGCAAGC-3′) and 5′-GCATTTCCGCGATCATAGTAT ATTTTAGACTCTAAGATTGAATAAAAGCTAGCTTGGCTG CAG-3′) and YDp-*TRP1* (Berben et al., 1991) as a template. The *bms1-ΔNLS* allele was first made in an untagged allele of *BMS1* and confirmed by sequencing, before transfer to pDG110 by gap repair to generate pDG116. A galactose inducible *GST-BMS1* plasmid, pDG98, was made by cloning the entire *BMS1* ORF (4,547-bp *Bam*HI-*Sph*I fragment from pDG94) into the 2μ, *LEU2*, *GAL1*:GST expression vector pTB328 (a kind gift of Michael Hall). pDG115 (2μ, *LEU2*, *GAL1*:*GST-bms1-K82A*) was created by cloning a 2,046-bp *Stu*I-*Nco*I fragment containing the K82A mutation from pDG114 into the same sites of pDG98. pJC10 (*CEN*, *LEU2*, *HA-bms1-P888L*) was created by gap repairing the *bms1-1* P888L mutation onto pDG110. pDG110 gapped with *Bgl*II and *Nde*I was cotransformed with a 4,454-bp *Stu*I-*Sph*I fragment from pJC1 containing the P888L allele into a wild-type yeast strain. Plasmids were rescued from yeast, transformed into *Escherichia coli* and correct clones were identified by diagnostic restriction enzyme digestion. pJC10 confers temperature-sensitive growth to *bms1*-Δ strains when present as the sole source of *BMS1*, similar to untagged or integrated *bms1-1* alleles.

A *TSR1* cognate clone in pRS416 (pYCG060; Casalone et al., 1999) was obtained from EUROSCARF (Frankfurt, Germany). pDG117 (2μ, *URA3*, *TSR1*) was made by cloning *TSR1* on a *Bam*HI-*Xho*I fragment from pYCG060 into pRS426. A clone for expression of Tsr1p tagged with GFP at its carboxyl terminus was made by cotransforming pYCG060 with a PCR product encoding GFP and 45 bp of flanking homology surrounding the stop codon of *TSR1* at each end to create pJB1. The PCR product was made with primers 5′-TACAA ACGTATGTGGCCCATGCCTTCGTTACCTTGGAATGGTATG CGGATCCCCGGGTTAATTAA-3′ and 5′-TCGACTTACCTT TATCAACAGTATCGTTGATACTATTTTATTAGCGAATTCG AGCTCGTTTAAAC-3′ using pFA6a-His3Mx6-GFP(S65T) (Longtine et al., 1998) as a template.

RNA methods

Total RNA was isolated from yeast cells using RNAeasy purification kits (Qiagen) according to manufacturer's instruc-

tions. Northern blots and primer extensions were performed essentially as in Venema et al. (1998). Sequences of probes used for northern blots and primer extension are identical to those used in Kressler et al. (1997). Probes for northern blots were labeled with polynucleotide kinase (Boehringer-Mannheim) and γ^{32}P-ATP (NEN) according to manufacturer's instructions and purified over Sephadex G-50 (Pharmacia) before use. For primer extensions, labeled oligonucleotides were gel purified before use.

Pulse-chase analysis of rRNA processing was performed as described in Venema et al. (1998) with the minor changes. Briefly, cultures were grown in synthetic medium lacking methionine (C-Met) or uracil (C-Ura) to log phase and shifted to 37 °C for 2 h before pulse-labeling. For SL4078 (*GAL1:TSR1*), cultures were grown in synthetic medium lacking methionine with galactose as the sole carbon source (C-Met+Galactose) and shifted to C-Met+Glucose for indicated times before pulse labeling. Log phase cells (2.5×10^8) were washed, resuspended in 7.5 mL medium, pulse labeled for 2 min with 150 μCi of [5,6-^{3}H]-uracil (specific activity 50 Ci/mmol, NEN) or 270 μCi [^{3}H-methyl]-methionine (85 Ci/mmol, NEN) and chased by addition of prewarmed unlabeled uracil (2 mM final) or methionine (5 mM final) for indicated times. One and a half milliliters of cells were rapidly collected by centrifugation and cell pellets were quick-frozen on dry ice/ethanol. RNA was isolated using RNAeasy kits (Qiagen) according to manufacturer's instructions for glass bead lysis using a mini beadbeater. Equal CPM of RNA were separated on 1.2% agarose/6% formaldehyde gels and transferred to Zeta-Probe (BioRad) membranes, then sprayed with En^{3}Hance (Amersham) and dried completely before exposure to film at −80 °C.

Polysome profiles

All procedures were performed at 4 °C except where indicated. Approximately 0.5–1 $\times 10^9$ cells were pelleted from log-phase culture, resuspended in 5 mL ice-cold 100 μg/mL cycloheximide (Calbiochem, La Jolla, California) for 1 min, and repelleted. Lysates were made by glass bead lysis in a Braun homogenizer for 3 min in 1.2 mL polysome buffer (100 mM KCl, 2 mM magnesium acetate, 20 mM HEPES, pH 7.4, 14.4 mM beta-mercaptoethanol, 100 μg/mL cycloheximide). The cell lysate was centrifuged at 5,000 RCF for 8 min in a microcentrifuge and the supernatant removed and respun. Ten A_{254} units were loaded onto a 16.2 mL 10–50% sucrose gradient made in 100 mM KCl, 5 mM $MgCl_2$, 20 mM HEPES, pH 7.4, and 2 mM DTT and centrifuged in a Beckman SW28.1 rotor at 27,000 rpm for 4.5 h. Gradients were collected with continuous monitoring at 254 nm using an ISCO UA-5 absorbance detector and 1640 gradient collector.

GST-Bms1p purification and crosslinking to ATP and GTP

The vacuolar protease-deficient strain, BJ2168, was transformed with pDG98 (GST-Bms1p) or pDG115 (GST-Bms1p-K82A). Cells were grown to log phase in synthetic glucose medium lacking leucine, harvested, and grown to log phase in synthetic raffinose medium lacking leucine to yield a raffinose-adjusted preculture. To induce expression of GST fusions, raffinose-adjusted cells were grown for at least 8 h in fresh selective raffinose medium to 0.1–0.2 $\times 10^7$ cells/mL and then induced for 10–12 h with 2% galactose. Approximately 5×10^9 cells were harvested, washed once in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 5 mM EDTA, 5 mM EGTA), and resuspended in 2.4 mL ice-cold lysis buffer containing protease inhibitors (1 mM PMSF, 100 μM TPCK, 1 mM benzamidine-HCl, 5 mM E64, 25 μM pepstatin A and 4 μM leupeptin). Cells were lysed for 3 min in a Braun glass bead homogenizer and the lysates were spun in a 4 °C microfuge for 10 min at 14,000 rpm. Cleared lysates were diluted to 4.5 mL with lysis buffer containing protease inhibitors and incubated for 3 h on an end-over-end rotator at 4 °C with 300 μL of a 50% slurry of glutathione-sepharose beads (Pharmacia). Beads with attached fusion proteins were washed twice with 5 mL of ice-cold lysis buffer and four times with 1 mL of low EDTA lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.1 mM EDTA) before being divided into three equal aliquots. One aliquot was separated by SDS-PAGE and processed for immunoblotting with anti-GST antibodies (1:400, Santa Cruz). The other two aliquots were used for crosslinking to α-^{32}P-ATP and α-^{32}P-GTP essentially as described in de Boer et al. (1991). Fifty microliters of GST-Bms1p fusion-bound glutathione-sepharose beads were incubated with 25 μCi of α-^{32}P-GTP or α-^{32}P-ATP (New England Nuclear; 0.625 μM final concentration of nucleotide) in 50 μL reaction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.1 mM EDTA, 10 mM $MgCl_2$, 50 mM potassium glutamate, 1 mM DTT, 10 μg/mL BSA) in an ultralow cluster microtiter dish (Costar) placed on a chilled lead brick surrounded by ice water. A hand-held UV lamp was placed directly on the microtiter dish and samples were irradiated for 30 min at 254 nm. Reactions were removed to fresh tubes containing 25 μL of 5× SDS sample buffer and each well was rinsed with 50 μL of reaction buffer and combined with the retrieved reaction. Samples were heated to 95 °C for 5 min and separated by SDS-PAGE (7.5% polyacrylamide). Gels were stained with Coomassie brilliant blue, destained, dried, and exposed to film to visualize ^{32}P-labeled bands.

Cell extracts and immunoblotting

Cells (5×10^7) were collected by centrifugation, resuspended in 300 μL of 4% SDS, and lysed by vortexing for 5 × 1 min in the presence of an equal volume of glass beads. Seventy-five microliters of 5× SDS-PAGE sample buffer were added to each sample before heating at 95 °C for 5 min. Fifty microliters of each sample were separated by SDS-PAGE (7.5% polyacrylamide), transferred to nitrocellulose, and immunoblotted with anti-HA antibodies (3F10, 1:1,000; Boehringer Mannheim) and detected with anti-horseradish peroxidase-conjugated goat-anti-rat IgG (Zymed) and chemiluminescence (ECL; Amhersham).

Immunofluorescence microscopy

Microscopy was performed with a Zeiss Axioplan-2 microscope equipped with Nomarski differential interference contrast optics using a plan-NEOFLUAR 100× objective (numerical aperture 1.3). Images were captured on a Hamamatsu

C4742–95 cooled CCD camera using QED software and manipulated with Adobe PhotoShop. Indirect immunofluorescence was performed as outlined in Gehrung and Snyder (1990). HA tagged proteins were detected using rat monoclonal 3F10 (1:300, Boehringer Mannheim), followed by Texas Red-conjugated goat anti-rat IgG (1:600, Molecular Probes). Gar1p was localized using *GAR1*-GFP expressed from plasmid pZUT3 (Trumtel et al., 2000). Tsr1p-GFP was localized in living cells grown overnight in the presence of DAPI (0.1 μg/mL, Sigma).

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