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Bms1p, a G-domain-containing protein, associates with Rcl1p and is required for 18S rRNA biogenesis in yeast

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ABSTRACT

Maturation of 18S rRNA and biogenesis of the 40S ribosomes in yeast requires a large number of *trans*-acting factors, including the U3 small nucleolar ribonucleoprotein (U3 snoRNP), and the recently characterized cyclase-like protein Rcl1p. U3 snoRNP is a key particle orchestrating early 35S rRNA cleavage events. A unique property of Rcl1p is that it specifically associates with U3 snoRNP, but this association appears to occur only at the level of nascent ribosomes and not with the U3 monoparticle. Here we report the characterization of Bms1p, a protein that associates with Rcl1p in multiple structures, including a specific complex sedimenting at around 10S. Like Rcl1p, Bms1p is an essential, evolutionarily conserved, nucleolar protein, and its depletion interferes with processing of the 35S pre-rRNA at sites A_0, A_1, and A_2, and the formation of 40S subunits. The N-terminal domain of Bms1p has structural features found in regulatory GTPases and we demonstrate that mutations of amino acids implicated in GTP/GDP binding affect Bms1p activity in vivo. The results indicate that Bms1p may act as a molecular switch during maturation of the 40S ribosomal subunit in the nucleolus.

Keywords: G proteins; GTPase; nucleolus; pre-rRNA processing; ribosome biogenesis; snoRNAs

INTRODUCTION

In eukaryotic cells, synthesis and processing of ribosomal RNAs (rRNAs) and assembly of ribosomes occur in the nucleolus and follow an unusually complex pathway. The 18S, 5.8S, and 25–28S rRNAs are synthesized as a single precursor (pre-rRNA), which contains additional sequences that are discarded during RNA maturation. The maturation process involves extensive modification of rRNA nucleotides, most of them guided by small nucleolar RNAs (snoRNAs), followed by multiple cleavage events resulting in the formation of different processing intermediates. The substrate for rRNA processing is a large ribonucleoprotein complex containing a multitude of ribosomal proteins and accessory nucleolar *trans*-acting factors that associate with the nascent pre-rRNA (reviewed by Kressler et al., 1999; Venema & Tollervey, 1999; Lewis & Tollervey, 2000). rRNA processing has been most extensively studied in the yeast *Saccharomyces cerevisiae* and many *trans*-acting factors, both proteins and ribonucleoproteins, required for the process have been characterized. These include, in addition to guide snoRNAs, the ribonucleoprotein RNase MRP, the essential snoRNAs U3, U14, snR30 and snR10, and many proteins, which either act in association with snoRNAs or function independently. Among the latter are putative ATP-dependent RNA helicases, Dim1p methylase, and endo- and exoribonucleases (Kressler et al., 1999; Venema & Tollervey, 1999).

Despite substantial progress in identification of the *trans*-acting factors required for pre-rRNA processing, their precise functions remain largely unknown. The factor best characterized to date is the U3 snoRNP. Based on results from yeast and vertebrate systems, it appears that U3 snoRNP plays a central role in the assembly of the machinery responsible for processing of 18S rRNA and biogenesis of the 40S ribosomal subunit (Beltrame & Tollervey, 1992; Mougey et al., 1993; Venema & Tollervey, 1999; Borovjagin & Gerbi, 2000, and references therein). U3 snoRNA base pairs with the 35S pre-rRNA within the 5′ external transcribed spacer (ETS) and the 5′ part of 18S rRNA and is required for early cleavages at the processing sites A_0, A_1, and A_2 (Beltrame & Tollervey, 1995; Sharma &

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Tollervey, 1999; Venema & Tollervey, 1999, and references therein). The yeast U3 snoRNP has recently been shown to contain five strongly associated structural proteins (Watkins et al., 2000). However, consistent with the central role of U3 snoRNP in rRNA maturation, immunoprecipitation (IP) experiments revealed many additional proteins associating with the particle. These include Sof1p, Mpp10p, Imp3p, Imp4p, Dhr1p, Lcp5p, and Rcl1p (Jansen et al., 1993; Dunbar et al., 1997; Wiederkehr et al., 1998; Lee & Baserga, 1999; Billy et al., 2000; Colley et al., 2000). Generally, these proteins are also required for pre-rRNA processing at sites A_0–A_2. Significantly, inactivation or depletion of many *trans*-acting factors other than the U3-linked components, including snoRNAs U14, snR30, and snR10, also interferes with pre-rRNA cleavage at A_0–A_2 or A_1–A_2 (Kressler et al., 1999; Venema & Tollervey, 1999). These observations indicate that coordinate processing at sites A_0–A_2 strictly depends upon accurate assembly of dozens of ribosomal proteins and *trans*-acting factors into a large ribonucleoprotein structure, often referred to as a nascent ribosome or processosome. How the formation of such a structure proceeds and what triggers the cleavage reactions upon assembly of the complex remains unknown. Similarly, nothing is known about the factors responsible for cleavages at sites A_0–A_2, although it is believed that processing at these sites is catalyzed by proteins rather than RNA (Venema & Tollervey, 1999).

Recently, we have identified a novel yeast nucleolar protein, Rcl1p, which is essential for pre-rRNA processing at sites A_0–A_2. Rcl1p is a member of an evolutionarily conserved family of proteins having strong sequence and structural similarity to the RNA 3′-terminal phosphate cyclases, the enzymes that catalyze conversion of the 3′-terminal monophosphate to the 2′,3′-cyclic phosphodiester in RNA. However, available evidence indicates that Rcl1p is not active as a cyclase and its catalytic function, if any, and precise role in pre-rRNA processing remain to be established. An interesting property of Rcl1p is that it specifically associates with U3 snoRNP, but is not a structural component of the U3 monoparticle. Most likely, the interaction between Rcl1p and U3 snoRNP takes place in large complexes of 70–80S, representing nascent ribosomes (Billy et al., 2000).

Here we report the characterization of Bms1p, a protein that associates with Rcl1p in multiple structures, including a specific complex sedimenting at around 10S. Like Rcl1p, Bms1p is an evolutionarily conserved nucleolar protein required for pre-rRNA processing at sites A_0, A_1, and A_2. Notably, the N-terminal domain of Bms1p has structural features common to the regulatory GTP/GDP binding proteins (G proteins), suggesting that Bms1p may act as a molecular switch or a proofreading factor during biogenesis of the 40S ribosomal subunit in the nucleolus.

RESULTS

Identification of Bms1p as a protein interacting with Rcl1p

To identify proteins interacting with Rcl1p, a yeast two-hybrid screen was carried out using a fusion of Rcl1p with the *GAL4* DNA binding domain as the bait. Sixty-four clones positive for *HIS3* gene expression and strongly activating β-galactosidase activity were selected. Of these, 31 contained prey plasmids harboring fragments of the same ORF, *YPL217C* (hereafter referred to as *BMS1*; see Discussion), fused in-frame with the *GAL4* activation domain. The shortest insert characterized encoded an internal fragment of 127 amino acids, extending from N535 to D661 of Bms1p. This region of Bms1p is highly enriched in charged amino acids, with acidic and basic residues constituting 25% and 20% of the sequence, respectively. Inserts present in all other characterized prey plasmids overlapped with the N535–D661 region.

To confirm the interaction between Rcl1p and Bms1p, a strain chromosomally expressing Bms1p fused to the Protein A (ProtA) tag at the C-terminus was constructed (BMS1-ProtA). An extract from this strain was used in IP experiments, using IgG-Sepharose beads. Extracts from strain BMA41-1a (hereafter referred to as wild-type or WT; see Materials and Methods) and strain LSM3-ProtA (Salgado-Garrido et al., 1999), expressing the nucleoplasmic protein Lsm3p fused to ProtA, were used as controls. The input material, and supernatants and pellets of the immunoprecipitation reactions were separated by SDS-PAGE and analyzed by westerns, using α-Rcl1p antibody (Ab) (Fig. 1A). The results indicated that Rcl1p coimmunoprecipitates with Bms1p and is absent from the IP pellets of the two control reactions.

BMS1p is an evolutionarily conserved G-domain-containing protein

BMS1 codes for a protein of 1,183 amino acids with a predicted molecular mass of 135.5 kDa and pI of 6.6. Proteins showing strong sequence similarity to Bms1p (amino acid sequence identities of 34–52%) are encoded in the genomes of *Homo sapiens*, *Drosophila melanogaster*, *Caenorhabditis elegans*, *Arabidopsis thaliana*, *Schizosaccharomyces pombe*, and *Plasmodium falciparum*. With the possible exception of *P. falciparum*, these proteins most likely represent orthologs of Bms1p (for more details, see Materials and Methods). Bms1p and related proteins from other organisms show a tripartite organization. The approximately 400 amino-acids-long N- and C-terminal domains (domains N and C) are the most highly conserved regions of the proteins. Moreover, these two domains show low but significant similarity to each other, suggestive of an

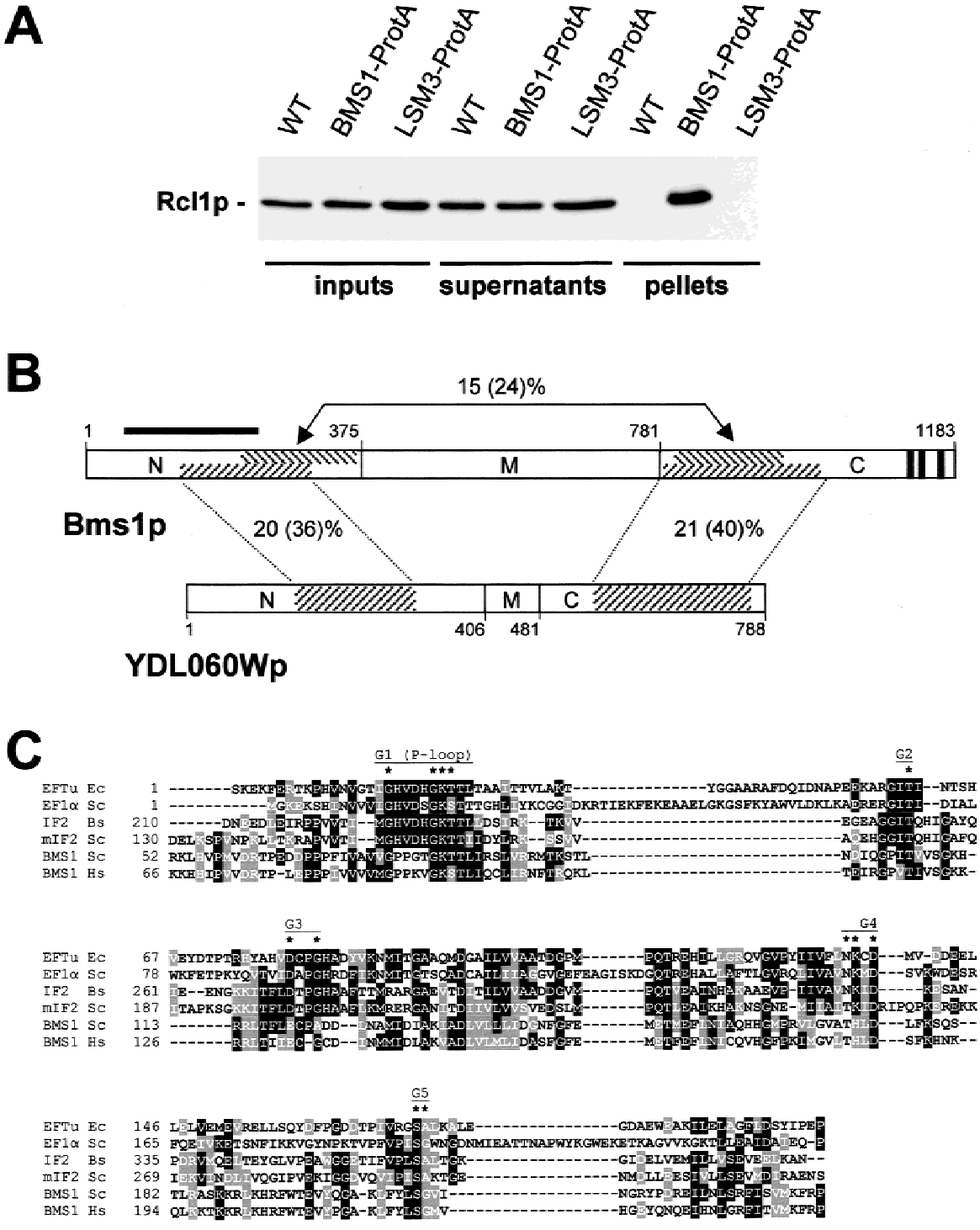

FIGURE 1. Coprecipitation of Bms1p and Rcl1p, and domain organization of Bms1p. **A:** Bms1p-ProtA immunoprecipitates Rcl1p. Inputs, supernatants, and pellets (loaded in a 1:1:10 ratio) from the IP reactions of ProtA-tagged proteins expressed in the strains indicated were analyzed by western blotting using α-Rcl1p Ab. The efficiency of Rcl1p coimmunoprecipitation was estimated as 20%. Incomplete recovery of Rcl1p may be explained by a low efficiency of Bms1-ProtA immunoprecipitation (about 50%; not shown), susceptibility of Bms1p to degradation (see Fig. 2C), and the fact that a substantial fraction of Rcl1p exists as a free form (Billy et al., 2000; this work). **B:** Domain organization of Bms1p and a comparison with the structurally related yeast protein, YDL060Wp. Percentages of sequence identity and similarity (in parentheses) between the N and C domains of Bms1p (in regions of similarity shown by down-sloping hatch), and between the two proteins (in regions of similarity shown by up-sloping hatch) are indicated. The black bar designates the G-like domain, and the black vertical lines the predicted nuclear localization signals. **C:** Alignment of the G-like domains of BMS1 proteins from *S. cerevisiae* (BMS1 Sc) and *H. sapiens* (BMS1 Hs) with the G domains of selected translation factors of different origin: elongation factors EF-Tu from *E. coli* (EFTu Ec; accession number CAA40370) and EF1-alpha from *S. cerevisiae* (EF1α Sc; CAA25798), and the initiation factor IF2 from *Bacillus subtilis* (IF2 Bs; CAB13536) and yeast mitochondria (mIF2 Sc; NP_014619). The five G motifs are indicated, with the most conserved residues marked with asterisks. The alignment was done manually and is based on available alignments of translation initiation and elongation factors, and also on results of the Blast searches carried out with BMS1 proteins.

evolutionary duplication event. In Bms1p, the conserved regions of domains N and C are 15% identical and 24% similar (Fig. 1B). Middle regions (domain M) of BMS1 proteins are much less conserved at the primary sequence level but are highly enriched in charged amino acids. In Bms1p, domain M contains 30% acidic and 13% basic residues. Interestingly, another gene in yeast, *YDL060W*, codes for a protein related in sequence and domain organization to Bms1p. The *YDL060W*-encoded protein is 788 amino acids long and its middle charged domain is much shorter than that of Bms1p (Fig. 1B). Homologs of YDL060W proteins are likewise encoded in the genomes of all eukaryotes examined, from *H. sapiens* to *S. pombe* (data not shown).

The N-terminal half of the N-domain of Bms1p, or its orthologs in other organisms, shows features characteristic of the G domain present in many regulatory GTPases, which act as molecular switches in different cellular processes such as translation, protein trafficking, and signal transduction (reviewed by Bourne et al., 1991; Sprang, 1997; see also the Discussion). Sequences of the G-like domains of BMS1 proteins are most closely related to the G domains of the eubacterial protein synthesis initiation and elongation factors IF2 and EF-Tu (Fig. 1C; data not shown). Indeed, the presence in YPL217Cp/Bms1p of a domain with strong three-dimensional structural similarity to the G domain of EF-Tu has been noted previously by Sanchez and Sali (1998), who carried out a genome-wide modeling of yeast proteins. G domains of nearly all regulatory GTPases have five conserved polypeptide loops designated G1 through G5, which form contact sites with the guanine nucleotide or coordinate the Mg^{2+} ion (Bourne et al., 1991; Kjeldgaard et al., 1996; Sprang, 1997; Song et al., 1999; Roll-Mecak et al., 2000; Sprinzl et al., 2000, and references therein). Sequences resembling G1 (consensus [G,A]X_4GK[S,T]; also known as a P-loop), G4 (consensus [N,T]KXD), and G5 (consensus for translation factors S[A,G]) are present in all BMS1 proteins, and either fully conform with the consensus or contain, at most, single conservative substitutions (Fig. 1C). The G2 motif (consensus for translation factors, GIT) is replaced in BMS1 proteins by the conserved sequence GP[I,V]T; importantly, the T residue involved in the coordination of the Mg^{2+} required for GTP hydrolysis (Sprang, 1997; Roll-Mecak et al., 2000, and references therein) remains conserved. The region in BMS1 proteins corresponding to the G3 motif diverges most from the consensus found in G proteins, DXXG; however, the D residue is replaced with the conserved E residue. The spacing between individual G-like motifs in BMS1 proteins is similar to that of known G proteins, particularly IF2 proteins (Fig. 1C). Domain C of BMS1 proteins, and domains N and C of the YDL060W-like proteins do not contain conserved sequence elements characteristic of G domains.

BMS1 is an essential gene

The *BMS1* gene was disrupted in the BMA41 diploid strain by the one-step PCR method (Baudin et al., 1993). The resulting heterozygous strain BMS1/Δbms1 was sporulated and 22 tetrads were dissected. All tetrads yielded, at most, two viable spores, which were auxotrophic for tryptophan. All unviable spores were unable to germinate. To exclude the possibility that the *BMS1* gene is essential only for germination, the BMS1/Δbms1 strain was transformed with the centromeric plasmid pFL38-BMS1, harboring the *BMS1* gene and the *URA3* marker. After sporulation, tetrads were dissected and spores prototrophic for tryptophan (which were all also prototrophic for uracil) were isolated. The resulting haploid strain Δbms1+pFL38-BMS1, bearing the *bms1::TRP1* allele in the genome, was unable to survive the loss of the pFL38-BMS1 plasmid during growth in a nonselective medium (YPD), as evidenced by the lack of colony formation on plates containing 5-fluoroorotic acid; this was in marked contrast to the original diploid strain BMS1/Δbms1 bearing the same plasmid. Altogether, these data demonstrate that the *BMS1* gene is essential for viability.

Conditional alleles of Bms1p

To investigate the function of Bms1p, a strain bearing the conditional allele *GAL::bms1* was constructed. This allele expresses Bms1p modified by the N-terminal addition of a destabilizing cassette containing ubiquitin and the influenza hemagglutinin (HA) tag; the gene is under the control of the *GAL1* upstream activating sequence (UAS_{GAL1}) and the *CYC1* promoter (together referred to as GAL promoter; Fig. 2A; Jenny et al., 1996). The strain GAL::bms1 grows in galactose-containing medium (YPGal) at the same rate as the WT strain (Fig. 2B). Following transfer to medium containing 6% glucose (YPD6%), its growth slows down considerably after 5 h, but does not stop completely even after 46 h; the doubling time is 180 min, compared with 90 min for the WT strain grown in YPD6%. Two other *BMS1* conditional alleles, placed on centromeric plasmids, were also evaluated. In the first, *BMS1* was cloned into the pGUR1 vector containing the *ADE2* marker (Jenny et al., 1996). In the resulting plasmid, pGUR-BMS1, *BMS1* is fused to the same destabilizing cassette as in *GAL::bms1*. In the second allele, *BMS1* is put under the control of the *MET25* promoter (plasmid pMET-BMS1); this promoter is repressed by the presence of 2 mM methionine in the medium. When compared to wild type, Δbms1 strains containing either pGUR-BMS1 or pMET-BMS1 showed only a moderate delay of growth in medium containing glucose and methionine, respectively. Significantly, the Δbms1 strain bearing plasmid pGUR-BMS1 (prototrophic for adenine and forming white colonies) never produced red

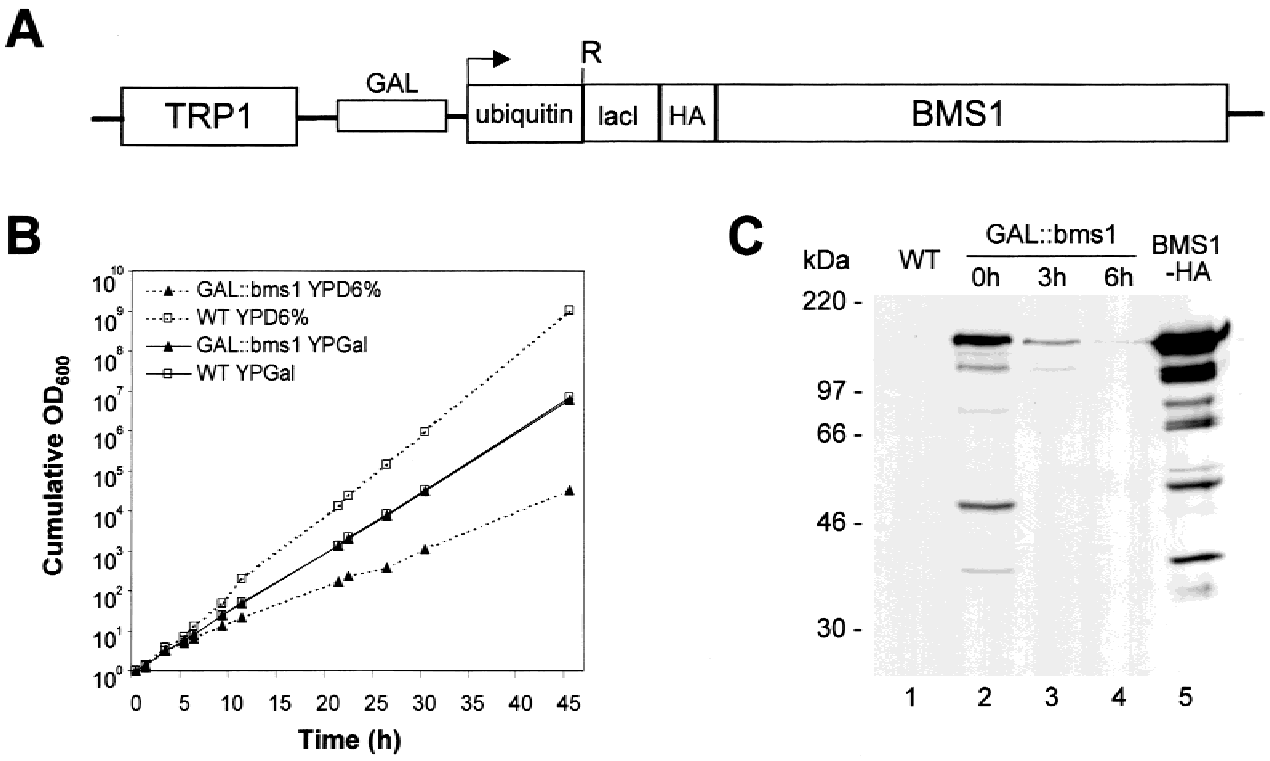

FIGURE 2. Characterization of the conditional mutant GAL::bms1. **A:** Scheme of the *GAL::bms1* allele. The BMS1 ORF is fused to ubiquitin, HA-tag, and LacI regions. Following removal of ubiquitin, the fusion protein starts with the destabilizing amino acid, ariginine (R). **B:** Growth curves of the GAL::bms1 and WT strains in YPGal and YPD6%. **C:** Expression of HA-Bms1p in the GAL::bms1 strain grown in YPGal (lane 2) or transferred to YPD6% for 3 and 6 h (lanes 3 and 4). Lane 1: extract from WT yeast; lane 5: extract from the BMS-HA strain, in which the C-terminally HA-tagged Bms1p (Bms1p-HA) is expressed from the chromosome under control of its own promoter. Cell extracts were prepared in denaturing conditions and proteins were subsequently precipitated by trichloroacetic acid according to Yaffe and Schatz (1984) to minimize protein degradation.

colonies or sectors, indicating that the plasmid could not have been lost. This further confirms that *BMS1* is an essential gene. Because the strain bearing the *GAL::bms1* allele integrated in the genome showed the most severe delay of growth under repressive conditions, it was chosen for further analysis of the effect of Bms1p depletion.

Western analysis, performed with the α-HA Ab, indicated that Bms1p is strongly depleted following 6 h of growth in YPD6% (Fig. 2C). This analysis also indicated that Bms1p is susceptible to proteolytic degradation. The cleavage products were visible not only in the case of the destabilized ubiquitin-fusion protein, but also for the C-terminally HA-tagged Bms1p, which is expressed from its own promoter in the BMS1-HA strain (Fig. 2C, see also legend).

Bms1p is a nucleolar protein and its depletion affects 40S subunit levels

Rcl1p was previously shown to be a nucleolar protein required for 18S rRNA processing and 40S ribosomal subunit biogenesis (Billy et al., 2000). To establish the localization of Bms1p, the cellular distribution of the C-terminally HA-tagged Bms1p expressed from the chromosome was examined by indirect immunofluorescence. Localization of the nucleolar protein Nop1p was performed as a control, and DNA was stained with 4,6-diamidino-2-phenylindole (DAPI) to localize the nucleus. As shown in Figure 3, treatment with both α-HA and α-Nop1p Abs showed a typical crescent-shaped staining, characteristic of nucleolar proteins including Rcl1p (Billy et al., 2000). Merging of all three images revealed colocalization of Bms1p-HA and Nop1p, whereas DAPI staining was mainly confined to the nucleoplasm. Similar results were obtained when localization of the N-terminally HA-tagged Bms1p was studied in the strain GAL::bms1 (data not shown).

To gain some insight into Bms1p function, we analyzed polysome profiles of the GAL::bms1 strain grown in either permissive or repressive medium. The polysome profile of GAL::bms1 grown in YPGal was similar to that of the WT strain (Fig. 4). On the other hand, depletion of Bms1p, following growth in YPD6% for 3 or 6 h, resulted in disappearance of 40S subunits and overaccumulation of free 60S subunits. Moreover, the

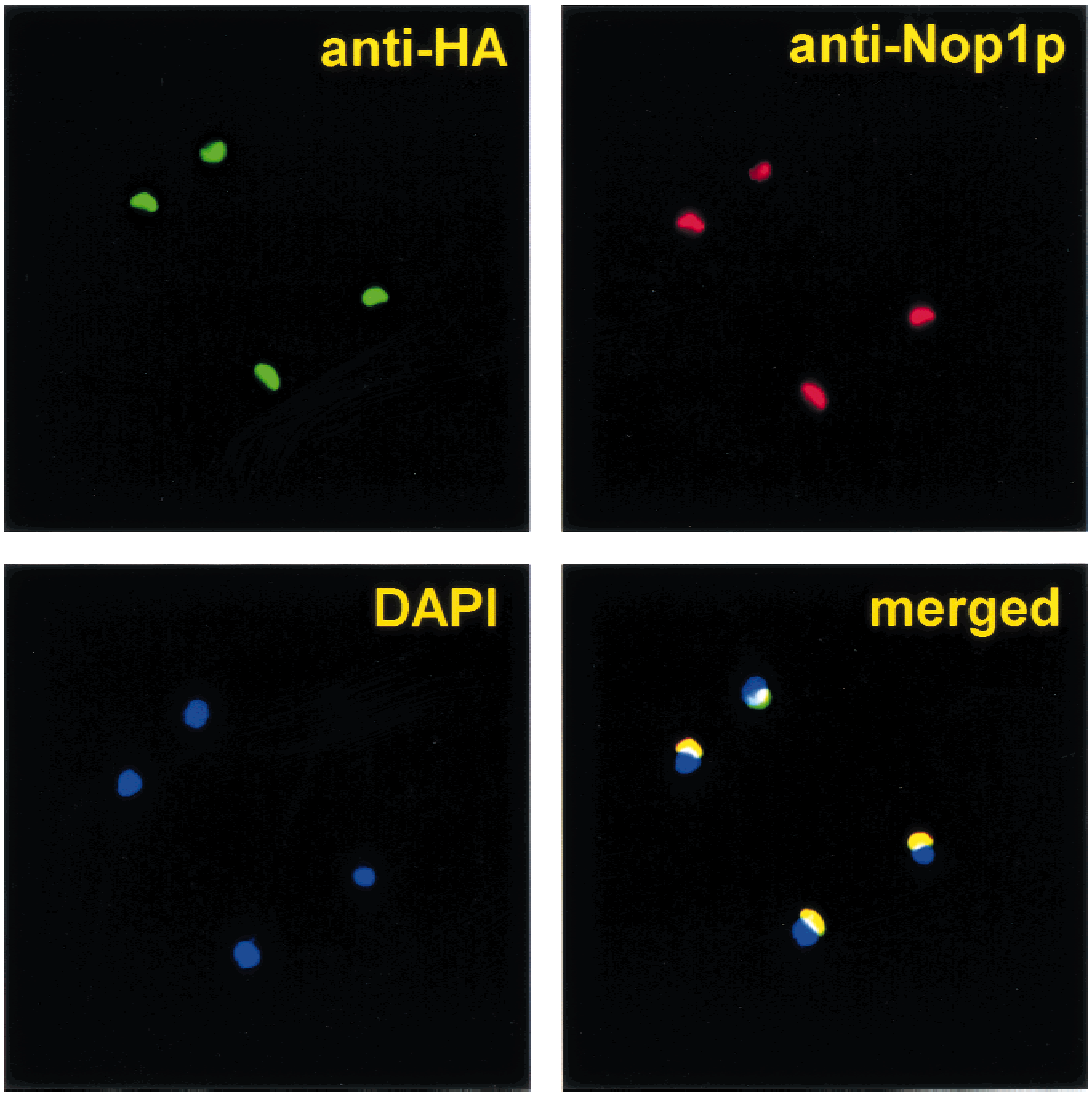

FIGURE 3. Bms1p is localized in the nucleolus. Bms1p-HA expressed in the BMS1-HA strain was detected by a rat 3F10 α-HA Ab followed by a goat FITC-coupled α-rat Ab. Nop1p was detected by mouse mAb A66 followed by a goat TR-conjugated α-mouse Ab. Chromatin was stained with DAPI. Merging of all three images is shown in the bottom right panel.

level of polysomes decreased gradually with time in cells depleted of Bms1p (Fig. 4). These effects are similar to those caused by depletion of Rcl1p (Billy et al., 2000).

Depletion of Bms1p causes defects of rRNA processing at sites A_0, A_1, and A_2

In yeast, the 35S pre-rRNA is initially cleaved at sites A_0, A_1, and A_2, leading to the formation of two processing intermediates, 20S and 27SA_2, containing sequences of mature rRNAs present in small (18S rRNA) and large (25S and 5.8S rRNAs) ribosomal subunits, respectively (see Fig. 5A). Processing at sites A_0–A_2, like most of the other cleavages, takes place in large ribonucleoprotein particles representing nascent ribosomes (reviewed by Kressler et al., 1999; Venema & Tollervey, 1999). To find out whether the decrease in the level of 40S subunits seen after depletion of Bms1p results from a defect in rRNA processing, steady-state levels of precursor rRNAs were analyzed by northerns. RNA isolated from GAL::bms1 and WT strains grown in either YPGal or YPD6% for different times was separated on a denaturing gel and probed with oligonucleotides complementary to different regions of the 35S pre-rRNA (a–g; see Fig. 5B). Depletion of Bms1p resulted in a pattern of RNA products characteristic of processing defects at sites A_0, A_1, and A_2: (1) The aberrant 23S RNA, extending from the 5′ end of the 35S precursor to the A_3 cleavage site, was detected with probes a, b, and c. The 23S RNA results from a cleavage at site A_3 in the absence of cleavages at sites A_0, A_1, and A_2. The 22S RNA, extending from A_0 to A_3, and readily detectable in yeast depleted of Rcl1p (Billy et al.,

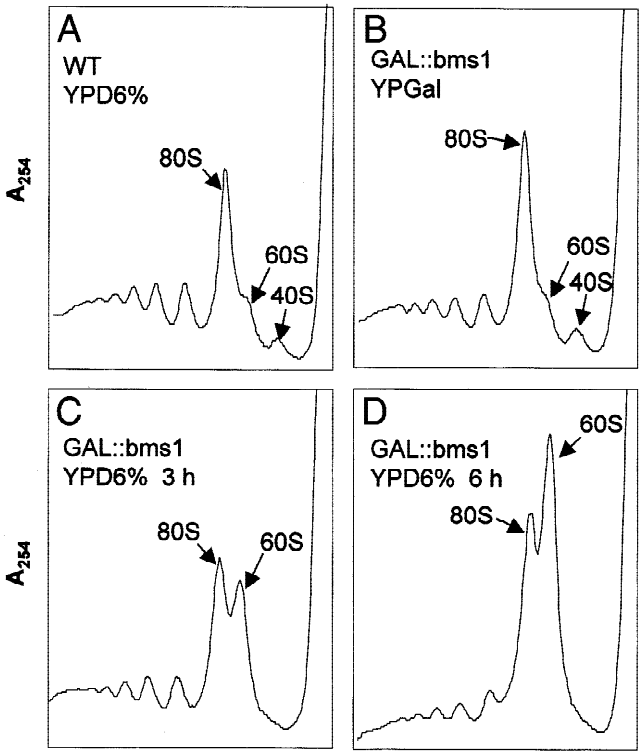

FIGURE 4. Polysome profiles of the WT strain grown in YPD6% (**A**) and the GAL::bms1 strain grown under permissive (YPGal) (**B**) or restrictive (YPD6%) conditions for 3 h (**C**) or 6 h (**D**).

2000), was only barely visible, suggesting that processing at A_0 is strongly inhibited. (2) As expected, a direct cleavage of the 35S pre-rRNA at A_3 resulted in the disappearance of the precursor $27SA_2$ in cells depleted of Bms1p (probe c), whereas the precursor 27SA/B, extending downstream from site A_3, was not affected (probe e). (3) Levels of 18S rRNA (probe g) and its immediate precursor 20S (probe b) were significantly decreased, whereas the level of mature 25S rRNA (probe f) was not changed in response to depletion of Bms1p.

To further investigate the processing defects at sites A_0–A_2, a primer extension analysis was performed (Fig. 5B, panel h). It revealed strongly decreased levels of cDNA extending to site A_2, consistent with the accumulation of 23S and the disappearance of $27SA_2$ RNAs, as identified by northerns. The amount of cDNA extending to site A_0 was not increased upon Bms1p depletion. This contrasts with the situation observed in cells deficient in Rcl1p (Billy et al., 2000) and is consistent with very low levels of the 22S RNA detected on northern blots.

In conclusion, Bms1p deficiency inhibits processing of the pre-rRNA at sites A_1 and A_2, similar to depletion of Rcl1p (Billy et al., 2000). However, the effect on cleavage at site A_0 is stronger in cells depleted of Bms1p. It should be noted that the processing defects at sites A_0–A_2 in strain GAL::bms1, although appearing early after the shift to YPD6%, do not intensify with time. This may be explained by the "leakiness" of the *GAL::bms1* allele and continued growth in this medium.

Rcl1p and Bms1p cosediment together in the 10S complex

Glycerol gradient analysis has previously shown that Rcl1p is present in several different structures sedimenting at approximately 10S, 40S, and 70–80S; a fraction of the protein was also found to sediment as a free form near top of the gradient (Billy et al., 2000). To analyze the distribution of Bms1p, an extract prepared from strain BMS1-HA was layered on glycerol gradients, which were centrifuged for either 315 min or 24 h. Fractions were collected and analyzed by westerns, using α-HA and α-Rcl1p Abs. As shown in Figure 6A, Bms1p-HA and Rcl1p cosedimented together in complexes of approximately 10S (right panel), and 40S and 70–90S (left panel).

We examined the effect of Bms1p depletion on the distribution of Rcl1p. Extracts from the GAL::bms1 strain grown in YPGal or in YPD6% for 6 h were analyzed by gradient sedimentation as described above. Rcl1p in the GAL::bms1 strain grown in YPGal showed a distribution similar to that in BMS1-HA (Fig. 6A) and WT (not shown) strains grown in YPD, except that little free Rcl1p was present (Fig. 6B, first row). Depletion of Bms1p caused a dramatic overaccumulation of the free form of Rcl1p and a decrease or disappearance of Rcl1p associated with all faster sedimenting structures (Fig. 6B, second row). The total level of Rcl1p was not affected by depletion of Bms1p (data not shown).

We investigated whether the effect on Rcl1p distribution results specifically from the depletion of Bms1p and not a general inhibition of pre-rRNA processing. For this purpose we used the GAL::imp3 strain, which conditionally expresses the protein Imp3p. Like Rcl1p, Imp3p associates specifically with the U3 snoRNP, and its depletion affects pre-rRNA processing at steps identical to those inhibited in the absence of Rcl1p or Bms1p (Lee & Baserga, 1999). We found that under permissive conditions the distribution of Rcl1p in the GAL::imp3 strain is similar to that seen with the BMS-HA strain (compare Fig. 6A, lower row, with Fig. 6B, third row) or the WT strain (not shown). Significantly, depletion of Imp3p, following 8 h of growth under repressive conditions, had no significant effect on the level of the 10S complex, and only marginally affected the level and the distribution of larger complexes containing Rcl1p (Fig. 6B, bottom row). We have verified that depletion of Imp3p in the GAL::imp3 strain grown in YPD6% for 8 h causes the same changes in the polysome profile, including overaccumulation of the free 60S subunits, as depletion of Bms1p (not shown). These results demonstrate that the effect of Bms1p depletion on the Rcl1p sedimentation profile is specific.

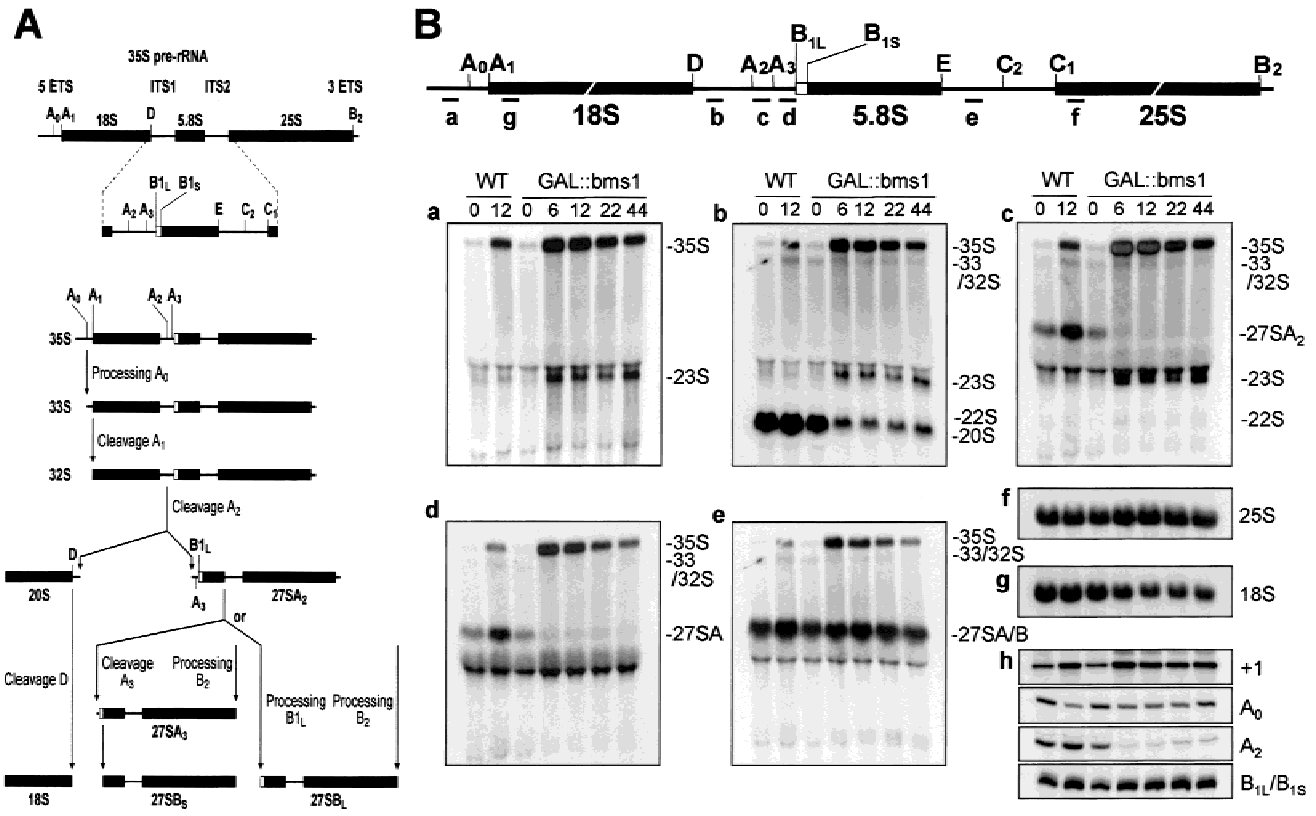

FIGURE 5. Depletion of Bms1p causes defects in rRNA processing at cleavage sites A_0, A_1, and A_2. **A:** Structure of 35S pre-rRNA (upper portion) and a simplified scheme of pre-rRNA processing in yeast (lower portion). Processing sites and intermediates are indicated. For more details, see Kressler et al. (1999) and Venema and Tollervey (1999). **B:** Northern blotting and primer extension analysis of RNA isolated from WT and GAL::bms1 strains grown in YPGal (lanes 0) or YPD6% for 6, 12, 22, or 44 h. The oligonucleotide probes, indicated on the scheme of the 35S pre-rRNA above the gels, were complementary to: 5′ETS, upstream of A_0 (**a**); ITS1, upstream of A_2 (**b**); ITS1, between A_2 and A_3 (**c**); ITS1, downstream of A_3 (**d**); ITS2, upstream of C_2 (**e**); mature 25S rRNA (**f**); and mature 18S rRNA (**g**). Positions of different processing intermediates and mature rRNAs are indicated**.** In **b**, the 22S RNA is only visible after prolonged exposure (not shown). Elevated levels of physiological precursors 35S and $27SA_2$ in the WT strain after transfer to YPD6% are likely caused by higher pre-rRNA production rates during growth in the rich medium containing 6% glucose and supplemented with 20 μg/mL adenine. **h:** Primer extension products ending at sites +1 (5′ end of the 35S precursor), A_0, A_2, and B_{1L}/B_{1S}.

Taken together, the data indicate that Rcl1p and Bms1p are components of the 10S complex, and also cosediment in association with structures representing nascent ribosomes.

Bms1p immunoprecipitates low levels of U3 snoRNA

Rcl1p was previously demonstrated to specifically associate with U3 snoRNP (Billy et al., 2000). To find out whether Bms1p interacts with snoRNAs, we carried out IP reactions using the strain BMS1-ProtA, which chromosomally expresses a Bms1p-ProtA fusion. Control strains expressing other ProtA-tagged proteins: Nop1p, a protein associated with U3 and other C/D box snoRNAs, Lsm3p, a protein associated with the U6 spliceosomal RNA, and Rcl1p, were analyzed in parallel. As shown in Figure 7, Bms1p-ProtA specifically precipitates low levels of U3 snoRNA but none of the other RNAs tested, representing members of either the C/D- or H/ACA-box snoRNA families (U14, snR190 and snR10, snR30, respectively). Similar results were obtained when IPs performed with the anti-HA antibody were carried out with extracts originating from strains expressing either Bms1p-HA or the HA-tagged Imp3p, another protein associating specifically with the U3 snoRNP (Lee & Baserga, 1999; data not shown). Interestingly, also in this case immunoprecipitation of U3 snoRNA with Bms1p-HA was much less effective than with Imp3p-HA. The significance of the less efficient precipitation of U3 snoRNA with Bms1p than with Rcl1p is not understood at present. Low recovery of U3 RNA may be related to the observed susceptibility of Bms1p to proteolytic degradation (see Fig. 2C). Alternatively, it is possible that interaction of the 10S complex with the nascent ribosome is accompanied by a structural rearrangement resulting in weakening of the interaction between Bms1p and Rcl1p and/or U3 snoRNP (see Discussion).

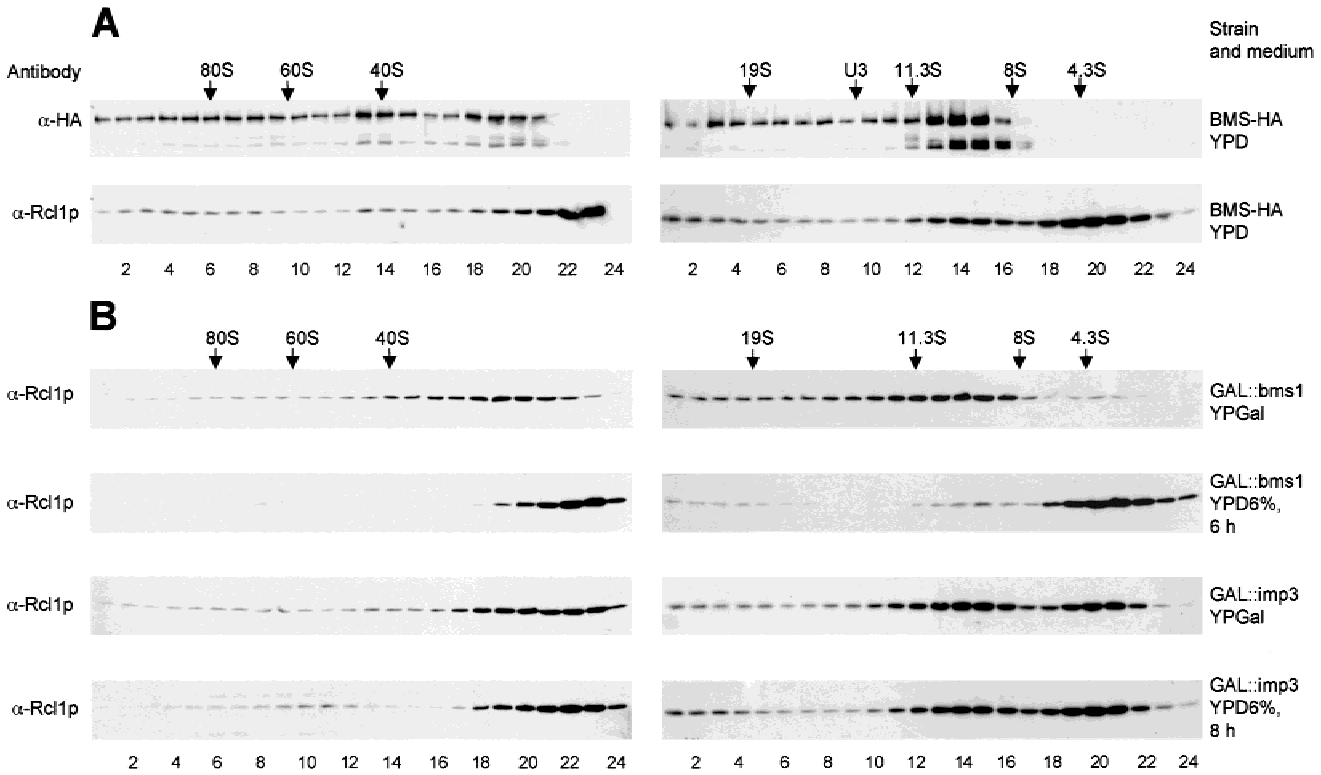

FIGURE 6. Bms1p and Rcl1p cosediment together in the 10S complex and in larger structures representing nascent ribosomes. **A:** Distribution of Bms1p-HA and Rcl1p was analyzed on a 10–30% glycerol gradients centrifuged for either 315 min (left panel) or 24 h (right panel). An extract was prepared from strain BMS1-HA and gradient fractions were analyzed by westerns using α-HA Ab (upper panel) and specific α-Rcl1p Ab (lower panel). Bands detected by the α-HA Ab represent the full-size Bms1p-HA and its degradation products (see Fig. 2C, lane 5). **B:** Sedimentation analysis of complexes containing Rcl1p in extracts prepared from the GAL::bms1 strain (two upper panels) and the GAL::imp3 strain (two lower panels). Yeast was grown in either YPGal or YPD6% as indicated on the right. Gradients were centrifuged for 315 min (left panels) or 24 h (right panels), and fractions were analyzed by westerns using α-Rcl1p Abs. Positions of 80S, 60S, and 40S ribosomes and protein markers (19S: 669 kDa thyroglobulin; 11.3S: 232 kDa catalase; 8S: 158 kDa aldolase; 4.3S: 67 kDa BSA; all from Pharmacia Biotech) run in parallel gradients are indicated. Position of the U3 snoRNP monoparticle was determined by hybridization, using RNA recovered from gradient fractions and the U3-specific oligonucleotide as a probe (Billy et al., 2000).

Amino acid substitutions in the G domain motifs affect biological activity of Bms1p

We have investigated whether motifs present in the G-like domain of Bms1p, implicated in GTP/GDP binding, contribute to protein function in vivo. Three mutants of Bms1p were constructed. In the mutant T83A/T84A, two threonine residues of the P loop are replaced with alanines. In mutants D175A and S208A, single aspartate and serine residues are changed to an alanine in the G4 and G5 motifs, respectively. In the established crystal structures of translation factors EF-Tu (Song et al., 1999, and references therein) and IF2/eIF5B (Roll-Mecak et al., 2000), threonines equivalent to T83 and T84 are involved in binding of Mg^{2+} (or both Mg^{2+} and phosphate) and a phosphate, respectively, whereas residues equivalent to D175 and S208 both interact with a guanine ring.

To study the effect of these mutations on the activity of Bms1p, the strain Δbms1 bearing the pGUR-BMS1 plasmid (this plasmid carries the ADE2 marker; see above) was transformed with centromeric URA3 plasmids expressing mutants of Bms1p: pFL38-BMS1-T83A/T84A, -D175A or -S208A. A plasmid carrying a WT copy of *BMS1*, pFL38-BMS1, and the empty vector pFL38 were transformed as controls. The resulting transformants were selected on a minimal medium lacking uracil and subsequently examined for the ability to lose the pGUR-BMS1 plasmid. Its loss should lead to the formation of red colonies due to the absence of Ade2p. Transformants bearing plasmids pFL38, pFL38-BMS1-T83A/T84A, and pFL38-BMS1-D175A formed only white colonies and thus were unable to lose the pGUR-BMS1 plasmid. In contrast, transformants bearing pFL38-BMS1 and pFL38-BMS1-S208A readily formed red colonies and sectors, indicative of the loss of the original pGUR-BMS1 plasmid (Fig. 8).

We conclude that mutations of the P-loop residues T83 and T84, and the G4 D175 residue affect activity of Bms1p but the mutation of S208 in the G5 motif has no

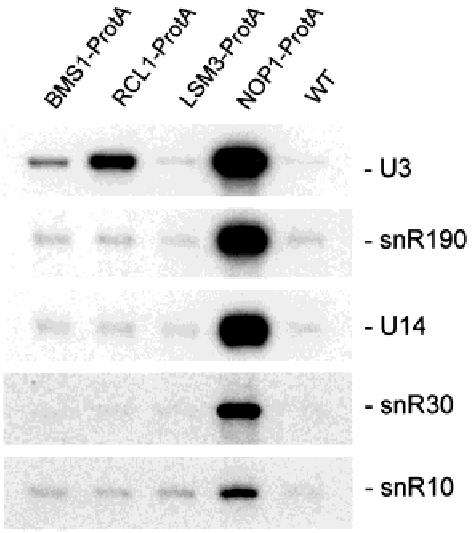

FIGURE 7. Bms1p immunoprecipitates low levels of U3 snoRNA. Extracts from the strains indicated were used for IP reactions of ProtA-tagged proteins. RNA isolated from the IP pellets was analyzed by northerns using oligonucleotides complementary to U3, snR190, U14, snR30, and snR10 snoRNAs. Nop1p specifically immunoprecipitates C/D class snoRNAs U3, snR190, and U14 (three upper panels); it also unspecifically precipitates low levels of H/ACA class snoRNAs, snR30 and snR10 (two lower panels; Ganot et al., 1997).

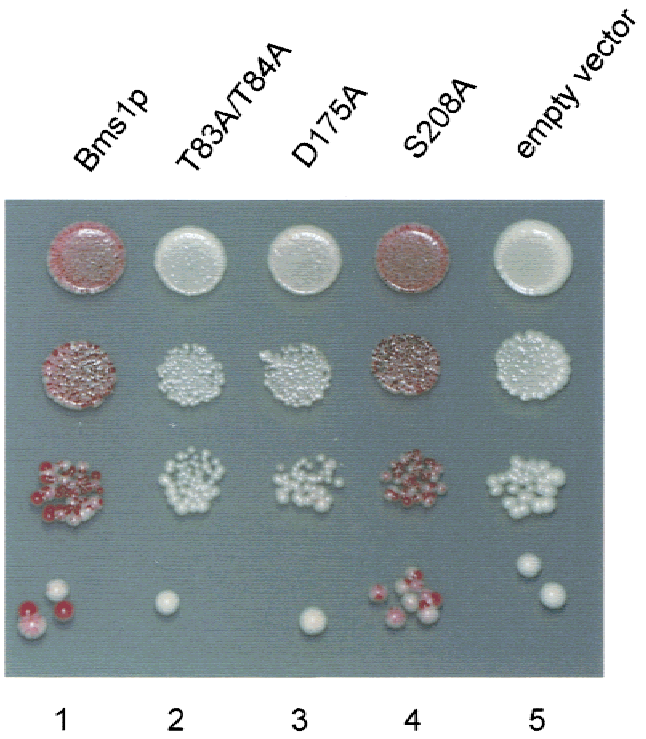

FIGURE 8. Mutations in the G1 P-loop and G4 motifs of Bms1p, T83A/T84A and D175A respectively, inactivate the protein. Centromeric *URA3* plasmids expressing Bms1p (lane 1) or its mutants indicated above the picture (lanes 2–4), and also the empty vector (lane 5) were introduced into the strain Δbms1 carrying the pGUR-BMS1 plasmid expressing WT Bms1p. pGUR-BMS1 carries the *ADE2* marker and its loss results in the formation of red colonies due to the absence of the *ADE2* gene product. Rows represent serial dilutions of the initial yeast culture.

effect, even when tested at 37 °C (result not shown). It is possible that the S208A substitution does inactivate Bms1p partially but this effect is compensated by overexpression of the protein from the centromeric plasmid.

DISCUSSION

In this work, we show that Bms1p is a protein partner of Rcl1p in the yeast *S. cerevisiae*. Like Rcl1p, Bms1p is an essential, evolutionarily conserved, nucleolar protein required for pre-rRNA processing at sites A_0, A_1, and A_2, and the formation of 40S ribosomal subunits. Bms1p and Rcl1p are components of a complex sedimenting on glycerol gradients at around 10S, which appears to associate with the U3 snoRNP at the level of nascent ribosomes. Bms1p contains an evolutionarily conserved G-protein-like domain, suggesting that the protein may play an important regulatory role in pre-rRNA processing in the nucleolus.

Several independent lines of evidence support the conclusion that Bms1p is a true partner of Rcl1p. The two proteins interact with each other in the yeast two-hybrid system and coprecipitate together in extracts prepared from a strain expressing ProtA-tagged Bms1p. Moreover, depletion of either Bms1p or Rcl1p results in a similar, though not identical, processing defect. Both proteins strongly affect cleavages at sites A_1 and A_2. However, the effect on processing at site A_0 is more pronounced in the absence of Bms1p than Rcl1p. Bms1p and Rcl1p are detected in complexes of similar sizes. Most significantly, association of Rcl1p with the prominent 10S complex is strongly diminished upon depletion of Bms1p, as is its association with structures sedimenting at ~40S and 70–90S that most likely represent nascent ribosomes at different stages of maturation. This effect is Bms1p-specific, as depletion of Imp3p, another protein required for pre-rRNA processing at sites A_0–A_2 (Lee & Baserga, 1999), does not result in any major change of Rcl1p distribution. Despite the fact that neither Rcl1p nor Bms1p cosediments on gradients with the U3 monoparticle, both proteins specifically immunoprecipitate the U3 snoRNA, though with very different efficiencies. Most probably, Rcl1p and Bms1p associate with U3 at the level of nascent ribosomes (Billy et al., 2000; this work). The observation that the association of Rcl1p with the 70–90S and 40S complexes depends on the availability of Bms1p strongly argues for the importance of the Bms1p-Rcl1p interaction for this association.

Are Rcl1p and Bms1p the only components of the 10S complex? We have carried out experiments with extracts prepared from yeast strains expressing either Rcl1p or Bms1p tagged with the calmodulin binding peptide and ProtA (TAP-tag; Rigaut et al., 1999). In

each case, affinity purification of the complex, followed by mass spectrometry, identified the expected protein partner, either Bms1p or Rcl1p. In addition, variable levels of several ribosomal proteins copurified. We are investigating whether they originate from the contaminating preribosomal particles or represent components of the 10S complex (D. Hess, E. Billy, T. Wegierski, & W. Filipowicz, unpubl. results). Rcl1p-like proteins are conserved among eukaryotes and the mouse ortholog, mRcl1, complements growth of a yeast strain depleted of Rcl1p (Billy et al., 2000). Likewise, proteins similar to Bms1p are expressed in other eukaryotes, and we have isolated a human Bms1p homolog while carrying out a two-hybrid screen using human Rcl1 as bait (our unpubl. results). Thus, the interaction between the two proteins seems to be evolutionarily conserved.

An intriguing finding of this work is that Bms1p, a protein required for pre-rRNA processing, contains an evolutionarily conserved region resembling the guanine nucleotide binding domain, or "G domain," found in many regulatory GTPases (Bourne et al., 1991; Sprang, 1997; Sprinzl et al., 2000). The presence in YPL217Cp/Bms1p of a domain with strong structural similarity to the G domain of the bacterial elongation factor EF-Tu has been noted previously by Sanchez and Sali (1998), who carried out a genome-wide modeling of yeast proteins. We have found that amino acid mutations in the two motifs involved in GTP/GDP and/or Mg^{2+} binding in known G proteins, inactivate Bms1p. Moreover, Gelperin and Lemmon (pers. comm.) have demonstrated specific crosslinking of GTP to the overexpressed protein. These data indicate that Bms1p is a true member of the G protein superfamily. Members of this family include regulatory GTPases involved in many different cellular processes, ranging from ribosomal protein synthesis (e.g., translation factors IF-2, EF-Tu, and EF-G), through signal transduction and membrane signaling (e.g., ras, and heterotrimeric G proteins), to protein traffic and cytoskeleton organization (e.g., Ran and Rho proteins). Common to the functioning of these proteins is that their G domains generally act as molecular switches, which are active in the GTP-bound form and inactive in the GDP-bound form. GTP hydrolysis and exchange of GDP for GTP to reactivate the protein are usually assisted by other regulatory factors (Bourne et al., 1991; Sprang, 1997; Sprinzl et al., 2000).

Hydrolysis of GTP bound to Bms1p might act as a signal to initiate pre-rRNA cleavage reactions following the correct assembly of the processing complex. In this context, the proposed interaction of Bms1p and Rcl1p with U3 snoRNP at the level of nascent ribosomes is particularly intriguing and makes Bms1p, either alone or in association with Rcl1p, very well suited to perform such a regulatory role. Much evidence exists that U3 is a key snoRNP required for 18S rRNA processing and 40S biogenesis (see the Introduction). Among several proteins that are specifically associated with U3 snoRNP and required for A_0–A_2 or A_1–A_2 processing (see the Introduction), are Sof1p and Dhr1p. Sof1p contains a repeated sequence found in the β subunit of the heterotrimeric G proteins and some other regulatory proteins (Jansen et al., 1993), whereas Dhr1p is a member of the DEAH subfamily of putative ATP-dependent RNA helicases; Dhr1p was shown to efficiently coprecipitate with Rcl1p (Colley et al., 2000). It is possible that Sof1p and Dhr1p also contribute to the establishment of the cleavage-competent processing complex.

The possible structural rearrangement, which might involve Bms1p, Dhr1p, and possibly other U3 snoRNA-associated proteins, could be formation of the central 18S rRNA pseudoknot. This evolutionarily conserved long-range interaction between the 5′-terminal and central regions of the mature 18S rRNA is mutually exclusive with the well-documented base-pairing of U3 snoRNA with the stem-loop structure at the 5′ end of the 18S rRNA sequence in the 35S pre-rRNA (Hughes, 1996; Mereau et al., 1997; Sharma & Tollervey, 1999). The 18S-U3 to pseudoknot rearrangement could constitute a final checkpoint before cleavages at sites A_1 and A_2 are initiated, and a role for Dhr1p in this process has recently been postulated (Colley et al., 2000). Interestingly, Fabrizio et al. (1997) have demonstrated that one of the protein components of the spliceosomal U5 snRNP, the Snu114p/116kD protein essential for splicing, is a G-domain-containing GTP binding protein similar to the translation factor EF-2/EF-G. It is possible that Snu114p/116kD mediates some structural rearrangements required for the splicing reaction. These latter findings together with our observations indicate that translational G proteins have been recruited during evolution to perform conformational transitions in ribonucleoprotein machineries other than mature ribosomes.

Our previous X-ray and structural modeling studies, performed with RNA 3′-phosphate cyclases and Rcl1p respectively, revealed that one of the two domains of these proteins consists of three repeats of a folding unit previously identified in the bacterial translation factor IF3. Notably, the triplicated IF3 fold also constitutes the catalytic domain of two enzymes that are not involved in nucleic acid metabolism (Palm et al., 2000). The propensity of the triplicated IF3 unit to serve as a catalytic domain in distinct enzymes prompted us to propose that RCL1 proteins have evolved into endonucleases catalyzing one or more cleavages required for the 40S subunit synthesis (Billy et al., 2000). However, demonstration that Rcl1p is a protein associated with the G protein Bms1p also merits investigation into whether Rcl1p plays some role in the GDP to GTP exchange or GTP hydrolysis.

Interestingly, a temperature-sensitive allele of *YPL217C*/*BMS1* was originally identified as synthetic lethal with the deletion of *BMH1* (hence its name: *BMH1* sensitive; D. Gelperin & S. Lemmon, pers. comm.). Bmh1p is one of two 14-3-3 proteins in yeast; these

proteins generally act as regulators or effectors of diverse intracellular signal transduction processes in eukaryotes (reviewed by Fu et al., 2000). Among pathways involving 14-3-3 proteins in yeast is the rapamycin-sensitive TOR kinase signaling pathway, which (among other processes) targets initiation of translation and, significantly, ribosome biogenesis at several different levels (Powers & Walter, 1999; Cosentino et al., 2000; Schmelzle & Hall, 2000, and references therein). Although the basis of the reported genetic interaction between Bms1p and Bmh1p is not understood, this observation raises the possibility that Bms1p may be the downstream target of the TOR kinase pathway in yeast. We have found, however, that the Gal::bms1 strain does not show increased sensitivity to rapamycin when grown on YPD (our unpubl. results).

A role for Bms1p in pre-rRNA processing and its similarity to G proteins have been independently reported by Gelperin et al. (2001, this issue).

MATERIALS AND METHODS

Strains, media, and genetic methods

The yeast strains used in this study are derivatives of BMA41 (*MAT* a/α *leu2-3,112/leu2-3,112 his3-11,15/his3-11,15 ade2-1/ade2-1 ura3-1/ura3-1 trp1*Δ*/trp1*Δ *can1-100/can1-100*): BMA41-1a (as BMA41 but *MAT* a; haploid strain referred to in this work as wild type or WT); BMS1/Δbms1 (as BMA41 but *BMS1/bms1::TRP1*); GAL::bms1 (as BMA41-1a but $UAS_{GAL1}P_{CYC1}$-*BMS1 TRP1*); BMS1-HA (as BMA41-1a but *BMS1-HA LEU2*); BMS1-ProtA (as BMA41-1a but *BMS1-CBP-ProtA TRP1-KI*); RCL1-ProtA (as BMA41-1a but *RCL1-ProtA URA3-KI*; Billy et al., 2000).

Strain BMS1/Δbms1 was constructed by transforming the BMA41 strain with a DNA fragment containing *TRP1* gene as a selection marker with flanks complementary to the immediate upstream and downstream noncoding regions of *BMS1*. The DNA was amplified by PCR using appropriate oligonucleotides as primers and plasmid pFL35 (Bonneaud et al., 1991) as a template. Other strains were constructed by transforming the BMA41-1a strain with a DNA fragment amplified by PCR using appropriate oligonucleotides as primers and plasmids (indicated in parentheses) as templates: GAL::bms1 (YIpGUR; Jenny et al., 1996), BMS1-HA (pYX242, Novagen), BMS1-ProtA (pBS1479; Rigaut et al., 1999). In the latter strain Bms1p is C-terminally fused with two consecutive tags, calmodulin binding peptide (CBP) and Protein A (jointly referred to as TAP-tag; see Rigaut et al., 1999). Strains LSM3-ProtA (Salgado-Garrido et al., 1999), IMP3-HA, and GAL::imp3 (Lee & Baserga, 1999) and ProtA-NOP1 (Ganot et al., 1997) were kindly provided by B. Seraphin, S. Baserga, T. Kiss, and M. Caizergues-Ferrer.

Genetic manipulations and preparation of standard yeast media followed established procedures (Brown & Tuite, 1998). YPGal medium was supplemented with 20 μg/mL adenine when used for growth of strains auxotrophic for adenine. YPD medium enriched in glucose (6%), referred to as YPD6%, was used for all experiments involving depletion of Bms1p and Imp3p in strains GAL::bms1 and GAL::imp3, respectively, to ensure maximum repression of the promoter in the *GAL::bms1* allele. This medium was also supplemented with 20 μg/mL adenine.

Construction of plasmids

A plasmid containing the *BMS1* gene was isolated from a YEp13-based yeast genomic library following colony hybridization. Plasmid pFL38-BMS1 contains the *BMS1* gene subcloned on a *Sal*I-*Pst*I genomic fragment into the vector pFL38 (centromeric, with *URA3* marker; Bonneaud et al., 1991). Plasmid pGUR-BMS1 contains the *BMS1* gene cloned as a PCR-amplified *Not*I-*Not*I fragment. Oligonucleotides used for amplification were TTA*GCGGCCGC*GATGGAGCAGTCTAA TAAACAGCACCG and ATAC*GCGGCCGC*TTACCTCCTCA TCTTACGTGGACG (*Not*I sites are in italics, sequences complementary to *BMS1* are underlined) and the fragment was cloned into the vector pGUR1 (Jenny et al., 1996).

Sequence analysis of BMS1 proteins

The alignment of BMS1 proteins (result not shown) included sequences from *H. sapiens* (accession number NP_055568), *D. melanogaster* (AAF49383), *C. elegans* (the sequence was originally proposed to represent two separate proteins, AAF60833 and AAF60834; after analysis of available cDNA clones and comparison with sequences of BMS1 proteins from other organisms, new splicing sites were predicted, resulting in the BMS1-like protein), *A. thaliana* (AAF63146), *S. pombe* (sequence reconstructed from two overlapping protein sequences CAB39140 and CAC01516), *S. cerevisiae* (NP_015107), and also *P. falciparum* (CAA70129). The alignment was performed with the ClustalW program using default settings. It is not certain that the *Plasmodium* protein represents an ortholog of Bms1p. It was identified as a member of the group of asparagine- and aspartate-rich proteins translocated from *Plasmodium* trophozoites into the red blood cell cytoplasm (Barale et al., 1997). The alignment is available upon request.

Two-hybrid screen

The yeast strain HF7c (Clontech) was transformed first with the bait plasmid pGBT9 (Clontech), containing the *GAL4* DNA binding domain fused in-frame with the full-length *RCL1* gene, and subsequently with a prey library containing the sonicated yeast genomic DNA cloned in the plasmid pACT2 (Clontech). Clones positive for activation of the *HIS3* gene were selected on a medium lacking histidine in the presence of 25 mM 3-aminotriazole. They were then assayed for β-galactosidase activity in the presence of X-Gal.

PCR mutagenesis

Mutants of the *BMS1* gene were generated by a two-step PCR approach. In the first step, DNA fragments were amplified by PCR using the following forward oligonucleotides containing desired mutations: CACCTGGAACAGGAAAG GC*AGCGCT*GATTCGGTCCCTCGTC (T83A/T84A), GGTG

TAGCTACACATCT*AGCGCT*GTTTAAATCTCAGTC (D138A), GCAAAATTATTTTACCTA*GCCGGC*GTGATTAATGGAAGG (S208A); triplets coding for substituting alanines are underlined and sites for *Eco*47III or *Ngo*MIV restriction enzymes introduced to facilitate cloning are in italics. Oligonucleotide ACACACCTCCGACATCGGACATTGG was used as a reverse primer and plasmid pFL38-BMS1 as a template. In the second step, the amplified products from the first step were used as reverse primers together with the "-20" oligonucleotide TGACCGGCAGCAAAATG as a forward primer, and pFL38-BMS1 as a template. The resulting products were cut with *Sal*I and *Bst*XI restriction enzymes and used to replace the corresponding WT fragments in pFL38-BMS1. The mutagenized inserts were verified by sequencing.

RNA extraction, northern blotting, and primer extension

RNA from yeast cells and IP pellets was isolated using a standard hot-phenol procedure. Northern analysis of small and large RNAs was performed as in Billy et al. (2000) using the following oligonucleotide probes complementary to pre-rRNAs: a: CGCTGCTCACCAATGG; b: GCTCTCATGCTCTTGCC; c: ATGAAAACTCCACAGTG; d: CCAGTTACGAAAATTCTTG; e: GGCCAGCAATTTCAAGTTA; f: GCTCTTTGCTCTTGCC; g: CATGGCTTAATCTTTGAGAC; and snoRNAs: U3: TAGATTCAATTTCGGTTT; U14: TCACTCAGACATCCTAGG; snR190: GTCGAATCGGACGAGG; snR10: AATTTGTTCTCCAGTCCAAGC; snR30: GCCGTTGTCCGAAGCGCC.

Primer extension was performed as previously described (Billy et al., 2000). Oligonucleotide ACCAGATAACTATCTTAAAAG, complementary to the 5′ETS/18S boundary, was used for the analysis of site A_0 and the 5′ end of the 35S pre-rRNA, and oligonucleotide e (see above) for the analysis of sites A_2, $B1_S$, and $B1_L$.

Immunoprecipitations

Immunoprecipitations were performed as previously described (Ganot et al., 1997). Buffers contained either 500 mM potassium acetate (for IP of RNA) or 250 mM sodium chloride (for IP of proteins) during binding and washing steps.

Immunolocalization

Indirect immunofluorescence analysis was performed as described in the Botstein's Lab Home Page (http://genome.stanford.edu/group/botlab/protocols). The following primary Abs were used: rat 3F10 α-HA (dilution 1/300), α-Nop1p mAb A66 (obtained from J. Aris; Aris & Blobel, 1988; dilution 1/5,000). Secondary Abs were FITC-conjugated α-rat (dilution 1/300), and TR-conjugated α-mouse mAb (1/300) (Jackson). The cell nucleus was stained with DAPI at a concentration of 50 ng/mL. Images were analyzed using a LEICA laser-based confocal microscope, and data were collected on an Applied Vision System, and processed by deconvolution.

Gradient analyses

Polysome profiles were analyzed as previously described (Billy et al., 2000). To analyze the distribution of Rcl1p and Bms1p-HA, 100 OD_{600} units of yeast cells were lyzed with glass beads by vigorous vortexing 6 $\times$ 30s in a buffer containing 20 mM Tris-HCl, pH 8, 5 mM $MgCl_2$, 150 mM NaCl, 0.2% Triton X-100, 1 mM DTT, and the Complete Protease Inhibitor cocktail (Roche Molecular Biochemicals). The extract was clarified by centrifugation at 10,000 $\times$ *g* for 5 min and layered on a 10–30% glycerol gradient prepared in the lysis buffer (with the concentration of Triton X-100 reduced to 0.1% and protease inhibitors omitted). Gradients were centrifuged at 36k rpm for 315 min (short run) or 24 h (long run) in a Beckman SW41Ti rotor at 4 °C.

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