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Late Cytoplasmic Maturation of the Small Ribosomal Subunit Requires RIO Proteins in *Saccharomyces cerevisiae*

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Numerous nonribosomal *trans*-acting factors involved in pre-rRNA processing have been characterized, but few of them are specifically required for the last cytoplasmic steps of 18S rRNA maturation. We have recently demonstrated that Rrp10p/Rio1p is such a factor. By BLAST analysis, we identified the product of a previously uncharacterized essential gene, *YNL207W*/*RIO2*, called Rio2p, that shares 43% sequence similarity with Rrp10p/Rio1p. Rio2p homologues were identified throughout the Archaea and metazoan species. We show that Rio2p is a cytoplasmic-nuclear protein and that its depletion blocks 18S rRNA production, leading to 20S pre-rRNA accumulation. In situ hybridization reveals that in Rio2p-depleted cells, 20S pre-rRNA localizes in the cytoplasm, demonstrating that its accumulation is not due to an export defect. We also show that both Rio1p and Rio2p accumulate in the nucleus of *crm1-1* cells at the nonpermissive temperature. Nuclear as well as cytoplasmic Rio2p and Rio1p cosediment with pre-40S particles. These results strongly suggest that Rio2p and Rrp10p/Rio1p are shuttling proteins which associate with pre-40S particles in the nucleus and they are not necessary for export of the pre-40S complexes but are absolutely required for the cytoplasmic maturation of 20S pre-rRNA at site D, leading to mature 40S ribosomal subunits.

Seen from a home economics point of view, making ribosomes is the main task of a living cell (36, 37). Synthesis of the ribosome constituents, their processing and assembly into mature particles, and the regulation of the entire process require the participation of numerous factors. Surprisingly, until relatively recently, few players involved in these processes had been identified. However, in the past decade, by means of genetic and biochemical tricks, and thanks to the tractability of the yeast *Saccharomyces cerevisiae* and, more recently, with in silico searches, dozens of factors (snoRNAs or protein factors) implicated in ribosome biogenesis have been characterized. For most of them, however, their molecular function in this process is not known.

During the past year, systematic analyses of protein complexes in *S. cerevisiae* by tandem affinity chromatography purification (3, 7, 11, 14, 16) as well as proteomic analysis of the nucleolus of human cells (1) and definition of putative modular transcriptional networks by computer assisted analysis of the transcriptional expression patterns (18, 20) led to a burst of new putative players. Through these approaches, new factors associated with the pre-60S particle, precursor of the large ribosomal subunit (LSU) and thus putatively involved in the maturation-assembly pathway of the LSU, have been identified. Likewise, factors associated with the early processing complexes containing the large 35S pre-rRNA have been characterized (11) and shown to include mostly proteins specifically required for small ribosomal subunit (SSU) production. In contrast, the nonribosomal proteins found in late nucleoprotein complex precursors of the SSU have not been identified yet by these methods. Nevertheless, criss-cross analysis of the different complexes described before (7, 16) allows us to infer a possible role in the processing-assembly pathway of the SSU for a few proteins (http://www.pre-ribosome.de; 27). In *S. cerevisiae*, the final maturation of the 20S pre-rRNA leading to the mature 18S rRNA of the SSU occurs in the cytoplasm, and few nonribosomal factors specifically involved in this event are known.

We recently reported that Rrp10p/Rio1p is absolutely required for this processing step (35). This protein is the archetype-founder of a family of proteins found in all sequenced archaeal and eukaryotic organisms (see below) (2, 35), which possess a so-called RIO domain overlapping protein kinase motifs. It has recently been demonstrated that Rio1p exhibits protein serine kinase activity in vitro and that mutations abolishing this kinase activity also abrogate Rio1p function in vivo (2).

In a BLAST search through the *S. cerevisiae* genome, we identified another open reading frame encoding a RIO protein, *YNL207W*. Considering our previous demonstration of the involvement of Rio1p in the late processing of the SSU, we investigated whether the product of *YNL207W* (referred to below as Rio2p) is also implicated in the processing of the 20S pre-rRNA. We show in this report that this is indeed the case: depletion of Rio2p prevents 20S pre-rRNA from being processed to 18S rRNA. Furthermore, we show that Rio1p and Rio2p cosediment with pre-40S particles and shuttle from the nucleus to the cytoplasm. Thus, both RIO proteins encoded in the *S. cerevisiae* genome are required for the processing of the 20S pre-rRNA into mature 18S rRNA.

MATERIALS AND METHODS

Yeast methods. Yeast medium used are standard YP supplemented with 2% glucose (YPD) or 2% galactose plus 2% sucrose (YPG) and YNB supplemented with amino acids and bases as required. Fluoroorotate-resistant clones were

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TABLE 1. *S. cerevisiae* strains used in this study

Strain	Genotype	Reference or source
YO296	*MAT*α *leu2-*Δ *trp1 his3-*Δ*200 gal2 gal-*Δ*108 HIS3*::*GAL*::*PROTA-RRP10*	35
YO396	*MAT***a**/*MAT*α *his3*Δ*1*/*his3*Δ*1 leu2*Δ*0*/*leu2*Δ *met15*Δ*0*/*MET15 lys2*Δ*0*/*LYS2 ura3*Δ*0*/*ura3*Δ*0 rio2*Δ::*KAN*/*RIO2*	Euroscarf strain Y22005
YO419	*MAT*α *trpI-1 his3-11 ura3-1 ade2-1 leu2-3 can1-100 crm1*Δ::*LEU2*/pKW457 (*HIS3 crm1-1*)	31
YO420	*MAT*α *trp1-1 his3-11 ura3-1 ade2 -1 leu2-3 can1-100 crm1*Δ::*LEU2*/pKW457 (*HIS3 crm1-1*)/ pNOPGFPA1A (*pNOP*::*GFP ADE2*)	This study, derived from YO419
YO470	*MAT***a** *his3*Δ*1 leu2*Δ*0 met15*Δ*0 lys2*Δ*0 ura3*Δ*0 rio2*Δ::*KAN*/pEV80 (*pGAL*::*RIO2-PROTA URA3*)	This study, derived from Euroscarf strain Y22005
YO471	*MAT*α *his3*Δ*1 leu2*Δ*0 met15*Δ*0 lys2*Δ*0 ura3*Δ*0 rio2*Δ::*KAN*/pEV80 (*pGAL*::*RIO2-PROTA URA3*)	This study, derived from Euroscarf strain Y22005
YO511	*MAT***a** *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0*	This study, derived from Euroscarf strain Y22005
YO558	*MAT*α *trp1-1 his3-11 ura3-1 ade3-1 leu2-3 can1-100 crm1*Δ::*LEU2*/pKW457 (*HIS3 crm1-1*)/pEV90 (*pNOP*::*GFP*::*RIO1*/*RRP10 ADE2*)	This study, derived from YO419
YO560	*MAT*α *trp1-1 his3-11 ura3-1 ade2-1 leu2-3 can1-100 crm1*Δ::*LEU2*/pKW457 (*HIS3 crm1-1*)/pEV71 (*pNOP*::*GFP*::*RIO2 ADE2*)	This study, derived from YO419
GFP-RIO1	*MAT*α *leu2-*Δ *trp1 his3-*Δ*200 gal2 gal-*Δ*108 HIS3*::*GAL*::*PROTA-RRP10*/pEV24 (*LEU2 pNOP*::*GFP*::*RIO1*)	35
GFP-RRP8	*MAT***a** *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0*/pJPG310 (2μm *LEU2 GFP*::*RRP8*)	This study, derived from YO511
YO566	*MAT*α *his3*Δ*1 leu2*Δ*0 met15*Δ*0 lys2*Δ*0 ura3*Δ*0 rio2*Δ::*KAN*/pEV71 (*pNOP*::*GFP*::*RIO2 ADE2*)	This study, derived from YO471

selected on supplemented YNB proline medium containing 0.6 g of 5-fluoroorotic acid per liter (26). *S. cerevisiae* cells were transformed as previously described (9). *Escherichia coli* DH5α was used for molecular cloning and propagation of plasmids. For Rio2p depletion, cells growing in YPG medium at 30°C were harvested in early exponential growth phase (optical density, 0.4 to 0.7) by centrifugation, washed, and resuspended in warm YPD or YPG medium. During growth, cells were periodically diluted with warm medium in order to be kept in exponential growth phase.

Plasmids. Plasmids pJPG310 (2μm *LEU2 GFP*::*RRP8*) and pEV24 (*CEN ARS LEU2 GFP*::*RIO1*) used in this study were described previously (4, 35). New plasmids used in this work were constructed as follows. The *RIO2* open reading frame from wild-type genomic DNA was PCR amplified (*Pfu* polymerase, Promega) with the following oligonucleotides introducing an *Nco*I restriction site (YNL207W-I-*Nco*I and YNL207W-II-*Nco*I) or oligonucleotides introducing a *Bgl*II restriction site (YNL207W-III-*Bgl*II and YNL207W-IV-*Bgl*II) (see Table 2). The 1.3-kb PCR product amplified with oligonucleotides YNL207W-I-*Nco*I and YNL207W-II-*Nco*I was digested with *Nco*I and cloned into *Nco*I-restricted vector plasmid pNOPPATA1L (*CEN ARS LEU2*) obtained from K. Hellmuth and E. Hurt, giving plasmid pEV69 (*CEN ARS LEU2 RIO2*::*PROTA*).

The PCR product amplified with YNL207W-III-*Bgl*II and YNL207W-IV-*Bgl*II was digested with *Bgl*II and cloned into *Bam*HI-restricted vector plasmid pHA114 (2μm *URA3*) (15), leading to plasmid pEV80 (2μm *URA3 GAL*::*RIO2*::*PROTA*). This *GAL*::*RIO2*::*PROTA* allele is thereafter referred to as *GAL*::*RIO2*. Plasmids directing the production of green fluorescent protein (GFP)-Rio2p or GFP-Rio1p fusion proteins were constructed as follows. The TEV-RIO2 *Pst*I fragment from pEV69 and the TEV-RRP10/RIO1 *Pst*I fragment from pEV24 (35) were inserted into the *Pst*I site of plasmid pNOPGFPA1L, obtained from K. Hellmuth and E. Hurt, giving plasmids pEV71 (*CEN ARS ADE, NOP*::*GFP*::*RIO2*) and pEV90 (*CEN ARS ADE2 NOP*::*GFP*::*RIO1*), respectively.

S. cerevisiae* strains.** Strains used in this work are listed in Table 1. A strain expressing a conditional allele of *RIO2* was constructed as follows. A diploid strain heterozygous for a disrupted copy of *RIO2*, obtained from Euroscarf (strain Y22005), was transformed with pEV80, and selected transformants were sporulated. Two meiotic segregants which were kanamycin resistant and 5-fluoroorotic acid sensitive and unable to grow in glucose-containing medium, YO470 (*MATa**) and YO471 (*MAT*α), were chosen for further functional analyses.

RNA methods. RNA isolation and Northern hybridizations were carried out as previously described (15, 32). The oligonucleotide probes used for Northern hybridizations (numbered 1 to 7 in Fig. 3A) are described in Table 2. Metabolic labeling of RNA was performed as described previously (33). Strain YO471 was grown at 30°C in YPG medium, shifted to YPD medium as described above, grown in this medium for 12 h, and then grown for 7 additional hours in YNB Glu medium without uracil (*GAL*::*RIO2*, Glu). The *RIO2*$^+$ strain (YO511) was grown at 30°C in YNB Glu medium without uracil. Labeling was initiated by addition of 150 μCi of [5,6-^{3}H]uracil to 5-ml samples of these cultures. After 4 min of labeling, 0.6 ml of a 2-mg/ml uracil solution were added. Then 1-ml samples were collected at 1, 3, 5, 10, and 20 min following chase and processed as previously described (4).

Fluorescence in situ hybridization. The 5′ part of the ITS1 was detected by fluorescence in situ hybridization with an oligonucleotide probe, as described previously (10).

Glycerol gradients. *S. cerevisiae* extracts were prepared as previously described (4) except for the method of grinding. From 5 to 20 g of *S. cerevisiae* cells were ground in an original Waring blender (Osterizer) in the presence of dry ice nuggets. Sedimentation profiles on 10% to 30% glycerol gradients were obtained as described previously (4). A total of 19 fractions (0.6 ml) were collected manually from the top of the gradients.

Immunoprecipitations. Immunoprecipitations and analysis of immunoprecipitated RNAs were done as previously described (35).

Western blotting. Proteins from total extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Fractions from glycerol gradients were trichloroacetic acid precipitated, and their protein content was analyzed by SDS-PAGE and Western blotting. Samples were transferred onto nitrocellulose membranes (Hybond C-super; Amersham/Pharmacia). Protein A was detected with the rabbit horseradish peroxidase-conjugated anti-protein A antibody (PAP; Dako SA) diluted to 1:5,000, GFP was detected with a mouse monoclonal anti-GFP antibody (Roche Mannheim) diluted to 1:1,000, ribosomal proteins rpL3, rpL30, and rpS2 were detected with a mouse monoclonal anti-rpL3 antibody diluted to 1:5,000 and a rabbit polyclonal anti-rpL30 antibody cross-reacting with rpS2 (kindly provided by J. Warner) diluted

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence
YNL207W-I-*Nco*I	5′-CCCCCCGCCATGGTGAAATTGGATAC TTCTCAT-3′
YNL207W-II-*Nco*I	5′-CCCCCCCCCATGGCAGTCGTCTAAACT AAACTA-3′
YNL207W-III-*Bgl*III	5′-CCCCCCAGATCTACTAGAAGAAATTG GATACTT-3′
YNL207W-IV-*Bgl*III	5′-CCCCCCAGATCTCTAGTATATAGTTTC CTAGTTTATCC-3′
Probe 1 (18S)	5′-CATGGCTTAATCTTTGAGAC-3′
Probe 2 (ITS1, D-A2)	5′-TTAAGCGCAGGCCCGGCTGG-3′
Probe 3 (ITS1, A2-A3)	5′-GATTGCTCGAATGCCCAAAG
Probe 4 (5.8S)	5′-TGCGTTCAAAGATTCGATG-3′
Probe 5 (ITS2, A3-C2)	5′-GGCCAGCAATTTCAAGTTA-3′
Probe 6 (25S)	5′-CTCACGACGGTCTAAACCC-3′
Probe 7 (ETS1, A0-A1)	5′-CACCCATTCCCTCTTGCTAG-3′

to 1:2,000, followed by enhanced chemiluminescence detection (Amersham/Pharmacia).

RESULTS

RIO protein family. BLAST (40) searches through the *S. cerevisiae* proteome with Rrp10p/Rio1p as the starting point disclosed that a previously uncharacterized essential gene, *YNL207W*, encodes a product which shows 19% sequence identity and 43% sequence similarity over its length with Rrp10p/Rio1p. *YNL207W* has thus been renamed *RIO2* and is referred to as such below. Rrp10p/Rio1p and Rio2p proteins show a central well-conserved core of approximately 200 amino acids referenced as the RIO domain in the protein domains database SMART (24) (Fig. 1A). Rio1p and Rio2p homologues were identified throughout archaeal and metazoan species.

The sequences of the two yeast proteins and their homologues were aligned and displayed as a phylogenetic tree (Fig. 1B). Based on phylogenetic distances, the proteins cluster in two distinct branches, the RIO1 protein family and the RIO2 family. Together these proteins constitute a larger superfamily, named the RIO family, containing a central RIO domain. The Rio1 proteins are duplicated in higher eukaryotes. Only one of the two copies contains a lysine-rich region at its C terminus, as does the Rrp10p/Rio1p yeast protein. Archaeal Rio1 proteins are smaller and restricted to the RIO domain. A distinguishing feature of the RIO2 protein family is the presence, upstream of the RIO domain, of a conserved 80-amino-acid N-terminal domain (referred as Pfam-B_3091 in the Pfam database) that has no equivalent among other members of the RIO family. A glutamic acid-rich region found at the C terminus (positions 362 to 405 in *S. cerevisiae* Rio2p) is specific to eukaryotic Rio2 proteins.

Rio2p is required for 18S rRNA production. We recently showed that Rrp10p/Rio1p is an essential cytoplasmic protein required for the processing of 20S pre-rRNA to 18S rRNA (35). Considering the homology between the two proteins, we tested the possible involvement of Rio2p in ribosome biogenesis. Since *YNL207W*/*RIO2* has been shown to be an essential gene (38), a conditional *RIO2* mutant allele was constructed (see Materials and Methods). This construct allows expression of the *RIO2* open reading frame, tagged at the 3′ end with a sequence encoding two protein A epitopes, under the inducible *GAL10* promoter (Fig. 2A). Transcription driven by the *GAL10* promoter leads to high expression of the gene in culture medium that contain galactose (YPG) but is repressed in culture medium containing glucose (YPD).

In order to deplete Rio2p, cells of strain YO471 (*rio2*Δ::*KAN*/*pGAL*::*RIO2*) were shifted from galactose medium (YPG) to glucose medium (YPD). This results in a severe reduction of the growth rate of the YO471 strain after 15 h of culture in the presence of glucose (Fig. 2B). An immunoblot, revealing the protein A tag, confirmed that the amount of Rio2p rapidly decreases and becomes barely detectable after 24 h of growth of strain YO471 in YPD (Fig. 2C).

We made use of this conditional *RIO2* allele to determine whether Rio2p is necessary for pre-rRNA processing. As shown in Fig. 3B, depletion of Rio2p (15 h in YPD medium) has no effect on the steady-state level of 25S rRNA but drastically affects 18S rRNA accumulation (Fig. 3B and 3C). To determine which step(s) of 18S rRNA processing is affected, total RNAs extracted from Rio2p-depleted cells were hybridized to specific oligonucleotide probes (Table 2, Fig. 3A). Hybridization to probe 2, complementary to the internal transcribed spacer 1 (ITS1 D-A2), discloses a strong increase of 20S pre-rRNA (Fig. 3C), concomitantly with 18S rRNA reduction. This result clearly shows that, in Rio2p-depleted cells, cleavage at site D is strongly inhibited, as previously observed in Rrp10p/Rio1p-depleted cells (35). Further effects of Rio2p depletion on pre-rRNA processing are a reduction in the level of 27SA2 pre-rRNA (probe 3 complementary to ITS1 A2-A3) and a very slight increase in the level of an aberrant intermediate of approximately 22S/23S detected with probes 2, 3, and 7 (not shown), indicating that this intermediate contains sequences extending from site A0 (or further upstream) to site A3 and results from cleavage of pre-rRNA molecules at site A3 in the absence of cleavage at site A2. The levels of 27SBS and 27SBL are not affected, as revealed by hybridization with probe 5, complementary to the internal transcribed spacer 2 (ITS2 A3-C2) (results not shown).

The kinetics of pre-rRNA processing and rRNA accumulation were analyzed in [^{3}H]uracil pulse-chase labeling experiments (Fig. 4). In a *GAL*::*RIO2* strain grown for 15 h in glucose-containing medium, 20S pre-rRNA is still present after 20 min of chase, while 18S rRNA is only faintly detectable at this time. In contrast, Rio2p depletion does not affect the time course of 27S pre-rRNA conversion to 25S rRNA.

Altogether, these data show that depletion of Rio2p strongly (and specifically) affects processing of the 20S pre-rRNA to 18S rRNA. Whether the minor alterations in A1 and A2 cleavages observed reflect the involvement of Rio2p in early processing events or are indirect consequences of this drastic defect in 20S pre-rRNA maturation is not clear.

Cells depleted of Rio2p accumulate 20S pre-rRNA in the cytoplasm. In order to visualize the intracellular fate of the 20S pre-rRNA seen to accumulate in the pulse-chase experiments and in Northern hybridizations, fluorescence in situ hybridization with a probe complementary to the 5′ part of the ITS1 (between sites D and A2) was performed on cells depleted of Rio2p (YO470 or YO471, *GAL*::*RIO2*, cells grown for 15 h in glucose). As shown in Fig. 5F, a strong ITS1 signal (red) was observed in the cytoplasm compared to the nondepleted cells or to wild-type cells (Fig. 5E and 5A). A comparable result was obtained with cells depleted of Rio1p (Fig. 5C and 5D), as previously shown by electron microscopy (35). Under similar hybridization conditions, ITS1 is detected in large amounts in the nucleus of cells bearing the thermosensitive allele *crm1-1* (Fig. 5B), consistent with published results showing the requirement for the exportin Crm1p in the nuclear export of the pre-40S particles (28). Thus, although necessary for the last processing step of 18S rRNA synthesis, Rio2p is not required for the nuclear export of the SSU precursor particles.

Rio2p is a nuclear and cytoplasmic protein cosedimenting with pre-40S particles. Subcellular localization of Rio2p was investigated with a GFP-Rio2p fusion protein. The GFP-Rio2p fusion was fully functional, as demonstrated by its ability to complement the lethality of the *RIO2* invalidation in strain YO566. In this strain expressing a *GFP*::*RIO2* fusion allele, the green fluorescence is detected in the cytoplasm and the nu-

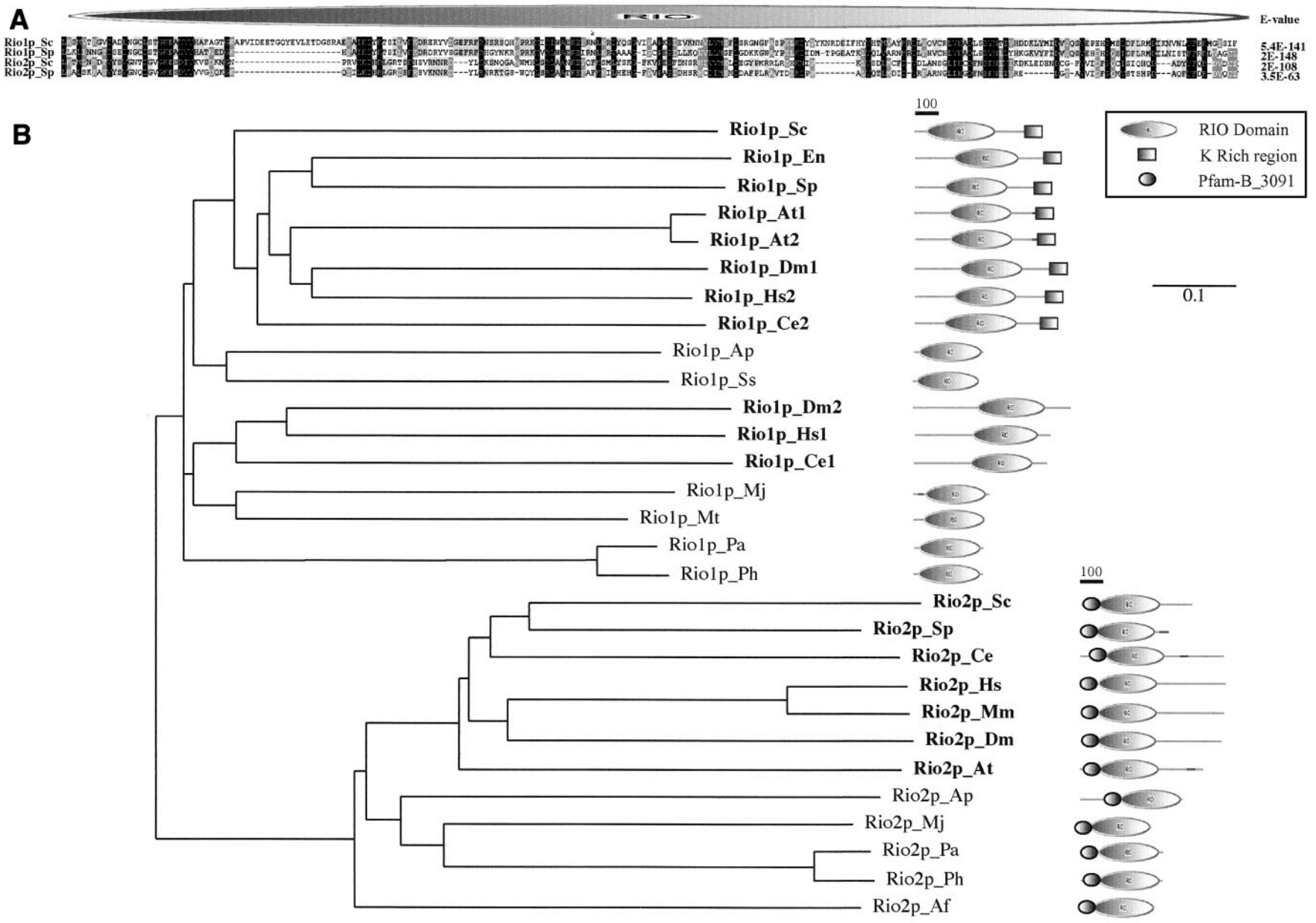

A
RIO
E-value
Rio1p_Sc
Rio1p_Sp
Rio2p_Sc
Rio2p_Sp
5.4E-141
2E-148
2E-108
3.5E-63
B
100
RIO Domain
K Rich region
Pfam-B_3091
0.1
Rio1p_Sc
Rio1p_En
Rio1p_Sp
Rio1p_At1
Rio1p_At2
Rio1p_Dm1
Rio1p_Hs2
Rio1p_Ce2
Rio1p_Ap
Rio1p_Ss
Rio1p_Dm2
Rio1p_Hs1
Rio1p_Ce1
Rio1p_Mj
Rio1p_Mt
Rio1p_Pa
Rio1p_Ph
100
Rio2p_Sc
Rio2p_Sp
Rio2p_Ce
Rio2p_Hs
Rio2p_Mm
Rio2p_Dm
Rio2p_At
Rio2p_Ap
Rio2p_Mj
Rio2p_Pa
Rio2p_Ph
Rio2p_Af

FIG. 1. Rio2p is a member of RIO family and is conserved from archaebacteria to humans. (A) Sequence comparison of the RIO domains of the *S. cerevisiae* proteins. The E-values of these domains in comparison with the consensus defined in the SMART database are indicated. Black shading highlights amino acids that are identical or similar in all sequences, and dark grey and light grey shading highlights those that are identical or similar in some sequences, respectively. (B) Phylogenetic tree and domain profile of all RIO family members. The phylogenetic tree was established with the ClustalW program and drawn with the Phylodendron program. The RIO domain (SMART) is represented by an oval, and Pfam-B_3091 domain by a circle, and lysine-rich regions by a squared box. Eukaryotes are noted in bold. Accession numbers of members of the RIO1/RRP10 family are: Rio1p_Sc, CAA65511; Rio1p_En, AAC26079; Rio1p_Sp, CAA15723; Rio1p_Ce1, P34649; Rio1p_Ce2, AAC17564; Rio1p_Dm1, AAF50965; Rio1p_Dm2, AAF50033; Rio1p_Ap, BAA79728; Rio1p_Mj, Q57886; Rio1p_Mt, AAB85501; Rio1p_Pa, CAB49514; Rio1p_Ph, BAA30679; Rio1p_At1, Q9FHT0; Rio1p_At2, Q9SK34; Rio1p_Hs1, O14730; Rio1p_Ss, CAC24438; and Rio1p_Hs2, Q9H2L9. Accession numbers of members of the RIO2 family are: Rio2p_Sc, NP_014192; Rio2p_Hs, FLJ11159; Rio2p_Ce; CAB60851.1; Rio2p_Sp, CAB66449.1; Rio2p_Dm, CG11859; Rio2p_At, T45758; Rio2p_Mm, NP_080210; Rio2p_Ap, E72539; Rio2p_Mj, H64433; Rio2p_Pa, B75068; Rio2p_Ph, C71164; and Rio2p_Aj, NP_071248. Species abbreviations: Sc, *Saccharomyces cerevisiae*; En, *Emericella nidulans*; Sp, *Schizosaccharomyces pombe*; At, *Arabidopsis thaliana*; Hs, *Homo sapiens*; Ms, *Mus musculus*; Ce, *Caenorhabditis elegans*; Dm, *Drosophila melanogaster*; Ap, *Aeropyrum pernix*; Mj, *Methanococcus jannaschii*; Mt, *Methanobacterium thermoautotrophicum*; Pa, *Pyrococcus abyssi*; Ph, *Pyrococcus horikoshii*; Ss, *Sulfolobus solfataricus*; Af, *Archaeoglobus fulgidus*. Bold bars on the right side of the figure, 100 amino acids; the bar at the upper right side measures the divergence index.

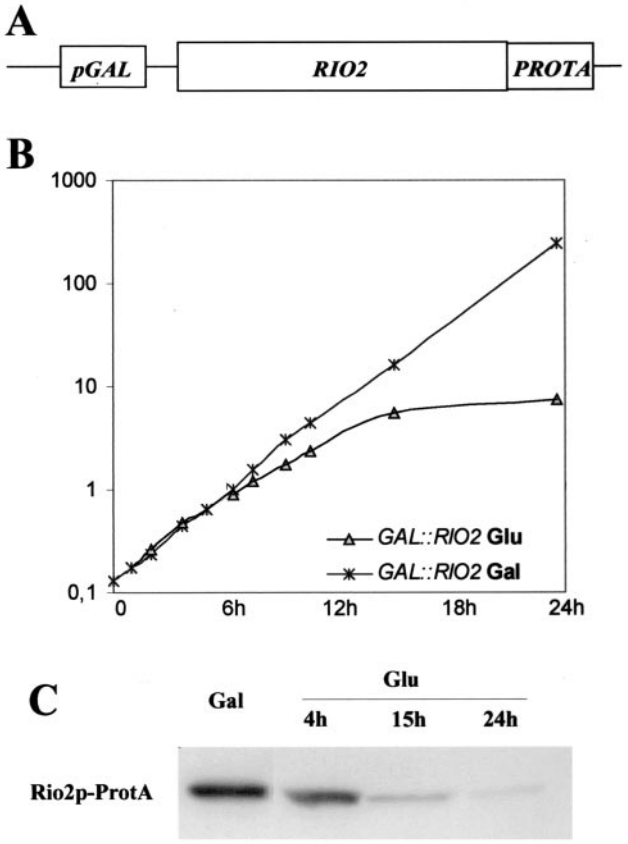
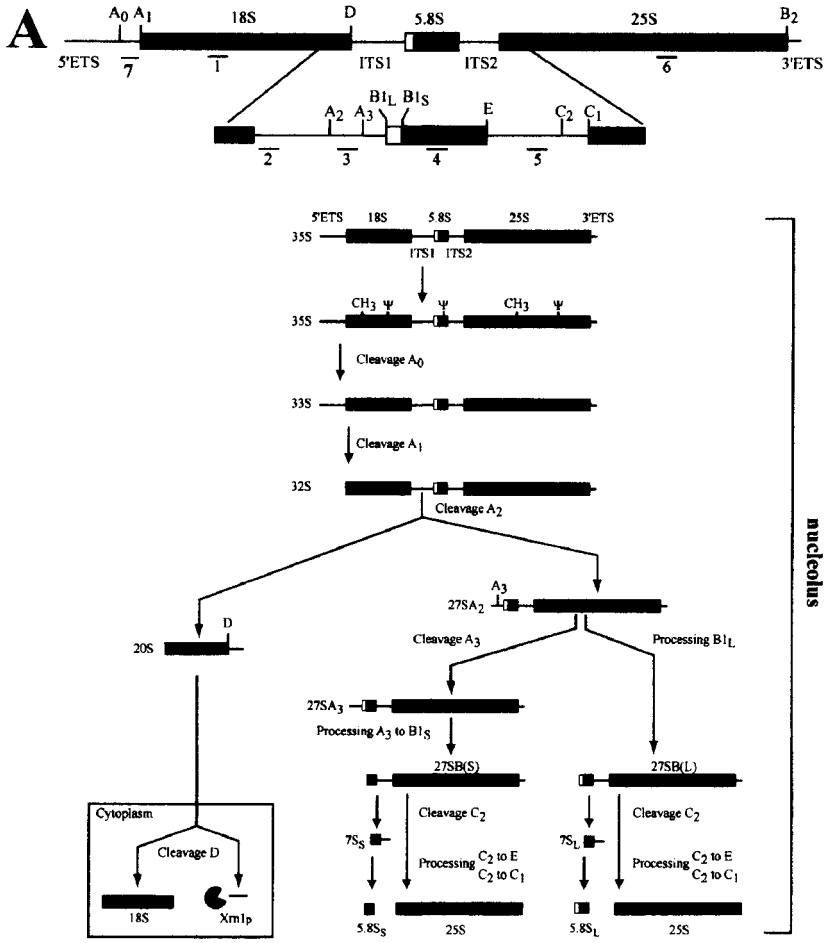


FIG. 2. *RIO2* gene expression under the control of the inducible *GAL10* promoter. (A) Schematic representation of the *GAL*::*RIO2*::*PROTA* allele. (B) Growth of strain YO471 carrying the *GAL*::*RIO2*::*PROTA* allele at 30°C. Cells from exponentially growing cultures in YPG medium (Gal) were harvested, washed, and resuspended either in YPD medium (Glu, triangles) or in galactose medium (Gal, crosses), and incubation was pursued for up to 24 h at 30°C. Cell density was measured at regular intervals, and the cultures were periodically diluted to keep them in the exponential phase of growth. The generation time in YPG is similar to that of an isogenic *RIO2*$^+$ strain. (C) Depletion of Rio2p during growth in YPD medium. Cell extracts of strain YO471 carrying the *GAL*::*RIO2*::*PROTA* allele were prepared from samples harvested at the indicated time points. Rio2p concentration was determined by Western blotting. Equal amounts of total proteins were loaded in every lane, as estimated by Coomassie staining of the gels.

cleus (Fig. 6A). In a strain expressing a *GFP*::*RIO1* allele, the green fluorescence is detected only in the cytoplasm (Fig. 6A), as previously shown (35), and in a strain expressing GFP-Rrp8p, the green fluorescence is detected in the nucleolus (Fig. 6A).

To find out whether Rio2p is associated with preribosomal particles, whole-cell lysates prepared from strains expressing protein A-tagged Rio2p or Rio1p were analyzed by glycerol gradient centrifugation. Presence of Rio2p-protein A, protein A-Rio1p, and ribosomal proteins rpS2 and rpL3 in the different fractions of the glycerol gradients was determined by immunoblotting (Fig. 6B). Rio2p-protein A and protein A-Rio1p peak in fractions of the glycerol gradients in which the 40S ribosomal subunit sediments. A pool of Rio2p-protein A is also found in the upper part of the gradients, which contains soluble proteins.



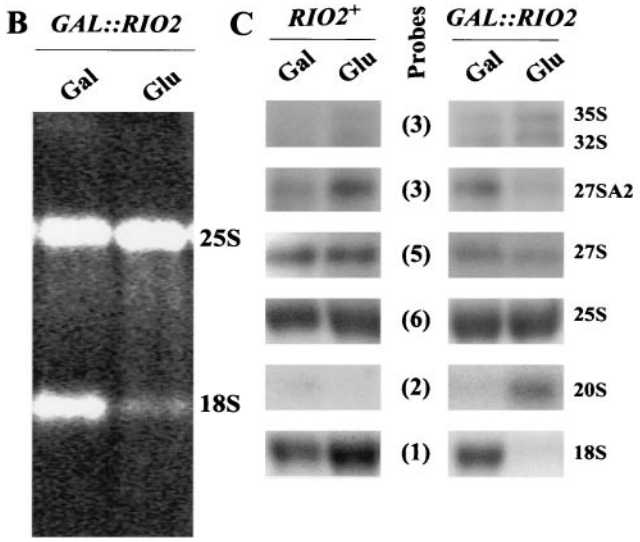


FIG. 3. (A) Pre-rRNA processing in *S. cerevisiae*. In the 35S pre-rRNA, the primary transcript, the sequences of the mature 18S, 5.8S, and 25S rRNAs are flanked by the external transcribed spacers (5′ and 3′ ETS) and separated by the internal transcribed spacers (ITS1 and ITS2). Cleavage sites are indicated by uppercase letters A to E, and oligonucleotide probes used in Northern blot hybridizations are indicated by the numbers 1 to 7. Pre-rRNA processing pathway: sequential cleavages of the 35S pre-rRNA at sites A_0 and A_1 generate the 33S and 32S pre-rRNAs. Cleavage of the 32S pre-rRNA at site A_2 in ITS1 yields the $27SA_2$ and 20S pre-rRNAs, which are precursors to the RNA components of the large and small ribosomal subunits, respectively. The $27SA_2$ precursor is either processed at site A_3 by RNase MRP (major pathway) and then to site B1s by the 5′-3′ exonucleases Rat1p and Xrn1p, or processed at site B_{1L} (minor pathway), yielding, respectively, the $27SB_S$ and $27SB_L$ pre-rRNAs. These two intermediates follow the same processing pathway to 25S and $5.8S_{S/L}$ through cleavage at site C_2 in ITS2, followed by 3′-5′ exonucleolytic digestion of $7S_S$ and $7S_L$ from site C_2 to E by the exosome complex, and 5′-3′ exonucleolytic digestion to the 5′ end of the 25S rRNA by the exonuclease Rat1p. The final maturation of the 20S pre-rRNA by an endonucleolytic cleavage at site D, occurs after pre-SSU export to the cytoplasm, and produces the mature 18S rRNA and a fragment D-A_2 (5′ ITS1). The D-A_2 fragment is then degraded by the 5′-3′ exonuclease Xrn1p. (B and C) Depletion of Rio2p specifically affects the steady-state level of mature 18S rRNA and results in 20S pre-rRNA accumulation. YO471 (*GAL*::*RIO2*) cells were grown in YPG medium (Gal) or in YPD medium (Glu) for 15 h. Total RNA was extracted and separated in 1% agarose–formaldehyde gels to analyze 35S, 32S, $27SA_2$, 25S, 20S, and 18S species. Equal amounts of total RNA (5 μg) were loaded in every lane and analyzed. (B) Ethidium bromide staining of the gel. (C) Northern blot analyses of pre-rRNA processing. After transfer to nylon membranes of electrophoresed material, the membranes were hybridized with different probes, as indicated.

Detection of Rio2p-protein A around the position of 40S particles suggests that Rio2p-protein A might be associated, at least transiently, with the 43S particle, precursor of the 40S ribosomal subunit. In order to assess such a physical association of Rio2p with pre-SSU particles, we determined if pre-

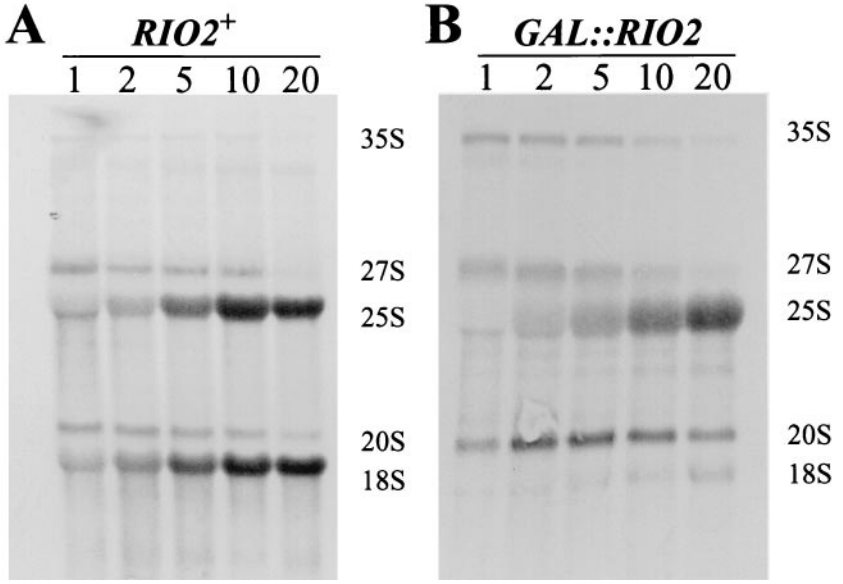


FIG. 4. Depletion of Rio2p-protein A results in reduced synthesis of 18S rRNA. (A) Strain YO511 (*RIO2*$^+$) was grown at 30°C in YNB Glu without uracil. (B) Strain YO471 (*GAL*::*RIO2*) was grown at 30°C in YPG medium (Gal), then shifted for 12 h to YPD medium (Glu), and finally grown for 7 h in YNB Glu without uracil. Cells were labeled for 4 min with [5,6-^{3}H]uracil and chased with a large excess of unlabeled uracil for 1 to 20 min.

rRNA coimmunoprecipitates with Rio2p. Whole-cell lysates from YO470 (*RIO2-PROTA*) and YO511 (*RIO2*$^+$) were incubated with immunoglobulin G-Sepharose beads, and the RNA molecules retained onto the immunoglobulin G beads were extracted, separated in an agarose gel, and probed with 18S, 25S, and ITS1(D-A2) probes. As shown in Fig. 6C, RNA coimmunoprecipitated with Rio2-protein A is selectively enriched for 20S pre-rRNA. No larger pre-rRNA processing intermediate was detected in these experiments (not shown), indicating that Rio2p is not associated with early pre-rRNA processing complexes.

Since both *S. cerevisiae* RIO proteins associate with 20S-containing pre-SSU and Rio2p has been found in complexes containing Dim1p (7), the protein required for dimethylation of the 18S rRNA adenosine residues A_{1779} and A_{1780} (numbering of *S. cerevisiae* mature 18S rRNA) (22), a late event in 20S pre-rRNA processing (5), we determined if the *S. cerevisiae* RIO proteins are required for this modification step. Primer extensions experiments with oligonucleotides complementary to ITS1 sequences as primers and total RNA extracted from either wild-type or Rio1p- or Rio2p-depleted cells as templates all revealed the same extension products characteristic of the presence of dimethyladenosine residues at positions A_{1779} and A_{1780} of the mature 18S rRNA (not shown). This indicates that this modification of the 20S pre-rRNA can still take place in the absence of either one of these proteins.

Since Rio2p-protein A and protein A-Rio1p are found in the same fractions and 20S pre-rRNA selectively copurifies with Rio1-protein A (35), we attempted to immunoprecipitate GFP-Rio1p with Rio2p-protein A. In strain YO470 transformed with plasmid pEV90 (*pNOP*::*GFP*::*RIO1 ADE2*), expression of the Rio2p-protein A fusion protein constitutes the unique source of Rio2p, while both GFP-Rio1p fusion protein and the endogenous Rrp10p/Rio1p protein are expressed. Under these conditions, Western blotting with a GFP antibody did not reveal any Rrp10p/Rio1p copurifying with Rio2p-protein A on immunoglobulin G-Sepharose.

Rio2p and Rio1p shuttle between the nucleus and the cytoplasm. Detection of Rio2p in the nucleus and the cytoplasm suggests that Rio2p could be a shuttling protein. To test this hypothesis, the distribution of GFP-Rio2p was analyzed in cells carrying the *crm1-1* mutation, which is known to affect the nucleocytoplasmic exchanges. Crm1p belongs to the karyopherin superfamily and is involved in the nuclear export of a large range of proteins. In addition, deficiency in Crm1p function results in the accumulation of pre-40S particles in the nucleus (28). In the *crm1-1* mutant cells grown at the nonpermissive temperature, a significant nuclear-nucleolar accumulation of GFP-Rio2p is observed (Fig. 7A). The same experiment performed with cells expressing GFP-Rio1p shows that it also accumulates in the nucleus (Fig. 7B), while in the same conditions GFP alone does not (Fig. 7C). These results clearly show that Rio1p and Rio2p are shuttling proteins.

To find out whether, in these conditions, Rio2p and Rrp10p/Rio1p are still associated with preribosomal particles, whole-cell lysates derived from *crm1-1* cells expressing GFP-tagged Rio2p or Rio1p and grown at the nonpermissive temperature were analyzed on glycerol gradients. The presence of GFP-Rio2p, GFP-Rio1p, and ribosomal proteins rpS2 and rpL3 in the different fractions of the glycerol gradients was examined by immunoblotting (Fig. 7D). GFP-Rio2p and GFP-Rio1p peak in fractions of the glycerol gradients in which the rpS2 protein sediments without rpL3. Accumulation of pre-40S particle is revealed by the larger amount of the rpS2 protein detected in these fractions (compare detection of rpS2 in Fig. 6B and Fig. 7D). These results indicate that when export of the SSU precursors is inhibited, both RIO proteins stay in the nucleus. Together, the nuclear localization of GFP-Rio1p and

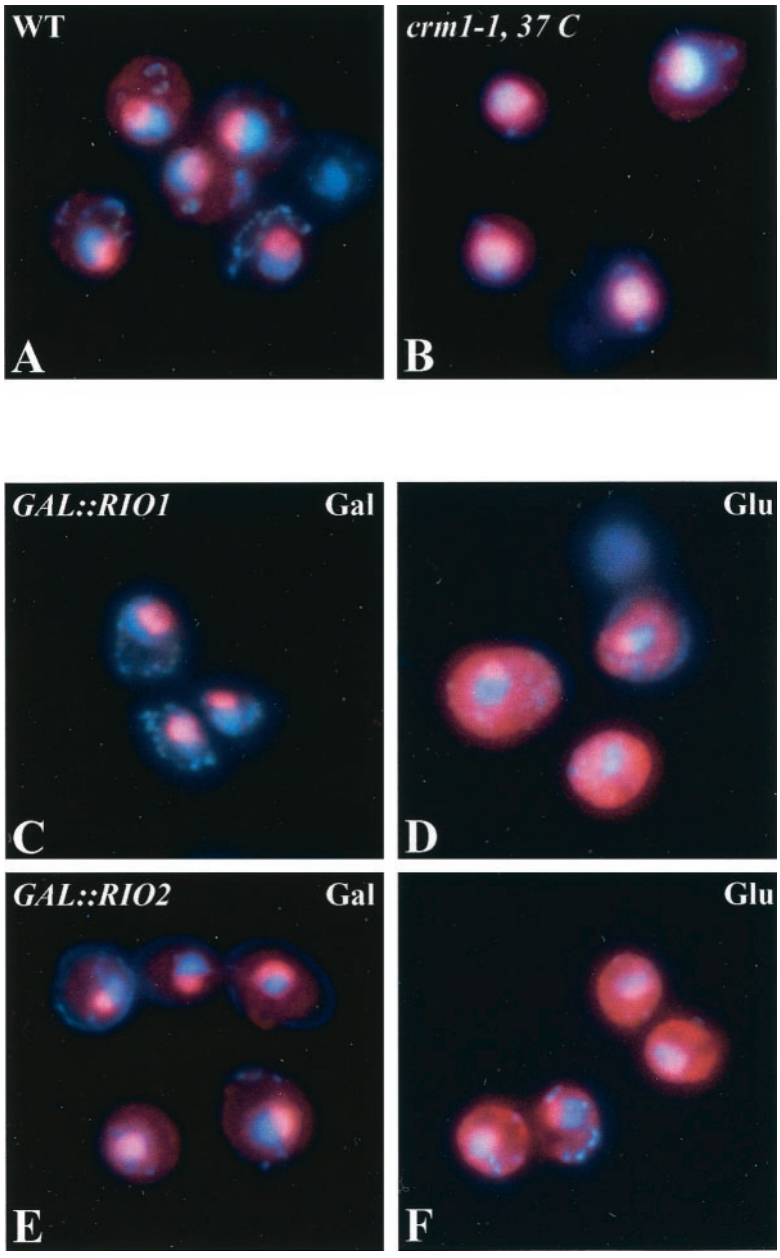


FIG. 5. Depletion of Rio2p results in accumulation of 20S pre-rRNA in the cytoplasm. The 5′ part of ITS1 (red) was detected by fluorescence in situ hybridization in *GAL*::*RIO1* cells (YO296) (C and D) and *GAL*::*RIO2* (YO470) cells (E and F). Cells were grown either in galactose medium or for 15 h in the presence of glucose. The nucleoplasm was counterstained with DAPI (blue). In wild-type cells (A), the ITS1 is mainly present in the nucleolus and is faintly detected in the cytoplasm. In contrast to *crm1-1* cells (YO419) shifted for 2 h at 37°C (B), which display a strong signal in the nucleus and no labeling of the cytoplasm, cells depleted of Rio1p or Rio2p show a build-up of the amount of 20S pre-rRNA in the cytoplasm (D and F).

GFP-Rio2p in *crm1-1* cells at the nonpermissive temperature, their cosedimentation with pre-40S particles, and association with 20S pre-rRNA suggest that these proteins associate with precursors of the SSU in the nucleus.

DISCUSSION

The functional analysis of Rio2p described above demonstrates that Rio2p is found in both the nucleus and the cytoplasm and that Rio2p-depleted cells, like Rio1p-depleted cells, exhibit a strong accumulation of 20S pre-rRNA. However, the level of 20S pre-rRNA accumulated does not reflect the 18S rRNA deficit (in samples from Rio2p-depleted cells, 20S pre-rRNA is barely detectable in ethidium bromide-stained gels, Fig. 3B). Thus, most of the unprocessed 20S pre-rRNA does not lead to immature SSU but is degraded. Such an accumulation of 20S pre-rRNA might stem from (i) defects of pre-40S subunit assembly impairing either its export from the nucleus or its final processing or both; (ii) alteration of elements of the export machinery, as previously shown for *prp20-1* and *yrb1-1*

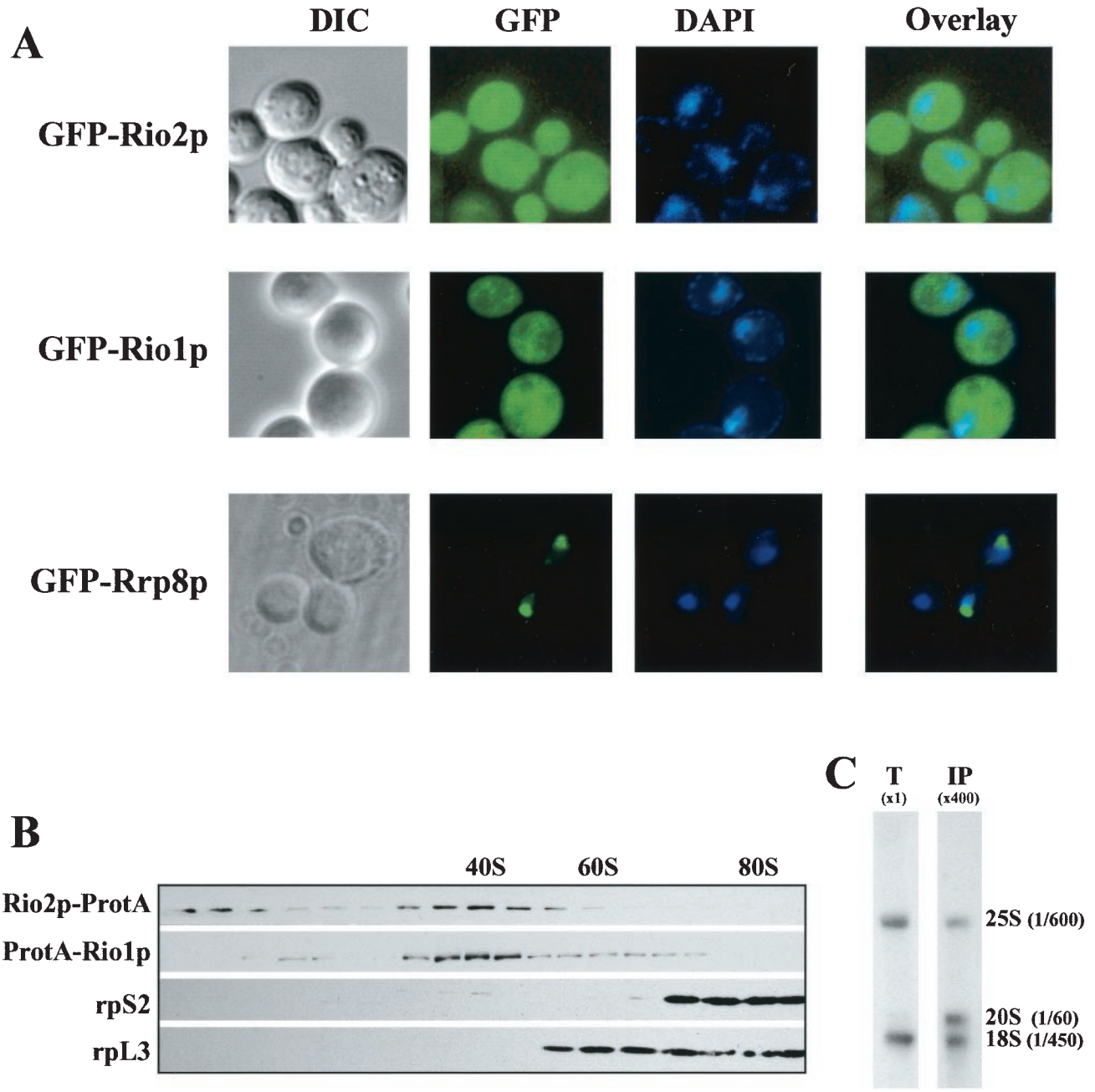
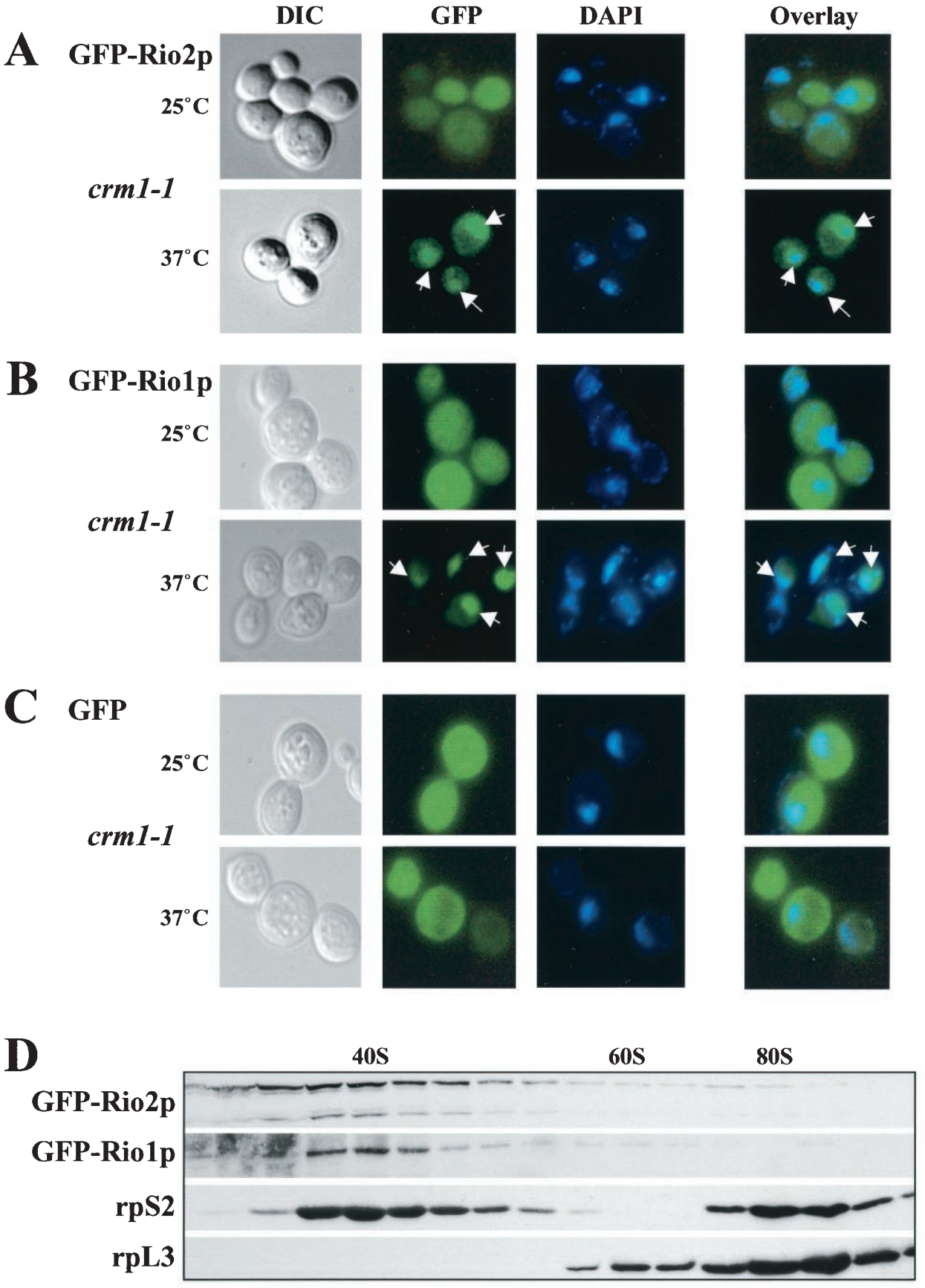


FIG. 6. Sedimentation profile of Rio2p-protein A, localization of GFP-Rio2p, and association of Rio2p with 20S pre-rRNA. (A) Subcellular localization of GFP-tagged proteins: GFP-Rio2p exhibits a cytoplasmic and nuclear pattern of fluorescence. GFP-Rio1p shows a cytoplasmic localization, and for Rrp8p-GFP, a punctate pattern characteristic of nucleolar staining is observed. Positions of nuclei were determined by DAPI staining (blue). Overlay images are shown by superposition of the blue and green stains. (B) Sedimentation profiles of Rio2p-protein A and protein A-Rio1p, small-subunit ribosomal protein rpS2, and large-subunit ribosomal protein rpL3 in glycerol gradients. A total extract was loaded on 10 to 30% glycerol gradients and subjected to centrifugation. Fractions were collected, and proteins in each fraction were precipitated with trichloroacetic acid, separated by SDS-PAGE, and revealed by Western blotting. Fractions containing the peak of 40S and 60S ribosomal subunits and 80S ribosomes are indicated. (C) 20S pre-rRNA selectively copurifies with Rio2-protein A. We probed 1/400 of the clarified cell extract (T) or the bulk of the immunoprecipitated RNA (IP) with a mixture of probes 1 and 6 shown in Fig. 3A (18S and 25S) and a D-A_2 probe prepared by multiprime labeling (20S). The fraction of each rRNA or pre-rRNA recovered in the immunoprecipitate was determined by phosphoimager quantification.

mutants (28); or (iii) direct inhibition of the endonucleolytic cleavage at site D.

Our data indicate that, as previously observed in Rio1p-depleted cells (35), 20S pre-rRNA is found in the cytoplasm of Rio2p-depleted cells, indicating that neither Rio1p nor Rio2p is required for the export of the pre-40S particles. We also show that in *crm1-1* cells grown at the nonpermissive temperature, in which the export of pre-40S particles is blocked, GFP-Rio2p and GFP-Rio1p are found mainly in the nucleus. Moreover, in extracts prepared from *CRM1*$^+$ cells as well as in


DIC
GFP
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A
GFP-Rio2p
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37°C
B
GFP-Rio1p
25°C
crm1-1
37°C
C
GFP
25°C
crm1-1
37°C
D
40S
60S
80S
GFP-Rio2p
GFP-Rio1p
rpS2
rpL3

TABLE 3. Ynl207p/Rio2p-associated factors

Name	Deletion phenotype	Protein localization	Function	Reference(s)
Enp1/Ybr247p	Lethal	Nucleolar	Unknown, associated with preribosomal particles.	11
Tsr1p/Ydl060p	Lethal	Nucleolar	GTPase, required for 40S ribosomal subunit biogenesis, depletion of Tsr1p leads to 20S pre-rRNA accumulation	8, 39
Nob1p/Yor056p	Lethal		Unknown, found in several complexes linked to small ribosomal subunit biogenesis, formerly associated with the 26S proteasome	7, 34
Yor145p	Lethal	Nucleolar	Unknown, found in several complexes linked to late small ribosomal subunit biogenesis, KH domain	7, 12
Dim1p/Yp1266p	Lethal	Nucleolar	rRNA dimethyltransferase, required for cytoplasmic formation of $m^{6,2}A_{1779}$, $m^{6,2}A_{1780}$, depletion results in inhibition of 18S maturation	22
Rrp12p/Yp1012p	Lethal		Found associated with several proteins involved in small ribosomal subunit biogenesis, depletion leads to 20S pre-rRNA accumulation	7, 39
Ltv1p/Yk1143p	Viable		Unknown, found in several complexes linked to late small ribosomal subunit biogenesis, required for viability at low temperature	7

extracts from *crm1-1* cells grown at the nonpermissive temperature, both Rio1p and Rio2p cosediment with pre-40S particles and 20S pre-rRNA is selectively coimmunoprecipitated with tagged Rio1p (35) and Rio2p (Fig. 6C). Taken together, these data show that both Rio1p and Rio2p are shuttling proteins required for the cytoplasmic processing of 20S pre-rRNA and suggest that they associate in the nucleus with pre-40S particles and are exported from the nucleus as elements of these particles.

Although depletion of Rio2p or Rrp10p/Rio1p causes the same phenotype, i.e., cytoplasmic accumulation of 20S pre-rRNA, they do not have overlapping functions: (i) both genes are essential for viability, and (ii) *RIO2* disruption is not complemented by overexpression of Rio1p or vice versa (our unpublished observations). In Rio2p-depleted cells, besides 20S pre-rRNA accumulation, minor effects on pre-rRNA processing are also observed: a slight accumulation of the aberrant 23S pre-rRNA and a small decrease in the level of the 27SA pre-rRNA. We attribute these defects to slowed processing at the A0, A1, and A2 cleavage sites. This could be related to the association of Rio2p with proteins present in the 90S preribosomes (Table 3) (7, 11). Such an effect is not seen in Rrp10p/Rio1p-depleted cells (35).

Localization of the two proteins is also somewhat different: in normal conditions, GFP-Rio1p is found exclusively in the cytoplasm, while Rio2p localizes in the nucleus and the cytoplasm. This nuclear localization is in good agreement with the recent identification of Ynl207p/Rio2p in different complexes (7). In tandem affinity purification tag experiments (30), with tagged Rio2p as the bait, different factors, some of them clearly involved in the biogenesis of the SSU, such as Tsr1p and Dim1p (8, 22) (Table 3), have been affinity purified (7). The functions and cellular localization of other ones are unknown, but some of them, like Enp1p, are found in 90S preribosomes (11). Rio2p as well as Enp1p, Ltv1p, Yor145p, and Rrp12p were also found in a multiprotein complex purified with tagged Tsr1p (Ydl060p) as the bait, a protein known to be required for SSU biogenesis (8). Furthermore, Ltv1p, a protein required for viability at low temperatures, which is found in these complexes, interacts in two-hybrid screens with rpS3, a protein of the SSU, and Crm1p, a protein required for the export of pre-40S particles. All these data clearly suggest that Rio2p is present in the nucleolus and the nucleus in complexes required for maturation and assembly of the SSU, as proposed in a recent review (6).

Certainly, due to its low abundance and its steady-state cytoplasmic localization, Rrp10p/Rio1p has not been referenced yet in these systematic purifications of multiprotein complexes. Compilation of all these results suggests that Rio2p may associate with pre-40S particles in the nucleus and earlier than Rio1p or with complexes of longer half-lives.

The RIO protein family has also been described by Koonin and coworkers (23) in an attempt to evaluate the entire repertoire of potential protein kinases in bacteria and archaea. These authors extensively scanned the sequences of available complete genomes for proteins with similarity to protein kinases. Retrieved putative protein kinases have been assigned to five distinct families, among them the RIO superfamily. More recently, by means of profile-matching procedures, conservation of important residues, and fold recognition techniques, Krupa and Srinivasan reported the occurrence of RIO-like proteins in eubacteria as well (21). They suggest that lipopolysaccharide kinases encoded in the genome of gram-negative bacteria are related to RIO proteins in eubacteria, archaea, and eukaryotes, pointing to the RIO domain as a possible evolutionary link between eukaryotic protein kinase-

FIG. 7. Localization and sedimentation profiles of GFP-Rio1p and GFP-Rio2p in a *crm1-1* genetic context. *crm1-1* strains used carry plasmids expressing either GFP-Rio2p (A), GFP-Rio1p (B), or GFP (C). Exponentially growing cultures in YNB medium at 25°C were divided in two parts; one part was shifted to 37°C for 2 h, while the other was kept at 25°C, and the subcellular localization of the tagged proteins was analyzed. Subcellular localization of GFP-Rio2p (A) and GFP-Rio1p (B) exhibited a wild-type localization pattern in *crm1-1* cells grown at 25°C, in contrast to cells shifted for 2 h at 37°C, in which GFP-RIO proteins display essentially a nuclear and nucleolar pattern of fluorescence. GFP (C) in the *crm1-1* strain at either 25°C or 37°C shows a normal cytoplasmic localization pattern. (D) Sedimentation profiles in a glycerol gradient of GFP-Rio2p, GFP-Rio1p, small-subunit ribosomal protein rpS2, and large-subunit ribosomal protein rpL3. A total extract from a *crm1-1* strain carrying plasmid pEV24 (*LEU2 pNOP::GFP::RIO1*) or pEV71 (*ADE2 pNOP::GFP::RIO2*) and grown at 37°C was loaded on a 10 to 30% glycerol gradient and processed as described in the legend to Fig. 6.

like sequences in prokaryotes and bona fide eukaryotic protein kinases.

The RIO domain defined in the SMART database (24) overlaps a putative kinase domain containing the key sequence motifs that are associated with catalytic activity and conserved among most protein kinases (corresponding to the canonical domains I to IX): the ATP binding site (conserved G, K, and E residues, positions 85 to 87 in Rio1p and 104 to 106 in Rio2p) and the catalytic D and N residues (D244 and N249 in Rio1p and D229 and N234 in Rio2p). Quite recently, a kinase activity has been demonstrated in vitro for Rrp10p/Rio1p (2). Moreover, mutations of conserved amino acid residues required for catalytic activity (i.e., within the ATP binding motif or catalytic domain) abrogate the kinase activity of the recombinant protein extracted from *E. coli* and Rio1p in vivo function.

Cytological analyses of Rio1p-depleted cells and of a strain carrying a weak *RRP10*/*RIO1* allele (D244E) suggest that Rrp10p/Rio1p could be involved in cell cycle progression. Likewise, in *Emericella nidulans*, mutations of *sudD*, the *RIO1* homologue, lead to an abnormal nuclear morphology (17). A recent systematic genetic analysis reveals that loss-of-function mutations or, in the case of essential genes, heterozygosity for loss-of-function mutations of numerous genes involved in ribosome biogenesis lead to altered average cell size. Further analysis disclosed that expression of most of these genes depends on the transcription factor Sfp1p (20). Some of these Sfp1p-regulated factors have established functional links to either Rio1p (Gar1p [35]) or Rio2p (Enp1p and Rrp12p [7]). Taken together, these data connect the involvement of Rio1p and Rio2p in ribosome biogenesis, as revealed by the drastic block in 20S pre-rRNA processing resulting from Rio1p or Rio2p depletion (35; this study), to cell cycle defects observed in Rio1p-depleted cells (2), or in *sudD* mutants of *E. nidulans* (17).

In this study we have shown that the RIO proteins in *S. cerevisiae* are shuttling proteins required for 20S pre-rRNA maturation. From our previous data (35) and the results reported herein, we infer that these proteins associate with pre-40S particles in the nucleus/nucleolus and are exported to the cytoplasm as part of 20S pre-rRNA containing precursors of the SSU. Recent proteomics data are in good agreement with this hypothesis (for a review, see reference 6). Several factors involved in preribosome assembly and pre-rRNA processing are phosphoproteins. Srp40p (Nopp140) interacts with and is phosphorylated by casein kinase II (25), and Nsr1p/nucleolin and nucleophosmin/B23 are also phosphorylated by casein kinase II and cdc2 (13, 25, 29), suggesting that the phosphorylation status of some *trans*-acting factors is involved in the control of ribosomes biogenesis. However, so far no kinase activity essential for ribosome biogenesis has been described among all the factors recently identified.

Considering their putative kinase activity, RIO proteins could themselves act as functional regulators of factors associated with the pre-40S complexes or required for 20S pre-rRNA maturation. In this respect, identifying the substrates of these putative protein kinases and disclosing how their activity and/or localization could be modulated should shed light on their function(s) in the late SSU processing pathway. The occurrence of RIO proteins or RIO-related proteins in eubacteria and archaea argues that these proteins are involved in highly conserved and ancient molecular processes.

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