Rrb1p, a Yeast Nuclear WD-Repeat Protein Involved in the Regulation of Ribosome Biosynthesis

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Ribosome biogenesis is regulated by environmental cues that coordinately modulate the synthesis of ribosomal components and their assembly into functional subunits. We have identified an essential yeast WD-repeat-containing protein, termed Rrb1p, that has a role in both the assembly of the 60S ribosomal subunits and the transcriptional regulation of ribosomal protein (RP) genes. Rrb1p is located in the nucleus and is concentrated in the nucleolus. Its presence is required to maintain normal cellular levels of 60S subunits, 80S ribosomes, and polyribosomes. The function of Rrb1p in ribosome biogenesis appears to be linked to its association with the ribosomal protein rpL3. Immunoprecipitation of Rrb1p from nuclear extracts revealed that it physically interacts with rpL3. Moreover, the overproduction of Rrb1p led to increases in cellular levels of free rpL3 that accumulated in the nucleolus together with Rrb1p. The concentration of these proteins within the nucleus was dependent on ongoing protein translation. We also showed that overexpression of RRB1 led to an increase in the expression of RPL3 while all other examined RP genes were unaffected. In contrast, depletion of RRB1 caused an increase in the expression of all RP genes examined except RPL3. These results suggest that Rrb1p regulates RPL3 expression and uncouples it from the coordinated expression of other RP genes.

Ribosome biogenesis is a complex process that requires the precise regulation of both the synthesis and assembly of its component parts in response to environmental stimuli (reviewed in references 39, 64, 66, and 69). In rapidly growing yeast cells, the expression of the ribosomal protein (RP) genes and ribosomal DNA consumes a major portion of the cell transcriptional activity (66, 69). Their products contribute to the production of ∼2,000 ribosomes per min (66). The rates of ribosome synthesis, however, can be quickly altered by changes in growth conditions. Although numerous steps in this regulatory process have been studied in detail, how these events are intertwined to coordinate ribosome assembly remains unclear.

In the yeast Saccharomyces cerevisiae, ribosome assembly is controlled by coordinated transcriptional events that regulate the expression of the RP genes and rRNA (for a review, see references 41 and 66). Expression of these components is influenced by a variety of factors, including nutrient availability, secretory activity, heat shock, and exposure to growth factors or signaling molecules (66). The cell’s responses to these cues are mediated by at least two kinase signaling pathways: the ras-cyclic AMP-protein kinase A pathway and the target-of-rapamycin or TOR pathway (38, 42, 52). Though distinct, both of these pathways converge to regulate the transcription of RP genes by a mechanism that is dependent on the DNA-binding protein Rap1 (28, 33, 34). Rap1 plays a role in the transcriptional regulation of a large number of genes, including the RP genes, exhibiting both activation and silencing activities depending on the loci to which it is bound (reviewed in references 41 and 45). For the RP genes, Rap1 acts as an activator of transcription, but it is also required for the suppression of their expression that occurs, for example, as a consequence of defects in the secretory pathway (33). The varied functions of this protein have led to the idea that Rap1 may play a general role in altering chromatin structure by making it accessible to other transcriptional regulators (35, 41).

The coordinated expression of the RP genes leads to the nearly equimolar production of each of the 78 ribosomal proteins (reviewed in references 66 and 69). Following their synthesis in the cytoplasm, most ribosomal proteins are actively transported into the nucleus. Ribosome assembly is believed to occur primarily in the nucleolus, where an ordered assembly of ribosomal proteins begins on a 35S rRNA precursor (27; for a review, see reference 58), leading to the formation of a 90S preribosomal particle (54). Among the early-assembling ribosomal proteins are rpL3 and rpL25. The 90S particle subsequently undergoes a series of processing steps that separate it into a pre-60S large subunit containing 25S and 5.8S rRNAs and a 43S small subunit precursor containing 20S rRNA (reviewed in reference 26). Both of these subunits are exported from the nucleus and are further modified to form mature ribosomal subunits.

The assembly of ribosomal proteins into ribosomal subunits is required to maintain their stability in the cell. By a mechanism that is not well understood, excess ribosomal proteins that fail to assemble into ribosomes are identified and are targeted for rapid degradation (30). This pathway was revealed, in part, on the basis of experiments examining the fate of individual ribosomal proteins including, among others, rpL3, rpL25, and rpL16 (rpL11A) (for the remainder of this paper the nomenclature described in reference 29 will be used for RP genes), produced by the overexpression of their genes (13, 30, 55). Their overexpression produces increased levels of mRNA that is efficiently translated; however, the excess proteins have extremely short half-lives of between 30 s and 3 min (66, 69). How
these excess proteins are identified and where their degradation occurs, in the nucleus or the cytoplasm, are not known. As a consequence of these and other studies, it is generally assumed that cellular levels of free ribosomal proteins are maintained at very low levels (69). Despite this, free ribosomal proteins do have functional roles outside the ribosome. For example, free rpL30 (7, 14, 59, 60) and rpS14 (16) both appear to act as feedback inhibitors of the splicing of their own mRNA.

Here we present data on the identification of an essential WD-repeat-containing protein, termed Rrb1p, that plays a role in the assembly of 60S ribosomal subunits and the regulation of RP gene expression. Rrb1p directly interacts with free rpL3 and it modulates the amount and localization of free rpL3 in the cell. Moreover, changes in the cellular level of Rrb1p alter RPL3 expression and uncouple it from the coordinated expression of other RP genes.

MATERIALS AND METHODS

Plasmids. The following plasmids were used in this study: pRS315, CEN/LEU2; pRS316, CEN/URA3 (47); pYEura3, CEN/URA3/GAL1-1/GAL10 (Clontech Laboratories, Inc., Palo Alto, Calif.); pYEUra3-RBB1-HA and pRS316-RBB1-HA, a DNA fragment encoding the hemagglutinin (HA) epitope GYPDYVPDYASG and a stop codon were inserted using PCR following the C-terminal amino acid codon of the RBB1 open reading frame (ORF), and the tagged gene was inserted into SacI sites in pRS315 and pRS316; pYEura3-RBB1, the complete ORF of RBB1 with a flanking 5′ XhoI site and a 3′ ClaI termination codon-XhoI fragment, synthesized by PCR and inserted following the GAL1 promoter in the plasmid pYEura3; pYEura3-RBB1-GFP, the GFP ORF containing flanking 5′ and 3′ XhoI sites, synthesized by PCR and inserted at the 3′ end of the ORF of RBB1 in pYEura3-RBB1; pYEura3-RBB1-HA, an MscI-BstXI fragment from pRS315-RBB1-HA containing the HA tag cloned into the MscI-XhoI sites in pYEura3-RBB1-pBUN100-DeRD-NOP1 (CEN LEU2) (kindly provided by Ed Hurt [University of Heidelberg, Heidelberg, Germany]). RPL3-GFP, RPL4A-GFP, and RPL25-GFP fusions were analyzed by flow cytometry using a FACscan (Becton Dickinson, San Jose, Calif.) and by immunofluorescence microscopy with anti-rpL3 MAb TCM1. Polyclonal rabbit antibodies directed against RPL3 (1454, 169) were generously provided by D. Rout (University of Heidelberg, Germany). Protein derived from approximately equal amounts of cells (for the experiment shown in Fig. 2B) or approximately equal amounts of total protein (for the experiments in Fig. 5A and B) were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (TransBlot; Bio-Rad Laboratories, Hercules, Calif.). Membranes were stained with amido black to visualize separated proteins and then blocked in Tris-buffered saline (20 mM Tris [pH 7.5] and 150 mM NaCl) containing 0.1% Tween 20 and 5% dried skim milk. Rrb1-HA was detected using the MAB 12CA5, and rpL3 was detected using the anti-rpL3 MAb (kindly provided by Jonathan Warner, Albert Einstein College of Medicine, Bronx, N.Y.). The Mabs were visualized with rhodamine-conjugated, goat anti-mouse antibodies (Amer sham Pharmacia Biotech, Baie d’Urée, Québec, Canada). Nuclear DNA was visualized by 4′,6-diamidino-2-phenylindole (DAPI) staining. GR1HA and RHA strains expressing RPL3-GFP, RPL4AGFP, and RPL25-GFP chimeras were grown in selection medium lacking leucine and were subjected to carbon source shift as described above. The distribution of GFP-fusion proteins was directly visualized in the fluorescein isothiocyanate channel. All slides were viewed under the 100× objective lens of an Olympus BX-50 microscope, and images were recorded using a Spot HRD600-NIC digital camera (Diagnostic Instruments Inc., Sterling Heights, Mich.).

Western blotting. In preparation for Western analysis, total cell extracts from the appropriate cultures were prepared as previously described (71). Briefly, cells were collected by centrifugation, washed with water, and then lysed with 7.4% β-mercaptoethanol in 1.85 N NaOH. Proteins were precipitated in 10% trichloroacetic acid and then solubilized in sodium dodecyl sulfate (SDS) sample buffer. Protein derived from approximately equal amounts of cells (for the experiment shown in Fig. 2B) or approximately equal amounts of total protein (for the experiments in Fig. 5A and B) were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (TransBlot; Bio-Rad Laboratories, Hercules, Calif.). Membranes were stained with amido black to visualize separated proteins and then blocked in Tris-buffered saline (20 mM Tris [pH 7.5] and 150 mM NaCl) containing 0.1% Tween 20 and 5% dried skim milk. Rrb1-HA was detected using the MAB 12CA5, and rpL3 was detected using the anti-rpL3 MAB TCM1. Polyclonal rabbit antibodies directed against rpL3 (which also cross-reacts with the ribosomal protein rpS2 [60]) were generously provided by Jonathan Warner. Polyclonal antibodies directed against Nup53p have been previously described (31). Antibody binding was detected with horseradish peroxidase-conjugated secondary antibodies (Amer sham Pharmacia Biotech) and the enhanced chemiluminescence (ECL) system. Quantification of Western blotting signals was performed using the ECL Plus System (Amer sham Pharmacia Biotech) and a Fluor Chem 8000 chemilumager scanner (Alpha Innotech Corp., San Leonardo, Calif.). Blots done on serial dilutions of cellular extracts were exposed for 5 min and quantification of the signal emitted from individual bands was performed on those bands empirically determined to be within a linear detection range.

Flow cytometry. G1GF and G1RF  cells were grown overnight to mid-logarithmic phase in YE media containing a mixture of galactose and glucose added in ratios of 70:30 or 60:40, for a 2% final concentration of sugar, as indicated. Cells were harvested, washed, and used to inoculate fresh medium containing either 2% galactose, 2% glucose, or the indicated mixture of both at an optical density at 600 nm (OD600) of 0.2 and then incubated for the indicated times. When necessary, cultures were diluted with fresh medium so that the OD600 would not exceed 0.7. The effects of altered Rbb1 expression on various cellular functions were examined as follows with the indicated modifications.

Fluorescence microscopy. Immunofluorescence microscopy was performed essentially as described previously (69). Rrb1-HA was detected with the monoclonal antibody (MAB) 12CA5 (Boehringer Mannheim, Laval, Québec, Canada). rpL3 was detected using the anti-rpL3 MAb (kindly provided by Jonathan Warner, Albert Einstein College of Medicine, Bronx, N.Y.). Nop1p was detected with an anti-Nop1 MAb (3); kindly provided by M. Rout). The Mabs were visualized with rhodamine-conjugated, goat anti-mouse antibodies (Amer sham Pharmacia Biotech, Baie d’Urée, Québec, Canada). Nuclear DNA was visualized by 4′,6-diamidino-2-phenylindole (DAPI) staining. GR1HA and RHA strains expressing RPL3-GFP, RPL4AGFP, and RPL25-GFP chimeras were grown in selection medium lacking leucine and were subjected to carbon source shift as described above. The distribution of GFP-fusion proteins was directly visualized in the fluorescein isothiocyanate channel. All slides were viewed under the 100× objective lens of an Olympus BX-50 microscope, and images were recorded using a Spot HRD600-NIC digital camera (Diagnostic Instruments Inc., Sterling Heights, Mich.).
separated on a 1.2% agarose gel. RNA was then transferred to Hybond-N+ nylon membrane (Amersham Pharmacia Biotech) and UV cross-linked. Membranes were then incubated for 2 h in prehybridization buffer (5 x Denhardt's solution, 5 x SSC [1 x SSC = 0.15 M NaCl plus 0.015 M sodium citrate], 50% formamide, 1% SDS) and then hybridized with a probe labeled with [32P]dCTP and washed with 2 x SSC at 70°C. Following hybridization at 37°C for 12 to 16 h, following washes in 0.2 x SSC and 0.1% SDS at 56°C, the blots were exposed to BioMax MR film (Eastman Kodak, Rochester, N.Y.). For the Northern analysis using the Y159 strain, cultures were grown as described above in a medium containing a 70:30 galactose:glucose mixture and then shifted to glucose- or galactose-containing medium for 12 h.

Probes for RBL1, RPL3, RPL30, and RPS26A were generated by PCR using genomic DNA as a template. The following CDNAs for PCR fragments were used to detect corresponding mRNAs: a BamHI-HindIII fragment from the plasmid pACTI for actin (kindly provided by D. Stuart, University of Alberta); an EcoRI-EcoRV fragment from the plasmid pRS314-L25-GFP (kindly provided by Ed Hurt, University of Heidelberg) for RPL25; a FokI-FokI fragment from pX242-RPS10A-GFP for RPS10A; and a Ball-Ball fragment from pX242-RPL4A-GFP for RPL4A. Each of the DNA fragments was labeled with [α-32P]dCTP using DNA labeling beads (Amersham Pharmacia Biotech). Quantification of Northern blots was performed using a Storm 840 phosphorimager (Molecular Dynamics, Sunnyvale, Calif.) and ImageQuant software, version 1.1.

**RESULTS**

**Identification of RRB1.** We have identified an uncharacterized ORF (YMR131c) in the *S. cerevisiae* genome whose deduced amino acid sequence displays characteristics that are consistent with a role in nuclear function. YMR131c displays a high degree of sequence identity with uncharacterized ORFs from various species, including *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Arabidopsis thaliana* (data not shown). It encodes a protein containing two extended acidic domains within its N-terminal half and five predicted tryptophan-aspartic acid (WD)-repeat motifs located in its C-terminal half. WD repeats are structural motifs present in a wide range of proteins that establish an interface to which other proteins bind (reviewed in reference 48). The acidic regions present in the N-terminal half of the protein are similar to those previously identified in a number of nucleolar proteins involved in ribosome biogenesis. Their presence has been suggested to reflect the intrinsic ability of a protein to shuttle between the nucleus and the cytoplasm (70). On the basis of its role in ribosome biogenesis described below, the protein encoded by YMR131c ORF has been termed Rrb1p, for regulator of ribosome biogenesis 1.

**Rrb1 encodes an essential nuclear protein.** The phenotype of cells lacking the *Rrb1* gene was determined by deletion of the gene and replacement with the HIS3 selectable marker in a W303 diploid strain. The heterozygous strain was sporulated and tetrads were dissected. All viable haploids lacked the HIS3 marker, indicating that the *Rrb1* gene is essential for cell viability (data not shown).

To examine the subcellular localization of Rrb1p, a plasmid-borne copy of its gene was tagged by inserting a DNA fragment encoding an HA tag following the C-terminal amino acid codon. An *rb1* null strain carrying this plasmid (RIHA) grew at wild-type rates, demonstrating that the Rrb1-HA protein is functional (data not shown). The subcellular distribution of this protein was examined by immunofluorescence microscopy using a MAb (12CA5) directed against the HA epitope. As shown in Fig. 1A, the Rrb1-HA protein was present throughout the nucleus but was concentrated in the nucleolus adjacent to the intensely DAPI-staining regions of the nucleus. This same distribution pattern was observed in cells producing an Rrb1-GFP fusion protein derived from a genomic copy of *Rrb1* tagged at the 3' end of its ORF with the GFP ORF (Fig. 1A). The localization of Rrb1p to the nucleus was further confirmed by subcellular fractionation. Immunoblotting of fractions derived from the RIHA strain detected a single protein species of ~70 kDa in a crude nuclear fraction (Fig. 1B). A second lower-molecular-mass species also cofractionated with an enriched nuclear fraction. The smaller species is thought to be a degradation product of Rrb1-HA, since it
gradually accumulated following isolation and storage of nuclei (data not shown).

Construction of a conditional RRB1 allele. We inserted the RRB1-HA ORF into the plasmid pYEura3 (CEN URA3) be-

hind the GAL1 promoter and introduced this plasmid into an rrb1 null strain by plasmid shuffling. The resulting strain (GR1HA; rrb1Δ GALL::RRB1-HA) grew on galactose-containing plates, but its growth was inhibited (upon repression of the GAL1 promoter) on plates containing glucose (data not shown). A similar strain (GR1GFP; rrb1Δ GALL::RRB1-GFP), in which the coding region for GFP was inserted in place of the HA tag, was also produced and it showed similar growth characteristics (data not shown). In these strains, the galactose-induced Rrb1-HA (Fig. 2A) and Rrb1-GFP (see Fig. 7A) chimera was accurately targeted to the nucleus and accumulated in the nucleolus.

The GAL1::RRB1 alleles were constructed to examine the effects of altering levels of Rrb1p on various cell processes. We first attempted to define conditions under which the GAL1-driven expression of RRB1-HA approximated that of wild-type cells and thus identified a starting point from which to over-express or deplete RRB1. We observed that GR1HA cells grown in galactose synthesized levels of Rrb1-HA significantly higher than those produced in the R1HA strain by the endogenous RRB1 promoter (Fig. 2B). To moderate the activity of the GAL1 promoter, GR1HA cells were grown in media containing raffinose or different combinations of galactose and glucose. We observed that GR1HA cells cultured in raffinose grew extremely slowly and expressed very low levels of Rrb1-HA, precluding its further use (data not shown). Consequently, we attempted to modulate the expression of the GAL1::RRB1 allele using media containing combinations of galactose and glucose. This approach was based on previous reports that the addition of glucose to cultures containing galactose causes a transient repression of GAL genes, which is followed by reduced levels of expression that are above those seen in media containing glucose alone (1, 23). We tested the effects of various combinations of galactose and glucose on the expression levels of GAL1::RRB1-HA or GAL1::RRB1-GFP by various techniques. As shown in Fig. 2B, increasing the ratio of glucose to galactose in the media for GR1HA caused a decrease in the amount of Rrb1-HA in total cell lysates. Moreover, when examined by fluorescence-activated cell sorter analysis, GR1GFP cells grown in increasing ratios of glucose to galactose showed a corresponding decrease in the level of Rrb1-HA expression in the overall cell population (Fig. 2C). Thus for several of the experiments that follow, the GR1HA and GR1GFP strains were grown in medium containing a mixture of galactose and glucose prior to shifting to media containing galactose or glucose alone. As shown in Fig. 2D, shifting these cells to glucose-containing medium arrested their growth.

Depletion of Rrb1p results in decreased levels of 60S ribosomal subunits and ribosomes. The concentration of Rrb1p within the nucleolus suggested a potential role for this protein in ribosome biogenesis. To address this possibility, the GR1HA (GAL1::RRB1-HA) strain was used to examine the effects of altering the cellular amounts of Rrb1p on the levels of cytoplasmic ribosomes. GR1HA cells were grown in medium containing a 60:40 galactose:glucose mixture and then shifted to medium containing glucose (to reduce levels of Rrb1p) or galactose (to increase levels of Rrb1p). Four hours later their ribosomal profiles were analyzed by sucrose-gradient fractionation. As controls, the same carbon source shifts were performed on the R1HA (RRB1 regulated by its endog-
enous promoter) strain. As shown in Fig. 3A, the levels of 60S subunits as well as 80S ribosomes and polysomes directly correlated with the levels of Rrb1p. The 60S subunit peak was reduced in GR1HA cells grown in medium containing a 60:40 galactose:glucose mixture, in which cellular levels of Rrb1p were reduced relative to the R1HA strain (Fig. 2B), and in

FIG. 2. GAL1 regulation of RRB1 expression. (A) GR1HA (GAL1::RRB1-HA) cells were grown in medium containing a galactose:glucose (60:40) mixture and then shifted to medium containing either 2% galactose or 2% glucose for 4 h. Ribosomal subunits, ribosomes, and polysomes derived from these cultures were then separated on 7-to-47% sucrose gradients and detected by their UV absorbance at 254 nm. The position of the 60S ribosomal subunit is indicated by an arrow. Half-mers are highlighted with an asterisk. (B and C) Pulse-chase analysis of rRNA. R1HA and GR1HA cells were grown in glucose-containing medium for 4 h, pulse-labeled with [5, 6-3H]uridine (B) or with [methyl-3H]methionine (C) for 3 min, and chased with medium lacking the labeling reagent. Following the indicated chase time, total RNA was isolated, resolved on agarose gels, and detected by autoradiography. The positions of the various rRNA species are indicated according to their sedimentation coefficients.

FIG. 3. The effect of depletion of Rrb1p on ribosome biosynthesis. (A) R1HA and GR1HA (GAL1::RRB1-HA) cells were grown in medium containing a galactose:glucose (60:40) mixture and then shifted to medium containing either 2% galactose or 2% glucose for 4 h. Ribosomal subunits, ribosomes, and polysomes derived from these cultures were then separated on 7-to-47% sucrose gradients and detected by their UV absorbance at 254 nm. The position of the 60S ribosomal subunit is indicated by an arrow. Half-mers are highlighted with an asterisk. (B and C) Pulse-chase analysis of rRNA. R1HA and GR1HA cells were grown in glucose-containing medium for 4 h, pulse-labeled with [5, 6-3H]uridine (B) or with [methyl-3H]methionine (C) for 3 min, and chased with medium lacking the labeling reagent. Following the indicated chase time, total RNA was isolated, resolved on agarose gels, and detected by autoradiography. The positions of the various rRNA species are indicated according to their sedimentation coefficients.

composed of the proportion of galactose (gal) and glucose (glu) indicated. Western blotting was performed on total cell lysates derived from equal amounts of cells, using MAb 12CA5 to detect Rrb1-HA or an antibody directed against the nuclear pore complex protein Nup53p (31). (C) Cultures of GR1GFP and R1GFP (RRB1-GFP controlled by the RRB1 promoter) were grown to early logarithmic phase and the mean fluorescent intensity (y axis) of 10^5 cells was determined by fluorescence-activated cell sorter analysis and plotted versus the percentage of galactose in the carbon source mixture. (D) GR1GFP, GR1HA, and R1HA cells were grown overnight in media containing a 60:40 mixture of galactose and glucose and then plated onto YP plates containing either galactose or glucose. Plates were incubated for 2 days at 30°C.
GR1HA cells shifted to glucose-containing medium to further repress RRB1. In contrast, these conditions had little effect on the levels of 40S subunits. Consistent with a depletion in 60S subunits, we also detected a shoulder on the 80S peak that likely represents a half-mer polysome containing a stalled 43S preinitiation complex attached to the same mRNA as the 80S ribosome. Such half-mers are often observed in strains defective in 60S subunit assembly (26, 69). In contrast, when the GR1HA cells were shifted to galactose for 4 h, the levels of the 60S subunits and ribosomes increased and no half-mers were visible (Fig. 3A). Moreover, GR1HA cells maintained in galactose-containing media exhibited subunit profiles that were indistinguishable from wild-type cells (data not shown). Finally, no significant changes were observed in the ribosomal profiles of R1HA cells grown under conditions identical to those described for the GR1HA strain (Fig. 3A).

The GAL1::RRB1-HA conditional allele was also used to examine the effects of Rrb1p depletion on the synthesis and accumulation of tRNA (Fig. 3B and C). tRNA is synthesized as a 35S precursor that is processed to yield mature 25S, 18S, and 5.8S species. The 25S and 5.8S RNAs are components of the 60S subunit, whereas the 18S RNA is part of the 40S subunit (58). For these experiments, GR1HA (GAL1::RRB1-HA) cells or the control strain R1HA was grown in medium containing a 60:40 galactose:glucose mixture and then shifted to glucose-containing medium for 4 h to suppress the expression of Rrb1p. Cells were then pulse-labeled with [5, 6-3H]uridine for 3 min to label the tRNA. Following a 5-min chase, various processing intermediates, including the 35S, 27S, and 20S species, were detected in control R1HA cells expressing endogenous levels of RRB1 (Fig. 3B). Processing of these intermediates was complete by 15 min, and similar amounts of the 25S and 18S species were visible. In comparison, the levels of precursor and mature forms of 25S tRNA, but not the 18S tRNA, were reduced in the Rrb1p-depleted cells and the appearance of 25S tRNA was delayed (Fig. 3B). As shown in Fig. 3C, the delay in the rate of 25S maturation upon RRB1 depletion was also observed in pulse-chase experiments in which tRNA was labeled with [methyl-3H]methionine. These results suggest that the formation of the 25S tRNA was reduced in these cells, consistent with the reduction of 60S subunits detected under similar conditions.

Rrb1p physically interacts with the ribosomal protein rpL3.

To further investigate Rrb1p’s function in ribosome biogenesis, we attempted to identify proteins that physically interact with Rrb1p. For these experiments, nuclei were isolated from cells expressing Rrb1-HA and the nuclei were extracted with buffer containing 1% Triton X-100 and 240 mM NaCl. Under these conditions, the majority of Rrb1-HA was released into a soluble supernatant fraction (data not shown). The soluble Rrb1-HA was then immunoprecipitated with MAb 12CA5. As shown in Fig. 4, three predominant polypeptides with apparent molecular masses of 70, 66, and 40 kDa were specifically detected in the bound fraction but not in similar fractions that were either lacking the HA-tag or had an HA-tagged version of the nuclear pore complex protein Pom152p (data not shown). This set of polypeptides was also observed when similar experiments were conducted using NaCl concentrations ranging from 150 to 450 mM (data not shown). Immunoblots revealed that both the 70- and 66-kDa species were derived from Rrb1-HA (Fig. 4). The identity of the 40-kDa band was determined by peptide microsequencing to be the essential 60S ribosomal subunit protein rpL3 (the product of the RPL3 gene [17]). This result was further confirmed by Western blotting using an anti-rpL3 antibody (Fig. 4).

Rrb1p modulates cellular levels of rpL3.

Our observation that rpL3 is the only ribosomal protein associated with the immunoprecipitated Rrb1p suggested that it may only interact with free rpL3, perhaps as a prelude to rpL3’s assembly into preribosomal 90S subunits. Moreover, as the half-life of free ribosomal proteins is short, between 30 s and 3 min (69), we hypothesized that Rrb1p may influence the half-life of rpL3. To further explore these possibilities, we examined the effects of altering RRB1 expression on the cellular levels of rpL3, its subcellular localization, and the expression of the RPL3 gene.

We first examined how the changes in RRB1 expression would affect the cellular levels of rpL3 in relation to other ribosomal proteins. GR1HA cells maintained in medium containing a 70:30 galactose:glucose ratio were shifted to either galactose- or glucose-containing medium for 6 h. Total cell lysates were then examined by Western blotting using various antibodies. As shown in Fig. 5A, the depletion of Rrb1p caused a slight decrease in the levels of rpL3. Alternatively, the induc-
tion of RRB1-HA led to a distinct increase in the levels of rpL3 relative to other proteins examined, including rpL30 (Fig. 5A, Gal). The increase in rpL3 was also observed in GR1HA cells grown in galactose and constitutively overexpressing RRB1. In these cells, steady-state levels of Rrb1-HA and rpL3 were ~3.7- and 1.5-fold higher, respectively, than those detected in R1HA strains (Fig. 5B). In contrast, rpL30 and the 40S subunit protein rpS2 were unaffected, suggesting that the overproduction of Rrb1p specifically increases cellular levels of rpL3.

As described above (Fig. 2A), excess Rrb1p accumulated in the nucleus. If the increased amounts of rpL3 produced in GR1HA cells were associated with Rrb1p, we would predict that excess rpL3 would also accumulate in the nucleus. To test this, immunofluorescence microscopy was performed using the anti-rpL3 antibody on the GR1HA strain after a shift to glucose- or galactose-containing medium for 6 h (Fig. 6A). Cells shifted to glucose-containing medium showed a diffuse cytoplasmic staining pattern similar to, albeit weaker than, that observed in R1HA cells. In contrast, cells overexpressing RRB1 showed both a cytoplasmic staining pattern similar to, albeit weaker than, that observed in R1HA cells. In contrast, cells overexpressing RRB1 showed both a cytoplasmic staining pattern similar to, albeit weaker than, that observed in R1HA cells. The nuclear staining was often most intense in crescent-shaped structures adjacent to the DAPI-staining regions of the nucleus, suggesting that rpL3, like Rrb1, concentrated in the nucleolus. This nuclear accumulation was not observed in R1HA cells grown in galactose (Fig. 6A).

To determine whether the recruitment of rpL3 into the nucleus by Rrb1p was specific for this ribosomal protein, we compared the distribution of rpL3 with two other large subunit proteins, rpL25 and rpL4A, in Rrb1p-overproducing cells. To visualize these proteins, GFP-tagged versions of each (which are capable of assembling into ribosomes [J. D. Aitchison and M. P. Rout, unpublished data]) were examined. As expected, each of these proteins was distributed throughout the cytoplasm in wild-type (data not shown) and R1HA cells grown in medium containing either galactose (Fig. 6B) or glucose (data not shown). A similar pattern was also seen in GR1HA cells grown in medium containing a 70:30 galactose:glucose mix (data not shown). However, when Rrb1-HA was overproduced in these cells, rpL3-GFP, but not rpL4A-GFP or rpL25-GFP, accumulated in the nucleus (Fig. 6B, GR1HA), again appearing to concentrate in the nucleolus. In contrast, repression of RRB1-HA expression resulted in the gradual fading of the cytoplasmic signal of each of the three ribosomal protein-GFP chimeras, consistent with the decreased levels of ribosomes observed in Rrb1p-depleted cells (data not shown).

**Nuclear localization of Rrb1p is dependent on protein translation.** A number of nucleolar proteins have been shown to shuttle between the nucleus and the cytoplasm (70). We asked whether Rrb1p might exhibit similar dynamics, in our effort to better understand its role in ribosome biogenesis. Classically, such studies involve examining the export and re-import of nuclear proteins under conditions that inhibit protein synthesis. During the course of our experiments, we surprisingly observed that protein synthesis inhibitors alone caused a rapid release of Rrb1p from the nucleus. Treatment of GR1GFP cells with cycloheximide (Fig. 7A) or sodium fluoride (data not shown), two well-characterized protein synthesis inhibitors (57), caused a redistribution of Rrb1-GFP to the cytoplasm. This effect was reversible. One hour after removal of the drug, Rrb1-GFP reaccumulated in the nucleus and was concentrated in the nucleolus (Fig. 7A), albeit at levels of intensity that were somewhat less than those seen prior to treatment with cycloheximide. These same dynamics were observed with the nuclear rpL3-GFP in GR1HA cells overpro-
ducing Rrb1-HA. In these cells, the protein synthesis inhibitors also caused a redistribution of the nuclear pool of Rp13-GFP to the cytoplasm (Fig. 7A). Moreover, like Rrb1p, removal of the drugs led to a progressive reaccumulation of rpL3-GFP in the nucleus. These results further suggest that the nuclear rpL3 detected in these cells was associated with Rrb1p.

The requirement of ongoing protein synthesis for the nuclear localization of Rrb1p was also tested using a thermosensitive mutant (prt1-1) containing a mutation in the PRT1 gene, which encodes a component of the eIF-3 translation initiation factor (37). At the restrictive temperature, the prt1-1 mutant is unable to form translational preinitiation complexes, causing rapid polysome runoff (19, 22). For our experiments, a plasmid-linked copy of RRB1-HA was introduced into the prt1-1 strain, and the Rrb1-HA protein was detected by immunofluorescence microscopy (Fig. 7B). At the permissive temperature (23°C), Rrb1-HA was predominantly nuclear. However, a shift to 37°C for 20 min caused a rapid redistribution of Rrb1-HA into the cytoplasm. Returning the cells to 23°C led to

![Image](image_url)

**FIG. 6.** rpL3 accumulates in the nuclei of cells overexpressing RRB1. (A) GR1HA (GAL1:RRB1-HA) and R1HA cells were grown in galactose:glucose (70:30)-containing medium and shifted to either galactose or glucose for 6 h. Cells were then fixed, permeabilized, and probed with the MAb TCM1 (α-rpL3). Nuclear DNA was visualized by DAPI staining. (B) GR1HA and R1HA cells containing a plasmid-borne copy of the gene fusion RPL3-GFP, RPL4A-GFP, or RPL25-GFP were grown in medium containing a galactose:glucose (70:30) mixture and then shifted to galactose-containing medium for 6 h. The GFP fusions were visualized directly by fluorescence microscopy. The position of the nuclei within these cells was determined by Nomarski optics (data not shown). Bar, 5 μm.

**FIG. 7.** Inhibition of protein synthesis causes a reversible relocation of Rrb1p from the nucleus to the cytoplasm. (A) GR1GFP (GAL1:RRB1-GFP) cells (Rrb1-GFP) and GR1HA (GAL1:RRB1-HA) cells expressing RPL3-GFP (rpL3-GFP) were grown in galactose-containing medium, and the GFP fusions were visualized by fluorescence microscopy (untreated). These cells were then treated with cycloheximide (100 μg/ml) for 1.5 h and reexamined by fluorescence microscopy (+CHX). Following treatment with cycloheximide, cells were washed, resuspended in fresh medium, and allowed to recover for 30 min at 30°C (CHX removed). (B) R1HA cells and the thermosensitive prt1-1 strain containing pRS316-RRB1-HA were grown at 23°C and then shifted to 37°C for 20 min. Cells harvested from both temperatures were fixed, permeabilized, and probed with either MAb 12CA5 (α-HA) or an MAb that specifically binds the nucleolar protein Nop1p (α-Nop1). Nuclear DNA was visualized by DAPI staining. Bar, 5 μm.
the reaccumulation of Rrb1-HA within the nucleus (data not shown). These same effects were also observed on a nuclear pool of rpL3-GFP in the prt1-1 strain overproducing Rrb1-HA (data not shown). By comparison, the temperature shift did not affect the localization of the nucleolar protein Nop1p or the integrity of the nucleolus in the prt1-1 strain (Fig. 7B). Finally, these effects were specific for the prt1-1 strain, as the temperature shift did not change the nuclear localization of Rrb1-HA in R1HA cells (Fig. 7B).

**RRB1 regulates the levels of ribosomal protein mRNAs.** The results described above suggested that Rrb1p binds to free rpL3 and that the overproduction of Rrb1p leads to the accumulation of free rpL3 within the nucleus. We have also observed that elevated levels of rpL3, induced by overexpressing RRB1, are accompanied by an increase in RPL3 mRNA (Fig. 8). Using Northern blot analysis, we examined the levels of RPL3 mRNA upon induction and repression of RRB1 expression. As shown in Fig. 8, the induction of RRB1-HA overexpression, stimulated in the GR1HA strain by a switch to galactose-containing medium, led to a striking increase in the amount of RPL3 mRNA. In contrast, shifting to glucose-containing medium had little effect on the levels of RPL3 mRNA within the time course examined.

The promoter regions of most RP genes are similar to one another (41), containing binding sites for the regulatory protein Rap1p. RPL3, however, is one of a few genes whose promoter has a different structure, which lacks Rap1p binding sites (69). Despite these differences, constitutive levels of expression are similar for all RP genes and it has been generally observed that they are coordinately regulated in response to different stimuli (see Discussion). With this in mind, experiments were performed to examine the effect of altering RRB1 expression on other RP mRNA levels, including those controlled by promoter elements similar to (RPS28A and RPL4B) or different from (RPL30, RPL25, and RPS10A) those controlling RPL3. The RP mRNAs selected for examination represented components of the 60S and 40S subunits (41). Unexpectedly, the depletion of Rrb1p, which was concurrent with a decline of ribosomal levels (Fig. 3), resulted in a significant increase in mRNA levels of all RP mRNAs examined except RPL3 (Fig. 8). These changes were specific, as no appreciable change was observed in the ACT1 mRNA control. Similarly, in GR1HA cells grown in medium containing a 70:30 galactose:glucose mix (Fig. 8A, t = 0), in which levels of Rrb1-HA are below wild-type levels, each of these mRNAs was more abundant than the RPL3 mRNA. These effects were reversed upon induction of GAL1::RRB1-HA. Increases in Rrb1p caused RP mRNA levels (except for RPL3) to decrease to approximately wild-type levels (Fig. 8A). In a separate set of experiments, the changes in the levels of mRNA were quantitated and normalized to levels of ACT1 mRNA (Fig. 8B). The results of these experiments suggest that the levels of RPL3 increased ~3-fold 6

**FIG. 8.** Rrb1p modulates RP mRNA levels. (A) GR1HA (GAL1:RRB1-HA) and R1HA (WT) cells were grown in galactose:glucose (70:30)-containing medium and then shifted to either galactose- or glucose-containing medium. At the indicated time points, total RNA was isolated. Equal amounts of RNA from each of these samples were separated on a 1.2% formaldehyde-agarose gel, transferred to a nylon membrane, and hybridized with the indicated 32P-labeled cDNA probe. Note that the RRB1 blot was exposed for a time period similar to that for the ribosomal protein mRNA blots and thus it is only visible here in the overexpressing samples. (B) Northern blots were performed as for panel A, and signals were quantitated using a phosphorimager. mRNA levels were normalized to ACT1 mRNA and wild-type levels of RP mRNAs were assigned a value of one. (C) Northern analysis was performed on RNA isolated from GR1HA cells expressing RPL25-GFP or RPL3-GFP driven by an exogenous promoter (see Materials and Methods). Both mRNAs were detected using a 32P-labeled RPL25 or RPL3 cDNA probe. (D) The effects of a GAL10::NOP1 conditional allele on the levels of RPL25 mRNA were examined in strain Y159. Y159 (GAL10::NOP1) and W303 (WT) cells were grown in galactose:glucose (70:30)-containing medium and then shifted to either galactose- or glucose-containing medium for 12 h. Northern analysis was performed on total RNA using a 32P-labeled RPL25 cDNA probe. The lane order is the same as that shown in panel C.
h after GR1HA cells were shifted to galactose-containing media. In contrast, a shift to glucose-containing media for 6 h caused a 2- to 4-fold increase in the other RP mRNAs examined.

The alterations in RP mRNA levels induced by changes in RRB1 expression were likely due to changes in the levels of transcription, as they relied on the presence of an endogenous promoter. For example, overexpression of RRB1 increased the levels of mRNA derived from the endogenous RPL3 gene but not from a plasmid-borne RPL3-GFP gene controlled by the triose phosphate isomerase promoter (Fig. 8C). Similarly, depletion of RRB1 only caused an increase in the levels of mRNA derived from the endogenous RPL25 gene but not from a plasmid-borne RPL25-GFP gene. Finally, the effects of Rrb1p levels on RP gene expression were specific and are not generally associated with defects in ribosome assembly. The suppression of expression of the nucleolar protein gene NOP1, which causes defects in rRNA processing and ribosome assembly, had no effect on the expression of the RPL25 gene (Fig. 8D).

**DISCUSSION**

Rrb1p is a member of a functionally diverse superfamily of WD-repeat-containing proteins (48). It is present throughout the nucleus but is concentrated in the nucleolus. The nucleolar concentration of Rrb1p and the fact that patterns of RRB1 gene expression mirror those of the RP genes during diauxic shift (9) suggested a possible involvement of Rrb1p in ribosome synthesis. To test this, we studied the effects of varying cellular levels of Rrb1p on the levels of ribosomes and ribosomal subunits. We observed that even moderate levels of Rrb1p depletion led to a decrease in the levels of 60S ribosomal subunits, 80S ribosomes, and polysomes (Fig. 3). This was accompanied by a significant inhibition in the production of 25S rRNA. In contrast, Rrb1p depletion had little effect on the levels of 40S subunits and these subunits were capable of forming 43S preinitiation complexes (half-mers). Moreover, unlike that of 25S rRNA, the formation of 18S rRNA was unaffected by reduced levels of Rrb1p. Similar results have been documented in a variety of mutants that are defective in 60S subunit formation, including those containing mutations in genes encoding constitutive 60S subunit proteins and factors affecting large-subunit assembly and maturation (26, 69). On the basis of these results, we conclude that Rrb1p plays a specific role in the assembly of the 60S subunit.

The function of Rrb1p in ribosome biogenesis is likely linked to its physical association with the ribosomal protein rpL3. We showed by immunoprecipitation of Rrb1p from nuclear extracts that it specifically interacts with rpL3, with no other ribosomal proteins being visibly bound to Rrb1p. These results suggest that Rrb1p interacts with free rpL3 at a point prior to its incorporation into ribosomes. This conclusion is further supported by the observation that excess Rrb1p causes a specific accumulation of rpL3 (but not other ribosomal proteins such as rpL4A or rpL25) within the nucleus (Fig. 6B). At what point following its synthesis rpL3 binds to Rrb1p is unclear. The two proteins could associate with one another either after rpL3 enters the nucleus or in the cytoplasm with the resulting complex being imported into the nucleus. The latter scenario is possible since Rrb1p is capable of shuttling between the nucleus and the cytoplasm (Fig. 7). Interestingly, we observed that Rrb1p’s steady-state localization to the nucleus is dependent on ongoing protein translation. Both chemical inhibitors (cycloheximide and sodium fluoride) and mutations (ptr1-1) that block translation (19, 57) caused Rrb1p to be released into the cytoplasm. Upon reinitiation of translation, Rrb1p again concentrated in the nucleus. Moreover, inhibition of the protein synthesis also led to the coordinated transport of a Rrb1p-rpL3 pool (see below) out of the nucleus (Fig. 7). Again, these proteins could be reimported into the nucleus after release of translational arrest, suggesting that a Rrb1p-rpL3 complex is capable of being imported into the nucleus. How the function of Rrb1p is linked to protein translation is yet to be investigated. However, the observation that a nucleolar protein’s localization is dependent on translation is, to the best of our knowledge, unprecedented.

Consistent with their physical association, we also showed that the overproduction of Rrb1p leads to a disproportionate increase in steady-state levels of rpL3 relative to other ribosomal proteins (Fig. 5). These data were surprising in light of numerous previous reports that the stoichiometric relationships between ribosomal proteins are tightly maintained and that excess unassembled ribosomal proteins are quickly degraded, having half-lives between 30 s and 3 min (66, 69). For example, it was shown that overproduced RPL3 mRNA accumulates in the cell (40) and is efficiently translated but that excess rpL3 is rapidly degraded (30). In contrast, in Rrb1p-overproducing cells, both following induction of the GAL1::RRB1 gene and in constitutively overexpressing cells, a surplus of rpL3 was detected (Fig. 5). The excess pool of rpL3 may be explained in two ways. First, overexpression of RRB1 stimulates the expression of RPL3 (see below), thus likely increasing the production of rpL3. Second, the overproduced Rrb1p sequesters rpL3 within the nucleus, concentrating it within the nucleolus (Fig. 6). Here it could directly protect rpL3 or segregate it from the proteolytic machinery that would normally degrade it. The latter mechanism is not unprecedented, as recent reports show that the sequestration of proteins within the nucleolus can protect them from degradation (50, 67) and regulate their activity (46, 49, 61).

Within the nucleolus, the Rrb1p-rpL3 complex could act as a precursor from which rpL3 is recruited into newly forming preribosomes. Since we have not detected Rrb1p in association with precursor or mature 60S subunits (data not shown), the binding of rpL3 to the 90S precursor is likely accompanied by the dissociation of Rrb1p. Such a mechanism would suggest a role for Rrb1p in the deposition of rpL3 on the 90S precursor. Our observation that Rrb1p depletion decreases the rate of 25S rRNA formation (Fig. 3) is consistent with a defect in rpL3 incorporation. Similar phenotypes have been observed when the incorporation of early assembly intermediates, including rpL3, onto pre-rRNA is altered (36; for a review, see reference 26). Interestingly, two other yeast proteins that contain WD-repeats have also been suggested to assist in the incorporation of ribosomal proteins onto ribosomal subunits. The cytoplasmic protein Sqt1p may play a role in depositing Qst1p-rpL10 on the 60S subunit during a late assembly step in the cytoplasm (11, 12). In another example, Rrp7p, a protein presumed to be nuclear, is required for rRNA processing through a mechanism that is proposed to involve the addition of two proteins, rpS27A and rpS27B, to the 43S precursor (6).
The coordinated increase in the levels of the rpl3 that accompanied Rrb1p overproduction prompted us to examine the effects of the RRB1 conditional allele on the expression of RPL3 and various other RP genes. A hallmark of RP gene transcription is that, under normal growth conditions as well as under conditions of stress including carbon source changes (20), heat shock (21), and alterations in protein secretion (28, 32, 56), the expression of all RP genes is globally coordinated (9, 10, 41, 66). The mechanics of this process, however, are not well understood. The majority of RP genes contain upstream sequences that bind the protein Rap1p. Rap1p has been shown to act as a transcriptional activator of RP gene expression, and it plays a necessary role in the repression of transcription induced by amino acid starvation and defects in secretion (33, 34). A few RP genes, including RPL3, lack the Rap1p-binding site and instead contain binding sites for the transcription factor Abf1p. Still, their expression is coordinated with the other RP genes under each of the various environmental conditions mentioned above (41).

Interestingly, we showed that varying the levels of Rrb1p uncouples the regulation of RPL3 mRNA levels from the coordinated control of other RP mRNAs, potentially through the control of their transcription. The overexpression of RRB1 leads to a robust increase in the levels of rpl3 mRNA, while all of the other RP mRNAs examined remained at or near wild-type levels (Fig. 8). In contrast, upon depletion of Rrb1p the levels of RPL3 mRNA appeared unaffected while mRNA levels of all other examined RP genes were increased. This includes RP genes whose promoters contain either Rap1p- or Abf1p-binding sites. Our analysis of the effects of RRB1 expression on the levels of RPL3 and RPL25 mRNAs (Fig. 8C) showed that the increase induced by overexpression (RPL3) or repression (RPL25) of RRB1 was dependent on the presence of RP's endogenous promoter. Thus, the increases in amounts of RP mRNAs that were detected upon depletion of Rrb1p may reflect an increase in transcription rather than a change in the half-life of these mRNAs. However, the latter possibility has not yet been tested.

The effects of Rrb1p on the levels of RPL3 mRNA may be linked to its physical association with rpl3. One scenario is that the state of Rrb1p, free versus bound to rpl3, would provide a means for Rrb1p to sense ongoing ribosome assembly and adjust RPL3 expression. For example, increased levels of free Rrb1p caused by a decrease in rpl3 could stimulate the expression of RPL3. Rrb1p also appears to play a more global function in ribosome biogenesis by, directly or indirectly, suppressing the expression of other RP genes. How Rrb1p can act as a transcriptional activator in the context of the RPL3 gene and a repressor for other RP genes remains to be investigated. Of note, the surge in the expression of RP genes observed upon depletion of Rrb1p is similar to that previously reported in mutants expressing truncations of Rap1p, which lack domains implicated in transcriptional silencing or activation (18). In both cases, the levels of RP mRNA significantly exceeded normal cellular levels detected in wild-type cells grown under the same conditions. This phenomenon is striking since normal levels of RP gene transcription already account for ~30% of RNA polymerase II-mediated transcription. The simplest explanation is that normal levels of transcription do not represent maximal levels.

While the functional links between the effects of Rrb1p and Rap1p are unclear, it is of interest that Rap1p appears to be functionally linked to a chromatin assembly complex that contains Msolp (15), a yeast protein exhibiting a high degree of sequence similarity to Rrb1p (data not shown). Both by its association with the chromatin assembly complex and, on a broader scale, as a consequence of its multiple effects on transcription regulation, Rap1p has been suggested to play a general role in chromatin remodeling (35). If Rrb1p, like Msolp, is functionally linked to Rap1p, it could function to modulate Rap1p activity at specific loci such as the RP genes.

Our results clearly indicate that Rrb1p plays a role both in the assembly of 60S ribosomal subunits and in the transcription of RP genes. Rrb1p is therefore positioned to link these two events and coordinate their activities. Moreover, the differential effects of Rrb1p on the expression of RPL3 as compared to other RP genes suggest that the coordinated regulation of RP gene expression is likely the result of independent, yet intertwined, regulatory pathways that together maintain similar levels of expression for all RP genes.

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