Identification of a role for *Saccharomyces cerevisiae* Cgr1p in pre-rRNA processing and 60S ribosome subunit synthesis

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***Saccharomyces cerevisiae* CGR1 encodes a conserved fungal protein that localizes to the nucleolus. To determine if this localization reflects a role for Cgr1p in ribosome biogenesis two yeast *cgr1* mutants were examined for defects in ribosome synthesis: a conditional depletion strain in which *CGR1* is under the control of a tetracycline-repressible promoter and a mutant strain in which a C-terminal truncated Cgr1p is expressed. Both strains had impaired growth rates and were hypersensitive to the aminoglycosides paromomycin and hygromycin. Polysome analyses of the mutants revealed increased levels of free 40S subunits relative to 60S subunits, a decrease in 80S monosomes and accumulation of half-mer polysomes. Pulse-chase labelling demonstrated that pre-rRNA processing was defective in the mutants, resulting in accumulation of the 35S, 27S and 7S pre-rRNAs and delayed production of the mature 25S and 5.8S rRNAs. The synthesis of the 18S and 5S rRNAs was unaffected. Loss of Cgr1 function also caused a partial delocalization of the 5'-ITS1 RNA and the nucleolar protein Nop1p into the nucleoplasm, suggesting that Cgr1p contributes to compartmentalization of nucleolar constituents. Together these findings establish a role for Cgr1p in ribosome biogenesis.**

Keywords: yeast, nucleolus, ribosome biogenesis

INTRODUCTION

Ribosome synthesis plays an essential role in the cellular adaptation to nutrient availability in all micro-organisms, allowing for the tight coordination of protein synthetic capacity with the demand for rapid growth (reviewed in Planta, 1997; Warner, 1999; Nomura, 1999). In eukaryotic organisms, ribosome synthesis takes place primarily in the nucleolus, a specialized compartment of the nucleus that is responsible for the transcription of the rDNA genes, the processing of pre-rRNA and the coordinated assembly of pre-rRNAs with ribosomal proteins (reviewed by Scheer & Weisenberger, 1994; Shaw & Jordan, 1995; Venema & Tollervey, 1999). Most of our understanding of eukaryotic ribosome synthesis has been obtained from the model yeast *Saccharomyces cerevisiae* (reviewed by Planta, 1997; Warner, 1999; Nomura, 1999). The yeast rDNA is transcribed by RNA polymerase I as a single

35S precursor RNA that is processed into three mature rRNAs (reviewed by Warner, 1982; Raué & Planta, 1991; Venema & Tollervey, 1999). The 35S pre-rRNA is flanked by two external transcribed spacers (ETs), 5'-ETS and 3'-ETS, and contains two internal transcribed spacers (ITSs), ITS1 and ITS2, that separate the sequences for the three mature rRNAs: 18S, 5.8S and 25S (see Fig. 1). The mature 5.8S and 25S rRNAs become incorporated into the 60S ribosome, and the mature 18S rRNA becomes part of the 40S ribosome. A fourth rRNA (5S) is transcribed independently by RNA polymerase III from a separate transcription unit and this RNA is incorporated into the 60S ribosome. The removal of the spacer sequences from the pre-rRNAs, and the final maturation of the rRNAs, is a complex process that involves a number of non-ribosomal proteins and small nucleolar RNAs (Raué & Planta, 1991; Kressler *et al.*, 1999).

Since ribosome synthesis is tightly linked to growth, the ability to target nucleolar proteins involved in ribosome biogenesis would be an effective therapeutic strategy against fungal infections. However, the ability to design

Abbreviations: ETS, external transcribed spacer; ITS, internal transcribed spacer.

Fig. 1. The major pre-rRNA processing pathways in *S. cerevisiae*. The 35S pre-rRNA is transcribed in the nucleolus and processed into the mature 18S, 5.8S and 25S rRNAs by a series of endonucleolytic and exonucleolytic steps. The 35S pre-rRNA is cleaved at site A₀ to generate the 33S pre-rRNA and site A₁ to generate the 32S pre-rRNA. The 32S pre-rRNA is then cleaved at A₂ to produce the 20S and 27SA₂ pre-rRNAs, thereby separating the RNAs destined for the small and large ribosomal subunits. Final maturation of the 20S precursor into the 18S rRNA occurs in the cytoplasm. The 27SA₂ precursor is processed by one of two alternative pathways into the mature 5.8S and 25S rRNAs. These pathways are distinguished by the synthesis of either a long or short version of the 5.8S rRNA (the long version is indicated by the black box). The 27S precursors of both pathways are eventually processed at sites C₁ and C₂ to yield the 7S pre-rRNA and the mature 25S rRNA. Subsequent 3'→5' exonucleolytic digestion of the 7S rRNA generates the mature 5.8S rRNA. Figure modified with permission from Hong *et al.* (1997).

selective inhibitors of fungal ribosome synthesis is limited by insufficient knowledge of the proteins involved in ribosome biogenesis in pathogenic fungi. We have previously identified a yeast nucleolar protein, Cgr1p, that is highly conserved among fungi. Species homologues have been cloned from *S. cerevisiae* (Sun *et al.*, 2001a), *Aspergillus nidulans* (Sun *et al.*, 2001b) and *Aspergillus fumigatus* (Boettner *et al.*, 2001), and putative homologues can be identified in the *Neurospora crassa*, *Candida albicans* and *Schizosaccharomyces pombe* databases. The *A. fumigatus* gene complemented a *S. cerevisiae* cgr1 mutant (Boettner *et al.*, 2001), suggesting that the two genes encode orthologous proteins with overlapping functions. The goal of this study was to use *S. cerevisiae* as a model to determine whether this function involves ribosome synthesis. Here we demonstrate that *S. cerevisiae* Cgr1p is involved in the processing of the pre-rRNA for the 60S ribosome subunit, thus establishing its role in ribosome synthesis and providing a foundation for future studies into ribosome synthesis as a potential drug target in *A. fumigatus*.

METHODS

Strains and culture conditions. The yeast strains used in this study are listed in Table 1. Yeasts were grown on either rich medium [YPD: 1% (w/v) yeast extract, 2% (w/v) peptone,

2% (w/v) dextrose], synthetic medium [SD: 0.67% (w/v) yeast nitrogen base without amino acids, 2% (w/v) dextrose] that was supplemented to meet auxotrophic requirements for each strain, or synthetic drop-out medium (Burke *et al.*, 2000). Generation times were calculated from cultures that were maintained in exponential growth for at least 30 h by diluting whenever the OD₆₀₀ reached 0.7. To measure antibiotic sensitivity, 1×10^7 exponentially growing cells were plated in a top agar overlay [1% (w/v) agarose in YPD] onto YPD plates. A 0.75 cm diameter glass filter disc was then placed on the surface of each plate (Brandel) and 10 µl of a dilution of either paromomycin (Sigma) or hygromycin (Sigma) was spotted onto each disc. The plates were cultured for 4 days at 30 °C and the diameter of the zone of growth inhibition around each filter was used as a measure of drug sensitivity.

Plasmids. A tetracycline-repressible *CGR1* expression plasmid was constructed by amplifying the *CGR1* ORF from yeast genomic DNA (strain YPH250) using the forward primer 5'-CGATGGATCCAGGAATATGGTGAATGAAACAGG-AG-3' and the reverse primer 5'-CGATATCGATCTAACGTTCTTTTAAAGCC-3' (restriction sites are in italic). The PCR product was cloned into the pCM183 plasmid (Gari *et al.*, 1997) at the *Bam*HI and *Cla*I sites of the polylinker to make pDA361 (Table 1). This construction placed the *CGR1* ORF downstream of a hybrid promoter comprising two copies of the *tet* operator sequence linked to the *CYC1* promoter, and upstream of the *CYC1* terminator.

Gel electrophoresis and blotting. For analysis of gene expression, RNA was isolated from yeast cultures by disruption with 0.5 mm glass beads (Fisher Scientific) according

Table 1. Strains and plasmids

Strain or plasmid	Genotype/construction	Source
Strains		
Cry1(Y300)	<i>MATa leu2-3,112 trp1-1 ura3-1 his3-11,15 can1-100</i>	Y. Sanchez, University of Cincinnati
YDA110	<i>MATa leu2-3,112 trp1-1 ura3-1 his3-11,15 can1-100 cgr1Δ81-120::kan^r</i>	This study
YDA128	<i>MATa leu2-3,112 trp1-1 ura3-1 his3-11,15 can1-100 cgr1Δ::kan^r(pDA361)</i>	This study
Plasmids		
pCM183	<i>P_{tetO-CYC1} TRP1 CEN</i>	ATCC
pDA361	pCM183, expressing <i>S. cerevisiae</i> <i>CGR1</i>	This study

to standard procedures. Twenty micrograms of total RNA was fractionated by formaldehyde gel electrophoresis as previously described (Li *et al.*, 1997), transferred to positively charged nylon membranes (MSI) and hybridized to ³²P-labelled DNA probes under stringent conditions in 50 % (v/v) formamide/5 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.6), 2 × Denhardt's solution, 10 % (w/v) dextran sulfate, 1 % (w/v) SDS. Hybridization intensity was quantified with a Phosphorimager (Molecular Dynamics) and normalized for loading by quantitating the relative levels of SYBR green (Molecular Probes) stained 18S rRNA on a Phosphorimager.

Genomic DNA was extracted by cell disruption in the presence of 0.5 mm diameter glass beads (Fisher Scientific) according to standard protocols. For genomic Southern blot analysis, 10 µg digested DNA was fractionated on a 1 % (w/v) agarose gel, transferred to a nylon membrane (MSI) and hybridized to a [³²P]dCTP-labelled probe under conditions of high stringency as previously described (Li *et al.*, 1997). Hybridization was monitored using a Phosphorimager (Molecular Dynamics).

Construction of a *cgr1* truncation mutant and a *cgr1* depletion mutant. A mutant expressing a C-terminal truncation of Cgr1p, YDA110, was constructed by one-step PCR-mediated gene disruption. The truncation cassette was obtained by PCR amplification of the kanamycin-resistance gene (*kan^r*) using plasmid pFA6-3HA-kanMX6 (Wach *et al.*, 1994) as the template and primers containing 50 bp *CGR1*-homologous sequences. The upstream primer was 5'-GATGAGAAGGAAGAAGCTCGTCAAGCTAAAAT-AACCATGTAAAGTGAGGCGCGCCACTTCTAAA-3' and the downstream primer was 5'-TATGAGCCTTCTAT-AATGCTTTATACCATTGTGCTTATCCGAATTGAGCTCGTTTAAAC-3' (sequences in italic are specific to pFA6-3HA-kanMX6). This construction places a stop codon in place of amino acid 81. The wild-type haploid yeast strain *cry1* (Table 1) was transformed with the PCR product, selected on YPD plates containing 200 µg G418 ml⁻¹ (Gibco-BRL) and the transformants were genotyped by PCR using a *CGR1* primer outside the recombination site (5'-ATAACTGTCTAGGGATGCCC-3') and the *kan^r*-specific primer (5'-ATC-GGGCTTCCCATAACAATC-3'). Confirmation of single-copy modification of *CGR1* was obtained by genomic Southern blot analysis of *EcoRI*-digested genomic DNA using *CGR1*- and *kan^r*-specific probes.

To generate a conditional *CGR1* depletion strain, the wild-type haploid strain *cry1* was transformed with pDA361 and

the chromosomal *CGR1* allele was deleted by transformation with a *CGR1* disruption cassette. The disruption cassette was constructed by PCR amplification of the kanamycin-resistance gene (*kan^r*) using plasmid pFA6-kanMX2 (Wach *et al.*, 1994) as the template with primers containing 50 bp *CGR1*-homologous sequences. The upstream primer was 5'-ACTGTCCATATTCATTGAAAGCAAAATAAAACA-TAACCAGGAAATCAGCTGAAGCTTCGTACGC-3' and the downstream primer was 5'-CTGTCTAGGGATGCCCT-TATTTTCTCCTTGTCAGGACTTAATAATGCATAG-GCCACTAGTGGATCTG-3' (sequences in italic are specific to pFA6-3HA-kanMX2). Following selection on G418 plates, the colonies were genotyped by PCR and deletion of the *CGR1* ORF was confirmed by genomic Southern blot analysis, using radiolabelled probes specific for the *CGR1* and *kan^r* genes. Down-regulation of the *tetO-CYC1* promoter in pDA361 was accomplished by supplementing the medium with 2 µg ml⁻¹ of the tetracycline analogue doxycycline, as described by Gari *et al.* (1997).

Pulse-chase analysis of rRNA processing. For the [methyl-³H]methionine pulse-chase experiments, wild-type and YDA110 cells were grown to a density of (1–2) × 10⁷ cells ml⁻¹ in methionine-free media at 25 °C. Wild-type cells containing pCM183 (Table 1) and YDA128 were grown to a density of (2–4) × 10⁷ cells ml⁻¹ in methionine-free media at 25 °C. The cells were diluted into media containing 2 µg doxycycline ml⁻¹ and cultured for 29 h. A total of 10⁸ cells was concentrated to a volume of 1 ml and pulse labelled with 250 µCi (9.25 MBq) [methyl-³H]methionine for 2 min (70–85 Ci mmol⁻¹, 5 mCi ml⁻¹, 185 MBq ml⁻¹; Amersham-Pharmacia Biotech). For the zero time point, 250 µl cells was transferred to a new tube, washed in 1 ml ice-cold media and the cell pellet frozen on dry ice. Chase was initiated by adding unlabelled methionine to the culture at a concentration of 1 mg methionine ml⁻¹. At time points of 2, 5 and 10 min of chase, 250 µl cells were removed and processed as described above. RNA was isolated by the hot acid phenol method (Ausubel *et al.*, 1997). A total of 20000 c.p.m. radioactivity was loaded per lane onto a 1.2 % (w/v) agarose-formaldehyde gel. RNA was transferred to a nylon membrane (Hybond-N⁺, Amersham-Pharmacia), UV-cross-linked and sprayed with En³hance (NEN). The membrane was exposed to film for 1 day at –80 °C.

For [5,6-³H]uracil pulse-chase experiments, wild-type and YDA110 cells containing pRS306 (*URA3* CEN) were grown to a density of 1 × 10⁷ cells ml⁻¹ in media lacking uracil at 25 °C. A total of 5 × 10⁸ cells was concentrated to a volume of 6 ml

and labelled with 100 μ Ci (3.7 MBq) [5,6- 3 H]uracil (30–50 Ci mmol $^{-1}$, 1 mCi ml $^{-1}$, 37 MBq ml $^{-1}$; NEN). After labelling for 3 and 6 min, 1 ml culture was removed, centrifuged, supernatants removed, and cell pellets frozen. Chase was initiated by adding unlabelled uracil to a concentration of 20 mg uracil l $^{-1}$. At time points of 5, 10, 30 and 60 min of chase (for the 6 min label), a 1 ml sample was processed as described above and 5 μ g RNA from each time point was loaded onto a 8% polyacrylamide, 8 M urea gel. RNA was transferred to Hybond-N $^{+}$ membranes by semi-dry electrophoresis (Owl Scientific) and visualized by fluorography as described above. The membrane was exposed to film for 15 days at -80°C .

Polysome profile analysis. Polysome profiles were performed as described by Kressler *et al.* (1997) with the following modifications. Cells were taken from the cultures used for the [methyl- 3 H]methionine pulse-chase experiments described above, and the lysate (4 A_{260} units) was layered onto 10 ml linear 7–49% sucrose gradients. Samples were centrifuged in a Beckman SW41Ti rotor for 2 h at 39000 r.p.m. at 4°C (Beckman Instruments). A Beckman fraction recovery system was used to pass the gradients through a Pharmacia UV-1 monitor to measure A_{254} . Analysis of dissociated ribosomal subunits was performed as described by Kressler *et al.* (1997). Cell extracts were prepared in 50 mM Tris/HCl (pH 7.5), 50 mM NaCl, 1 mM DTT, and 2 absorbance units at 260 nm were layered onto 10–30% sucrose gradients prepared in the same buffer and centrifuged as described above.

Subcellular localization of Nop1p and 5'-ITS1. Nop1 was localized with anti-Nop1 monoclonal antibodies as previously described (Moy & Silver 1999). 5'-ITS1 rRNA was localized by fluorescence *in situ* hybridization as described by Amberg *et al.* (1992) with the following modifications. An oligonucleotide complementary to the first 50 bases of ITS1 rRNA, 5'-ATGCTCTTGCCAAACAAAAAATCCATTTTCAAATTATTAATTTCTT-3', was synthesized with the Cy3 fluorophore at its 5' end (IDT). Samples were hybridized with this oligonucleotide at a concentration of 50 nM.

RESULTS

Mutants of *CGR1* are growth impaired

We have previously reported that *CGR1* is essential in the YPH274 background (Sun *et al.*, 2001a). To date, we have microdissected a total of 64 tetrads from a *CGR1/cgr1 Δ* heterozygote constructed in the YPH274 strain, each indicating that spore viability segregates with the wild-type allele. A slow-growing *cgr1 Δ* mutant has recently been reported using meiotic segregation analysis by the yeast deletion project (Winzeler *et al.*, 1999), suggesting that Cgr1p may be non-essential in some strains. As a first step to determine if Cgr1p has a role in ribosome synthesis in *S. cerevisiae*, we constructed two haploid mutants that were deficient in Cgr1p function: a conditional Cgr1p depletion mutant and a C-terminal *cgr1* truncation mutant. The depletion strain, YDA128, was created by deleting the chromosomal *CGR1* ORF in a haploid strain that contained a plasmid-borne *CGR1* gene under the control of a tetracycline-repressible promoter (see Methods). Genomic Southern blot analysis of YDA128 showed homologous targeting of the *CGR1* allele (data not shown). Northern blot analysis was used to confirm that YDA128

Fig. 2. Doxycycline-induced repression of *CGR1* expression in YDA128. Wild-type cells (containing the empty pCM183 plasmid) and YDA128 cells were seeded at an OD_{600} of 0.1 in SD-trp medium and maintained in exponential phase by diluting into fresh medium whenever the culture density reached an OD_{600} of 0.7. RNA was isolated at the indicated times and analysed for *CGR1* expression by Northern blot analysis. The first lane (WT) contains RNA from an exponential-phase culture of wild-type cells. The remaining four lanes contain equivalent amounts of RNA isolated from exponential cultures of YDA128 grown either in the absence of doxycycline (0) or in medium containing 2 μg doxycycline ml $^{-1}$ for 6, 15 and 30 h. Phosphorimager analysis of the SYBR-green-stained gel showed intact rRNA in all lanes, and the relative levels of the 18S rRNA were used to normalize hybridization intensity for RNA loading.

cells treated with doxycycline were expressing levels of *CGR1* that were below the levels seen in wild-type cells. In the absence of doxycycline, exponential cultures of YDA128 expressed 17-fold more *CGR1* RNA than wild-type cells (Fig. 2, compare wild-type with the zero time point for YDA128). However, within 6 h of treatment with doxycycline, *CGR1* expression was repressed (Fig. 2), falling over 10-fold below the levels observed in exponentially growing wild-type cells. When overnight cultures of wild-type and YDA128 cells were diluted into fresh medium containing doxycycline, and maintained in exponential phase by diluting whenever the culture reached an OD_{600} of 0.7, the cultures initially grew with the same generation time of 2.3 h. However, after 12–15 h in doxycycline, the generation time of YDA128 was increased to 5.5 h (Fig. 3). Doxycycline had no effect on the growth rate of wild-type cells at the concentrations used in this experiment (data not shown). Since extended exposure of the Northern blot shown in Fig. 2 showed that some *CGR1* expression remained in the doxycycline-treated YDA128 cells, this promoter was not completely repressed in the presence of doxycycline. However, the results indicate that wild-type levels of *CGR1* mRNA are required for optimal growth, thus providing an opportunity to determine how reduced *CGR1* mRNA affects ribosome synthesis.

A second mutant strain, YDA110, was constructed by introducing a stop codon into the chromosomal *CGR1* gene, thereby truncating the expressed protein at amino acid 81. Homologous targeting of the *CGR1* allele was confirmed by genomic Southern blot analysis (data not shown). This mutant was also growth impaired (generation time 3.8 h), but to a lesser extent than doxycycline-treated YDA128 cells (generation time 5.5 h), suggesting that the truncated protein has residual activity. These results indicate that a truncated Cgr1p is

Fig. 3. Cgr1p deficiency impairs growth. Overnight cultures of wild-type and YDA128 cells were seeded into YPD medium containing $2 \mu\text{g}$ doxycycline ml^{-1} and diluted whenever the culture attained an OD_{600} of 0.7 to maintain exponential growth. Wild-type: *cry1* cells containing the empty pCM183 plasmid (Table 1).

Fig. 4. Cells expressing a truncated Cgr1p show increased sensitivity to translational inhibitors. Filter paper circles containing $10 \mu\text{l}$ of an antibiotic concentration were placed onto a YPD plate containing 1×10^7 wild-type (a and c) or YDA110 (b and d) cells. (a) and (b) contain hygromycin (top left, 0 mg ml^{-1} ; top right, 50 mg ml^{-1} ; bottom left, 100 mg ml^{-1} ; bottom right, 250 mg ml^{-1}). (c) and (d) contain paromomycin (top left, 0 mg ml^{-1} ; top right, 50 mg ml^{-1} ; bottom right, 200 mg ml^{-1} ; bottom left, 500 mg ml^{-1}). Wild-type, Cry1.

insufficient to support optimal growth, thus providing an independent mutant in which to assess the consequences of impaired Cgr1p function on ribosome synthesis.

Fig. 5. Cgr1p is required for normal synthesis of 60S ribosomal subunits. Cells were harvested from exponential-phase cultures and polysome profiles were resolved in sucrose gradients. Peaks corresponding to free 40S and 60S subunits, 80S couples and polysomes are indicated. Arrows indicate the presence of half-mers in YDA110 cells.

***cgr1* mutants are hypersensitive to translation inhibitors**

Yeast mutants deficient in ribosome assembly or function often display increased sensitivity to aminoglycoside antibiotics (Zanchin *et al.*, 1997; Benard *et al.*, 1998; Ho & Johnson, 1999; Dresios *et al.*, 2000). We have previously shown that a strain expressing *CGR1* from a glucose-repressible promoter is hypersensitive to the aminoglycoside paromomycin under repressing conditions (Sun *et al.*, 2001a). To determine whether the *cgr1* truncation mutant also confers increased susceptibility to translational inhibitors, the sensitivity of YDA110 cells to paromomycin and hygromycin was examined. Glass filter discs containing various concentrations of each antibiotic were placed onto the surface of a plate of confluent cells and cultured for 4 days at 30°C . As shown by the zone of growth inhibition surrounding the filter paper discs, strain YDA110 was more sensitive to both of these antibiotics than wild-type cells (Fig. 4), indicating that inhibitors of translational fidelity synergize with loss of Cgr1 function to inhibit growth.

***cgr1* mutants are deficient in the synthesis of 60S ribosomal subunits**

To determine if Cgr1p has a role in ribosome synthesis, the polysome profile of each *cgr1* mutant was analysed on sucrose density gradients and compared to that of wild-type cells. As shown in Fig. 5, wild-type cells showed the typical profile of 40S and 60S subunits, the 80S monosome, and polysome peaks representing 2–8 ribosomes. By contrast, YDA110 cells showed a reduction in the level of the 60S subunit relative to free 40S subunits, a reduction in the level of the 80S monosome peak, an overall decrease in the mean number of ribosomes in polysomes, and the appearance of half-mer polysomes (Fig. 5). Half-mer polysomes, evident as

Fig. 6. Pulse-chase labelling indicates a role for Cgr1p in pre-rRNA processing. Cells were pulse-labelled with [methyl- ^3H]methionine for 2 min and then chased with unlabelled methionine for 2, 5 and 10 min. Total RNA was extracted, separated on an agarose-formaldehyde gel and visualized by fluorography following transfer to a nylon membrane. (a) Wild-type (Cry1) and YDA110 cells grown in YPD. (b) Wild-type (Cry1 cells containing pCM183) and YDA128 cells grown for 29 h in YPD containing 2 μg doxycycline ml^{-1} . The positions of the mature rRNAs and their precursors are indicated.

small peaks following the 80S and first two polysome peaks, represent 43S pre-initiation complexes composed of mRNA, the 40S subunit and initiation factors (Helser *et al.*, 1981). The reduction in the level of the 60S subunit, and presence of half-mers, suggested a stoichiometric imbalance between 60S and 40S subunits. This was confirmed by examining the relative amounts of total 60S and 40S subunits under conditions that cause dissociation of the subunits. A normal ratio of 60S:40S subunits of around 2 was observed for wild-type cells. By contrast, the ratio of 60S:40S subunits in YDA110 cells was approximately equivalent, confirming a disruption in 60S:40S stoichiometry (data not shown). YDA128 cells depleted of *CGR1* by growth in doxycycline for 24 h showed the same polysome profile as YDA110 cells (data not shown), indicating that either Cgr1p truncation, or a decrease in the levels of the wild-type protein, adversely affects the synthesis of the 60S subunit.

Cgr1p is required for normal pre-rRNA processing

The polysome profiles of the *cgr1* mutant shown in Fig. 5 indicated that Cgr1p is required for the maintenance of normal 60S ribosome subunit levels. Since a decrease in the level of a ribosome subunit is often caused by a defect in pre-rRNA processing and/or assembly of ribosomal subunits (Kressler *et al.*, 1999; Venema & Tollervey, 1999), we examined the synthesis and processing of pre-rRNA in each of the mutants. The major pre-rRNA processing pathways in *S. cerevisiae* are depicted in Fig. 1. Since the 18S and 25S rRNAs are highly methylated, their synthesis and processing can be readily followed by metabolic labelling with [methyl- ^3H]methionine (Warner, 1991). Exponentially growing cultures in medium lacking methionine were pulse-labelled for 2 min with [methyl- ^3H]methionine and chased for 2, 5 and 10 min with an excess of cold methionine. Total RNA was extracted from the cells, fractionated by formaldehyde-agarose gel electrophoresis and analysed for the presence of pre-rRNA species as described in Methods. As expected, the wild-type 35S and 32S precursors disappeared within 2 min of the chase (Fig. 6a), indicating normal and rapid processing

to the 27S and 20S intermediates. After 5 min chasing, the majority of the label was present in the mature 25S and 18S rRNAs, and by 10 min only the mature rRNAs were evident (Fig. 6a). By contrast, the 27S pre-rRNA accumulated in the YDA110 strain and only a small amount of this intermediate was processed into the 25S rRNA, even after 10 min chasing (Fig. 6a). Consistent with this delay in 27S pre-rRNA processing was a slight reduction in the steady-state levels of the mature 25S rRNA (data not shown). The processing of the 35S pre-rRNA was also slightly delayed, but since there was no accumulation of the 20S pre-rRNA, and the appearance of the 18S rRNA occurred with the same kinetics as wild-type, we conclude that the truncated Cgr1 protein was interfering with processing of the 27S rRNA.

To determine if the effect on 25S rRNA synthesis was due to a general loss of Cgr1 function or to an abnormal effect of the truncated protein, the same experiments were performed on cells depleted of Cgr1p. Exponential cultures of YDA128 grown in the presence of doxycycline for 29 h were pulse-chase labelled with [methyl- ^3H]methionine and analysed for processing of the 35S pre-rRNA as described above. As shown in Fig. 6(b), wild-type cells containing the empty tetracycline-repressible plasmid processed the 35S pre-rRNA with the same kinetics as wild-type cells, indicating that doxycycline treatment did not adversely affect pre-rRNA processing. By contrast, YDA128 cells depleted of Cgr1p showed the same defect in the processing of the 27S pre-rRNA as YDA110 cells, with normal processing of the 20S pre-rRNA (Fig. 6b). A small amount of the 23S rRNA may be increased in YDA110 and YDA128 after 5 min chasing (Fig. 6a, b). The 23S pre-rRNA is an unusual processing intermediate leading to the production of the 18S rRNA and is generated by cleavages A_0 and A_3 (Russell & Tollervey, 1992). This pathway is infrequently used in wild-type yeast, but increased levels of the 23S rRNA have been associated with depletion of other proteins involved in pre-rRNA processing (Tollervey *et al.*, 1991; Girard *et al.*, 1992; Hong *et al.*, 1997). Taken together, these results indicate that the primary defect associated with loss of Cgr1p function, either through protein truncation or depletion, is in the

Fig. 7. Pulse-chase labelling of pre-rRNA processing in YDA110 using [^3H]uracil. Wild-type and YDA110 cells were pulsed with [^3H]uracil for 3 min (p3') or 6 min (p6'), followed by a chase with unlabelled uracil for 5, 10, 30 and 60 min (with the 6 min label). Total RNA was extracted, separated on a polyacrylamide-urea gel and visualized by fluorography following transfer to a nylon membrane. The positions of the pre-rRNA, mature rRNAs and tRNAs are indicated.

formation of the 25S rRNA from the 27S pre-rRNA intermediate.

The defect in 27S pre-rRNA processing in YDA110 and YDA128 predicted that the formation of the 5.8S rRNA should also be adversely affected (Fig. 1). We therefore examined 5.8S rRNA synthesis in YDA110 cells by pulse labelling with [^3H]uracil for 3 or 6 min followed by a chase with an excess of cold uracil for 5, 10, 30 and 60 min (for the 6 min pulse). In wild-type cells, both forms of the 5.8S rRNA were evident within 5 min of chasing (Fig. 7), indicating a normal rate of processing. By comparison, the formation of the 5.8S rRNA was delayed in YDA110 cells (Fig. 7), consistent with the defect in 27S pre-rRNA processing that was observed in this strain (Fig. 6). The processing of the 7S pre-rRNA was also delayed in YDA110 (Fig. 7), suggesting that Cgr1p may have a role in the processing of this intermediate into the mature 5.8S rRNA. The 5S rRNA is transcribed from a separate transcription unit by RNA polymerase III (Raué & Planta, 1991) and although it forms a stable complex with rpL1, and is incorporated into the 60S subunit (Deshmukh *et al.*, 1993), this experiment showed no major defects in 5S rRNA synthesis in YDA110 cells (Fig. 7).

Cgr1p contributes to nucleolar compartmentalization

Current evidence indicates that the nucleolar compartment is formed by the recruitment of processing factors by direct or indirect interactions with rDNA or the nascent RNAs (reviewed by Melèse & Xue, 1995;

Fig. 8. Loss of Cgr1p function impairs nucleolar localization of Nop1p and 5'-ITS1. (a) Anti-Nop1p staining of wild-type and YDA110 cells shows increased levels of Nop1p in the nucleoplasm of YDA110 cells compared to wild-type (arrows). (b) Localization of 5'-ITS1 RNA by *in situ* hybridization of wild-type and YDA110 cells shows increased amounts of 5'-ITS1 in the nucleoplasm of YDA110 cells relative to wild-type (arrows). The DAPI-stained nucleus and Nomarski image for each figure is shown for comparison. Wild-type: Cry1.

Carmo-Fonseca *et al.*, 2000). To determine whether Cgr1p has a role in maintaining the integrity of the nucleolar compartment, we examined the localization of the nucleolar protein Nop1p in the *cgr1* truncation mutant. Nop1p is an essential nucleolar protein involved in pre-rRNA processing and ribosomal subunit assembly (Tollervey *et al.*, 1993) and has been shown to co-localize with Cgr1p in the nucleolus (Sun *et al.*, 2001a). In wild-type cells, the vast majority of Nop1p localized to the nucleolar region of the nucleus, with very little of the protein in the nucleoplasm (Fig. 8a). Although Nop1p was also found in the nucleolus in YDA110 cells, there was a notable increase in the amount that was present in the nucleoplasm (Fig. 8a, arrows), suggesting that the ability of Nop1p to accumulate within the nucleolus was partially impaired in this mutant.

To determine whether this impaired localization was specific to Nop1p, we examined another nucleolar

marker, 5'-ITS1. In *S. cerevisiae*, the 20S rRNA is assembled as part of the 43 pre-ribosome in the nucleolus prior to export to the cytoplasm. Once in the cytoplasm the 20S rRNA is cleaved by an endonuclease to the mature 18S rRNA and the released 5'-ITS1 spacer sequence is rapidly degraded by Xrn1p (Stevens *et al.*, 1991). The location pattern of the 5'-ITS1 RNA is an indicator of the assembly and nuclear export of the small ribosomal subunit and 5'-ITS1 can be detected by *in situ* hybridization with a Cy3-labelled 5'-ITS1 probe (Moy & Silver, 1999). In wild-type cells, the majority of the 5'-ITS1 RNA was found in the nucleolar crescent (Fig. 8b). By contrast, YDA110 cells showed a relative increase in the amount of 5'-ITS1 RNA in the nucleoplasm (Fig. 8b, arrows). Taken together, these results suggest that loss of Cgr1p function adversely affects the compartmentalization of these nucleolar constituents.

DISCUSSION

Although rDNA transcription, rRNA processing and ribosome assembly are the best characterized functions of the nucleolus, there is increasing appreciation for this organelle as a site for other cellular activities. The nucleolus has been implicated in the nuclear export of mRNAs (Kadowaki *et al.*, 1994; Schneider *et al.*, 1995; Tani *et al.*, 1995), in the processing of tRNA (Bertrand *et al.*, 1998) and in the synthesis of the RNA components for the signal recognition particle and the enzyme telomerase (Fang & Cech, 1995; Jacobson & Pederson, 1998; Grosshans *et al.*, 2001). Furthermore, at least two yeast cell cycle regulatory proteins are sequestered in the nucleolus away from their known targets, thereby restricting functional activity to specific points in the cell cycle (San-Segundo & Roeder, 1999; Shou *et al.*, 1999; Visintin *et al.*, 1999). Finally, recent evidence has shown that some yeast nucleolar proteins are potent regulators of the pace of cellular senescence and in the silencing of rDNA (Gotta *et al.*, 1997; Kennedy *et al.*, 1997; Kaeberlein *et al.*, 1999; Gartenberg, 2000). The nucleolus is thus a plurifunctional organelle and the proteins that localize to this compartment may have functional activities outside the nucleolus.

To investigate the possibility that the nucleolar localization of *S. cerevisiae* Cgr1p is due to its role in yeast ribosome synthesis, we have examined mutants deficient in Cgr1 function for abnormalities in ribosomal subunits and pre-rRNA processing. Each of the two *cgr1* mutants generated for this study was deficient in the steady-state levels of 60S ribosome subunits, resulting in abnormally high levels of the 40S subunit and an increase in half-mer polysomes. Similar profiles have been described for mutants defective in components required for pre-rRNA processing or 60S subunit assembly (Ripmaster *et al.*, 1992; Hong *et al.*, 1997; de la Cruz *et al.*, 1998a; Basu *et al.*, 2001), suggesting that Cgr1p is also involved in this pathway.

Analysis of pre-rRNA processing showed that the deficit in 60S subunits in the *cgr1* mutants was attributable to a

primary defect in 27S pre-rRNA processing, thereby affecting the synthesis of the mature 25S and 5.8S rRNAs. The role of Cgr1p in 25S/5.8S rRNA synthesis appears to be specific because synthesis of the 18S rRNA and 5S rRNAs was unaffected in either of the *cgr1* mutants. This places Cgr1p in a category of proteins that influence the production of the 25S rRNA (Moritz *et al.*, 1991; Russell & Tollervey, 1992; Deshmukh *et al.*, 1993; Berges *et al.*, 1994; Fabian & Hopper 1997; Hong *et al.*, 1997; Basu *et al.*, 2001). However, in addition to the major effects on the synthesis of the 25S/5.8S rRNAs, we also observed delayed processing of the 35S pre-rRNA and accumulation of the 7S intermediate. This suggests that Cgr1p may influence several steps in the pathway to ribosome synthesis, for which there is precedence among factors involved in pre-rRNA processing and ribosome assembly (Kressler *et al.*, 1999), although it is also conceivable that the effect is indirect. It has been suggested that a delay in processing of 35S pre-rRNA may be a consequence of a negative feedback mechanism to slow production of the 18S rRNA whenever the formation of the 25S/5.8S rRNA is inhibited (Zanchin *et al.*, 1997; de la Cruz *et al.*, 1998b). However, the delay in 35S pre-rRNA processing observed in these *cgr1* mutants had little effect on the overall synthesis of the 18S rRNA.

The phenotype of the *cgr1* mutants resembles the phenotype described for a mutant of Dob1p, a putative ATP-dependent RNA helicase. Similar to *cgr1* mutants, the *dob1* mutant has a defect in the synthesis of the 5.8S and 25S rRNAs, a reduction in 60S subunits and an increase in 7S rRNA (de la Cruz, 1998b). The processing of the 7S rRNA involves the activity of a complex of 3'→5' exonucleases called the exosome, and Dob1p acts as a cofactor for this complex (Allmang *et al.*, 1999). The similarity between the *dob1* and *cgr1* mutants raises the possibility that Cgr1p has a direct or indirect effect on the activity of the exosome.

The yeast nucleolus occupies a crescent-shaped region of the nucleus that is approximately one-third of the total nuclear volume (Warner, 1982). The assembly of the nucleolus is thought to involve the recruitment of various processing factors through direct or indirect interactions with rDNA, nascent rRNAs, or nucleolar proteins that already reside in the nucleolus (Melèse & Xue, 1995; Carmo-Fonseca, 2000). For example, Net1p preferentially binds to rDNA and is required for the recruitment of the silencing protein Sir2p into the nucleolus (Straight *et al.*, 1999). In *net1Δ* cells, Nop1p redistributes over the nucleus, suggesting that Net1p is required to maintain the integrity of the nucleolar compartment. Our results indicate that cells deficient in Cgr1p are unable to retain all Nop1p and 5'-ITS1 in the nucleolar region and this would also be consistent with a role for Cgr1p in nucleolar integrity.

In contrast to highly conserved nucleolar proteins such as Nop1p (Tollervey *et al.*, 1991, 1993), there are no clear homologues of Cgr1p in higher eukaryotes, including *Drosophila melanogaster* and *Caenorhabditis elegans*, raising the possibility that Cgr1p is either fungal

specific or highly divergent in metazoan species. If the *A. fumigatus* orthologue of Cgr1p proves to be essential for the growth of *A. fumigatus*, its apparent divergence in higher eukaryotes would make it an attractive antifungal target. Further understanding of the role for Cgr1p in fungal ribosome synthesis awaits mechanistic detail on how this protein contributes to pre-rRNA processing.

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