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The Yeast Nucleolar Protein Cbf5p Is Involved in rRNA Biosynthesis and Interacts Genetically with the RNA Polymerase I Transcription Factor *RRN3*

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Yeast Cbf5p was originally isolated as a low-affinity centromeric DNA binding protein (W. Jiang, K. Middleton, H.-J. Yoon, C. Fouquet, and J. Carbon, Mol. Cell. Biol. 13:4884–4893, 1993). Cbf5p also binds microtubules in vitro and interacts genetically with two known centromere-related protein genes (*NDC10/CBF2* and *MCK1*). However, Cbf5p was found to be nucleolar and is highly homologous to the rat nucleolar protein NAP57, which coimmunoprecipitates with Nopp140 and which is postulated to be involved in nucleolar-cytoplasmic shuttling (U. T. Meier, and G. Blobel, J. Cell Biol. 127:1505–1514, 1994). The temperature-sensitive *cbf5-1* mutant demonstrates a pronounced defect in rRNA biosynthesis at restrictive temperatures, while tRNA transcription and pre-rRNA and pre-tRNA cleavage processing appear normal. The *cbf5-1* mutant cells are deficient in cytoplasmic ribosomal subunits at both permissive and restrictive temperatures. A high-copy-number yeast genomic library was screened for genes that suppress the *cbf5-1* temperature-sensitive growth phenotype. *SYC1* (suppressor of yeast *cbf5-1*) was identified as a multicopy suppressor of *cbf5-1* and subsequently was found to be identical to *RRN3*, an RNA polymerase I transcription factor. A *cbf5*Δ null mutant is not rescued by plasmid pNOY103 containing a yeast 35S rRNA gene under the control of a Pol II promoter, indicating that Cbf5p has one or more essential functions in addition to its role in rRNA transcription.

Cbf5p of the yeast *Saccharomyces cerevisiae* was originally isolated as one of the major low-affinity centromeric DNA (*CEN*) binding proteins (23). *CBF5* is an essential gene encoding a highly charged protein with a domain containing ten tandem KKE/D repeats. These repeats are homologous to portions of microtubule-associated proteins 1A and 1B in the domain responsible for microtubule binding (40), and Cbf5p has been shown to bind microtubules in vitro (23). Yeast cells containing C-terminal truncated *CBF5* genes delay, with replicated genomes, at the G_2/M phase of the cell cycle, with the replicated DNA being located at the bud junction (23). Overexpression of Cbf5p suppresses the *ndc10-1* temperature-sensitive (ts) mutation in the gene specifying the 110-kDa subunit (Cbf2p/Ndc10p) of the multisubunit yeast centromere DNA binding complex, CBF3, and overexpression of meiosis and centromere regulatory kinase Mck1p suppresses a ts mutation in the gene for either Cbf2p or Cbf5p (22). These genetic interactions support a direct or indirect link between Cbf5p and centromeres. However, both Cbf5p and a homologous protein (NAP57) from rats were found to be nucleolar proteins (21, 33). While a functional relationship between a nucleolar protein and centromeres is not obvious, some proteins are associated with both the nucleolus and the centromere in higher eukaryotes. It has been known for some time that centromere autoantigens associate with the nucleolus (43), and experiments with autoimmune sera have also shown that a set of nucleolar proteins and ribonuclear proteins relocate around chromosomes during mitosis (16, 17).

The nucleolar location of Cbf5p may be indicative of a function unrelated to centromeres and chromosome segregation, since the major role of the nucleolus is the transcription, maturation, and packaging of rRNAs into ribosomal particles, (see references 35 and 49 for reviews). Within nucleoli, rRNA is transcribed, primary transcripts are processed to mature 25S, 18S, and 5.8S rRNAs, and ribosomal proteins are assembled with these rRNAs and the 5S rRNA to ultimately form the near-mature 40S and 60S ribosome subunits. The nucleolus also contains many nonribosomal RNA and protein components that can generally be grouped as structural proteins, rRNA processing proteins, or shuttle proteins (35, 49).

Cbf5p is a highly conserved protein with homologs now identified in 13 organisms, although the sequences of only ten of these are currently available in databases. The homolog in rats (NAP57) is 71% identical to Cbf5p over 384 amino acids and was isolated by coimmunoprecipitation with the nucleolar shuttling protein, Nopp140 (33, 34). Detailed immunolocalization experiments with rat cells, including immunofluorescence and immunoelectron microscopy, showed NAP57 to be localized to extranucleolar dots which were identified as coiled bodies. Coiled bodies store small nuclear ribonuclear proteins (snRNPs) and are sometimes associated with the nucleolus (1, 6). As yet, no specific function has been assigned to coiled bodies, but snRNPs are involved in various RNA-processing reactions in the nucleoplasm and the nucleolus (10, 14, 45). The nucleolus itself is a membrane-free organelle which can be described morphologically in mammalian cells as consisting of one or more fibrillar centers, each bounded by a dense fibrillar component (DFC) surrounded by the granular component which makes up the bulk of the nucleolus (48). Immunoelectron microscopy studies revealed that NAP57 was mostly concentrated in the DFC of the nucleolus surrounding the fibrillar center, which was devoid of gold particles (33). The DFC is the site where the early stages of rRNA processing and protein-RNA assembly are thought to occur. Interestingly, gold particles fused to either anti-NAP57 or anti-Nopp140 antibodies were occasionally seen on "curvilinear tracks" extending from the nucleolus to the nuclear envelope, suggesting a role for

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NAP57, as well as for Nopp140, in nucleocytoplasmic transport (33, 34). Because of NAP57's location and its association with Nopp140, a role as a chaperone of ribosomal proteins and/or a role in preribosome assembly has been proposed for it (33). And because of the high degree of sequence conservation between NAP57 and yeast protein Cbf5p, Cbf5p may have similar functions.

Several prokaryotes have been shown to contain putative sequence homologs of Cbf5p (42). These proteins compose the TruB family of enzymes, which show homology to various uridine-binding proteins (27). TruB in *Escherichia coli* specifies a pseudouridine (Ψ) synthase for nucleotide 55 in tRNA (42). The role of Ψ in tRNA and rRNA is unclear, but in tRNA Ψ bases may serve to stabilize RNA structure (2). In rRNA, Ψ residues are clustered at or near the peptidyltransferase center of the ribosomes, suggesting that they carry out a function in peptide bond formation (4, 5).

In eukaryotes, rRNA genes are transcribed in the nucleolus by RNA polymerase I (pol I) to produce a large precursor rRNA that must undergo several exo- and endonucleolytic cleavage events that yield mature rRNA species. In yeast, the 35S pre-rRNA is processed to mature 18S rRNA for the 40S ribosomal subunit and to 25S and 5.8S rRNAs that are incorporated into the 60S ribosomal subunit (55). The 5S rRNA is transcribed separately by pol III (55). rRNA is also subjected to specific methylation on both riboses and bases, as well as to pseudouridylation, with both occurring largely prior to cleavage events (5, 55). The nucleolar localization of Cbf5p prompted us to examine the in vivo synthesis of rRNA and ribosomal subunits in a yeast strain containing the *cbf5-1* ts mutation. We present evidence here, based on several types of in vivo rRNA-labeling studies at the restrictive temperature, that yeast cells bearing a ts mutation in the gene for Cbf5p are defective for the biosynthesis of rRNA produced by pol I but not for the synthesis of 5S rRNA or tRNA produced by pol III. The processing (cleavage) of pre-rRNA and tRNA appears to be unaffected in the mutant. The *cbf5-1* cells are also deficient in steady-state levels of mature cytoplasmic ribosomes, as judged by ribosome profile experiments.

In addition, a genetic approach was used to identify proteins interacting with Cbf5p. A screen for multicopy dosage suppressors of *cbf5-1* identified a yeast gene (*SYC1*, for suppressor of yeast *cbf5-1*) that, when overexpressed, relieves the *cbf5-1* ts growth defect. The *SYC1* gene is essential for yeast viability and recently was found to be identical to *RRN3*, a gene specifying a pol I transcription factor (56). The *RRN* group of genes is specifically required only for pol I-dependent rRNA transcription, in that loss of function is relieved by the presence of 35S rRNA genes driven by a pol II-dependent promoter (41). The group consists of at least 12 members, and the proteins characterized to date include pol I subunits and various transcription factors. In vitro, *SYC1/RRN3* binds pol I directly, independent of the DNA template, and serves to recruit pol I to the promoter (56). Our experiments indicate that Cbf5p is not a member of the *RRN* group of genes, although our molecular and genetic experiments indicate it is required for efficient rRNA biosynthesis. Therefore, Cbf5p must have an additional function in vivo that is essential for cell viability.

MATERIALS AND METHODS

Plasmids and yeast strains. Plasmids used include the following: pBluescriptII KS− (Stratagene), pNEB193 (New England Biolabs), pYES2 (Invitrogen; 2μm plasmid with *GAL1* promoter and *URA3* marker), pRS315 and pRS316 (*CEN6* with either *LEU2* or *URA3* marker, respectively), pBS-URA3 (pBluescriptII KS− with *URA3* on a *Hin*dIII fragment), pSEY18 and pYHY18-CBF5 (described in reference 22), 4B2 (the original *SYC1*-containing plasmid isolated from the YEp13-based library screen for *cbf5-1* ts suppressors), and pSub1 (2.7-kb *SYC1*-containing fragment from the 4B2 minilibrary in vector YEp13; isolated in a secondary screen for suppression). Plasmid pSub2 was created by cutting a 2.3-kb fragment containing *SYC1* from 4B2 by using the *Bam*HI site in the vector and the first *Xba*I site downstream of the *SYC1* open reading frame and ligating the fragment into pNEB193 by using the same cleavage sites; pSub3 was created by cutting the 2.3-kb fragment containing *SYC1* from pSub2 by using the *Bam*HI-*Sph*I sites in the polylinker and ligating the fragment to YEp13 digested with the same restriction enzymes; pSubχ was prepared by isolating the 390-bp fragment after digesting 4B2 with *Xba*I and ligating it into pBluescriptII KS− digested with *Xba*I; pKO/TRP1 was created by replacing the *Eco*RV-*Nde*I fragment of *SYC1* in pSub2 with the 980-bp *Bam*HI-*Sal*I *TRP1* gene from pJJ246 (24) after both the vector and the insert were blunted with Klenow polymerase plus dNTPs; pSYC1-YES2, pSYC1-315, and pSYC1-316 have the *SYC1* gene on a 2.3-kb *Bam*HI-*Xba*I fragment from pSub2 cloned into the *Bam*HI-*Xba*I sites of pYES2, pRS315, and pRS316, respectively. Plasmids pDK16 and pNOY103 have a 35S rRNA gene fused to either a *CUP1* promoter (30) or a *GAL7* promoter (41), respectively. The *syc1* ts plasmids and alleles are described in a later section.

Yeast strains used are as follows. YPH274 (*MAT***a**/*MAT*α *ura3-52/ura3-52 lys2-801*^{amber}/*lys2-801*^{amber} *ade2-101*^{ochre}/*ade2-101*^{ochre} *trp1-Δ1/trp1-Δ1 his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1* [Yeast Genetic Stock Center]), CG379 (*MAT*α *ade5 his7-2 leu2-3 leu2-112 trp1-289*^{amber} *ura3-52* [Yeast Genetic Stock Center]), YHY64α1 (*MAT*α *ura3-52 lys2-801*^{amber} *ade2-101*^{ochre} *trp1-Δ1 his3-Δ200 leu2-Δ1 cbf5-1*), and YHY64a1-*MAT***a** (isogenic with YHY64α1 except for mating type) were described previously (22); YWJ64-ts (*MAT***a**/*MAT*α *ura3-52/ura3-52 lys2-801*^{amber}/*lys2-801*^{amber} *ade2-101*^{ochre}/*ade2-101*^{ochre} *trp1-Δ1/trp1-Δ1 his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1 cbf5-1/cbf5-1*) was created as described previously (22); YCC32 (*MAT*α *ade5 his7-2 leu2-3 leu2-112 trp1-289*^{amber} *ura3-52 syc1::TRP1*) with plasmid pSYC1-YES2 was created in this study; YCC90 and YCC91 are isogenic with YCC32 except that the pSYC1-YES2 plasmid carrying the wild-type *SYC1* gene has been cured by selection on 5′-fluoro-orotic acid (5′-FOA) plates and that the plasmid with either the *syc1-8* ts allele (pTS-8) or the *syc1-16* ts allele (pTS-16), respectively, has been introduced by transformation; YCC95 and YCC96 are isogenic with CG379 except that they contain the *syc1-8* or *syc1-16* ts alleles, respectively, integrated at the *SYC1* locus in the genome; YHY11-3 (*MAT***a** *ura3-52 lys2-801*^{amber} *ade2-101*^{ochre} *trp1-Δ1 his3-Δ200 leu2-Δ1 cbf5::HIS3*) was described previously (22); YCC109 (*MAT***a** is isogenic with YHY11-3 except that it carries plasmid p64-FAT10; YHYK17-8 (*MAT***a**/*MAT*α *ura3-52/ura3-52 lys2-801*^{amber}/*lys2-801*^{amber} *ade2-101*^{ochre}/*ade2-101*^{ochre} *trp1-Δ1/trp1-Δ1 his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1 CBF5/cbf5::HIS3*) is the diploid strain heterozygous for *CBF5* that was sporulated to obtain YHY11-3; NOY397 (*MAT***a**/*MAT*α *ade2-1/ade2-1 ura3-1/ura3-1 his3-11/his3-11 trp1-1/trp1-1 leu2-3,112/leu2-3,112 can1-100/can1-100*) (57) was used as a parent strain to produce YCC130 (*MAT***a**/*MAT*α *ade2-1/ade2-1 ura3-1/ura3-1 his3-11/his3-11 trp1-1/trp1-1 leu2-3,112/leu2-3,112 can1-100/can1-100 CBF5/cbf5::TRP1*); and 272 *MAT*α *lys1* and 272 *MAT***a** *lys1* were used as mating tester strains.

^{3}H pulse-chase studies. The tRNAs of the mutant *cbf5-1* strain (YWJ64-ts) and the *CBF5* wild-type strain (YPH274) were analyzed by a [5,6-^{3}H]uracil pulse-chase procedure carried out essentially as described previously (19), followed by isolation of RNA, electrophoresis, and fluorography of the labeled RNA as described previously (18). The only variation was that the cells were incubated for 1 h at 38°C, pulse-labeled for 1 h at 38°C, and chased with an excess of cold uracil (final concentration of 40 μg/ml) for 1 h at 38°C. Equal amounts of radioactivity were loaded onto the gel for each sample.

Pulse-chase labeling of pre-rRNA was done essentially as described previously (52), except that 4 ml of YPH274 or YWJ64-ts cells growing at an optical density at 600 nm (OD_{600}) of ~0.6 to 0.7 were preincubated at 38°C for 1 h and then 133 μCi of either L-[*methyl*-^{3}H]methionine (80 Ci/mmol) or [5,6-^{3}H]uracil (50 Ci/mmol) was added. Pulse time was 3 min at 38°C, and then either a large excess of unlabeled methionine (final concentration, 5 mM) or uracil (final concentration, 240 μg/ml) was added. The chase times were extended to 30 min, and 1-ml samples were taken at 1, 10, 20, and 30 min after addition of the chase. Cells were pelleted for 10 s in a microcentrifuge, the media were aspirated off, and the cells were immediately frozen on dry ice until all samples were collected. RNA extraction using hot phenol and glass beads with vortexing was carried out as described previously (31). Approximately equal amounts of RNA were separated on 1.2% agarose–formaldehyde gels and transferred to Magnacharge nylon membranes (MSI). The membranes were treated with En^{3}Hance according to the instructions of the supplier (Du Pont, NEN) and exposed to X-ray film.

In vivo labeling of RNA. The long-term uptake and incorporation of [^{3}H]uracil into rRNA by strains YPH274 and YWJ64-ts, both bearing plasmid pYES2 to permit extended growth in media lacking uracil, were examined. The strains were grown in SD complete medium (25) lacking uracil to an OD_{600} of approximately 0.5 before 11 ml of each culture was shifted to 38°C for 1 h. Next, 330 μCi of [5,6-^{3}H]uracil (50 Ci/mmol) was added to each culture at 38°C. One-milliliter samples were taken at 5 min and at 1, 2, 3, and 4 h after addition of the label, and the OD_{600} of each sample was determined. The cells were spun down in a microcentrifuge for 10 s, the media were aspirated off, and the cell pellets were frozen immediately on dry ice. RNA isolation, electrophoresis, and transfer to nylon membranes were as described above for the rRNA pulse-chase studies. Equal volumes of the RNA preparations were loaded onto a 1.2% agarose–formaldehyde gel, and the dye front was run until it was 12 cm from the wells. The RNA was transferred to a nylon membrane, and after drying, a Bio-Rad

Molecular Imager system (model GS-525) was used to detect ^{3}H on the blot. Bio-Rad's Molecular Analyst software was used to image and quantitate the ^{3}H incorporated in the 25S and 18S rRNA species.

Sucrose gradient analysis of cytoplasmic ribosomal subunits. Our protocol for ribosomal profile analysis was as described by Baim et al. (3) with the following modifications. One-hundred-milliliter yeast cultures (YWJ64-ts or YPH274) were grown at room temperature or shifted to 38°C for 3 h, after which the log-phase cells were incubated with 1 mM NaN_3 for 15 min prior to being harvested (37). Cells were then disrupted by being vortexed in 2.5 ml of lysis buffer (100 mM NaCl, 30 mM $MgCl_2$, 0.2 μl of diethylpyrocarbonate per ml) and 0.25 ml of glass beads (1/10 volume). Aliquots of supernatant corresponding to 20 A_{260} U were layered over 35-ml linear sucrose gradients (7 to 47% [wt/vol]). The sucrose gradients were centrifuged at 28,000 rpm for 4 h at 13°C in an SW28 rotor (Beckman Instruments, Inc., Fullerton, Calif.), and A_{254} was monitored in an ISCO (Lincoln, Neb.) gradient fractionator (model 640) with a 60-ml syringe and upward displacement with Flurinet. The gradients were eluted with a chart speed of 15 cm/h and at a flow rate of 1 ml/min.

Tests for pNOY103 suppression of the *cbf5* mutants. Three different approaches were attempted to test for suppression of a *cbf5*Δ null allele by pNOY103. First, a diploid yeast strain heterozygous at the *CBF5* locus (*CBF5*/*cbf5::HIS3*) was transformed with pNOY103. Two of the transformants were sporulated (25), and tetrads were dissected on rich galactose (YPGal) plates (25) and incubated at room temperature. Viable spores were streaked on plates lacking either histidine or uracil to monitor the segregation of *cbf5::HIS3* and the pNOY103 plasmid. A yeast strain better suited for this strategy (YCC130) was created by disrupting one copy of *CBF5* in diploid strain NOY397 by transformation with the *Eco*RI fragment containing the *CBF5* open reading frame largely replaced by the *TRP1* gene, as described previously (23). The same sporulation procedure as that described above was employed with this new yeast strain.

A second approach was to transform plasmid pNOY103 into haploid yeast strain YCC109 which has the *cbf5::HIS3* null allele covered by plasmid p64-FAT10 (2μm *TRP1*). Transformants were grown for many generations in galactose media lacking uracil but containing tryptophan, thereby selecting only for the pNOY103 plasmid. Dilutions of cells were spread on galactose plates lacking uracil to obtain single colonies and then replica plated onto galactose plates lacking tryptophan to determine if any clones had lost the original plasmid. Clones capable of growing on plates without uracil but incapable of growing on plates without tryptophan were checked for their ability to grow on glucose to score for growth resulting from recombination of plasmids or gene conversion events.

The third approach employed was the use of a plasmid shuffle strategy to determine if plasmid pDK16, which has a copy of the 35S rRNA gene under the control of the *CUP1* promoter (30), can suppress the *cbf5*Δ null allele. Haploid yeast strain YHY11-3, which has the *cbf5::HIS3* null allele covered by plasmid pYHY18-CBF5 (2μm *URA3*), was transformed with plasmid pDK16. Several transformants were streaked onto plates containing 5′-FOA and 700 μM Cu^{2+} to select against plasmid pYHY18-CBF5 and to induce the *CUP1* promoter. Growth was scored after plates were incubated at 30°C for 4 to 5 days.

To determine if pNOY103 could suppress the *cbf5-1* ts allele, the yeast strain with the *cbf5-1* ts mutation (YWJ64-ts) was transformed with either plasmid pNOY103 or control plasmid pRS316 on plates lacking uracil. Transformants were streaked on plates that were incubated at 38°C or room temperature, and growth was scored after 4 to 5 days.

***SYC1* isolation.** To identify yeast genes that when overexpressed can rescue the conditional *cbf5-1* mutation, the YHY64α1 strain was transformed with a yeast genomic library constructed in 2μm-based *LEU2* vector YEp13 (7). The transformants were directly incubated at 38°C for 5 days on media lacking leucine. Of approximately 100,000 transformants screened, 16 were capable of growing at 38°C. All 16 showed loss of the suppression phenotype that correlated with the segregation of the library plasmid. However, only seven clones reproduced the rescue phenotype when these plasmids were introduced back into the *cbf5-1* mutant strain, YHY64α1. Two of the seven candidate suppressor plasmids were found to contain the *CBF5* gene by Southern hybridization with a 1.8-kb *Nde*I fragment containing the *CBF5* sequence. The remaining five plasmids contained identical 5.5-kb DNA inserts (plasmid 4B2). Plasmid 4B2 was unable to suppress *cbf5*Δ null mutants, indicating that the suppression was not due to a total bypass of Cbf5p functions.

***SYC1* gene disruptions.** Diploid yeast strain YPH274 was transformed with the 1.6-kb *Bam*HI-*Xba*I fragment of pKO/TRP1 containing the yeast *TRP1* gene cloned into the *Eco*RV-*Nde*I sites within the *SYC1* gene. Tryptophan prototrophs were selected, and the disruption of one copy of *SYC1* was verified by Southern hybridization of *Pvu*II-*Sac*I-digested yeast genomic DNA with a 467-bp *Bgl*II-*Eco*RV fragment of *TRP1* from pJJ246 as a probe. After this strain was sporulated on plates containing 1% potassium acetate and required nutritional supplements, tetrads were dissected and analyzed. No Trp^+ spores could be obtained out of 20 tetrads, and the gene was concluded to be essential.

An additional *SYC1* gene disruption was made, this time in haploid strain CG379, which carried plasmid pSYC1-YES2. The 1.6-kb pKO/TRP1 fragment described above was used to transform the strain, selecting for tryptophan prototrophy. Transformants were replica plated onto media lacking tryptophan, with or without 5′-FOA, and incubated at 25°C. Those clones that were unable to be cured of the pSYC1-YES2 plasmid were analyzed by Southern hybridization to confirm integration of the *TRP1* gene at the *SYC1* locus. The strain was designated YCC32.

Generation and integration of *syc1* ts alleles. The procedure for generating *syc1* ts alleles is similar to that described for *skp1* ts alleles (12) except that the PCR mutagenesis was carried out as described elsewhere (9). A gapped plasmid was prepared by digesting pSYC1-315 with *Eco*47III and *Pme*I, followed by agarose gel purification. PCR primers corresponding to the DNA at the N-terminal and C-terminal ends of *SYC1* were synthesized and used to amplify 10 ng of template in mutagenic PCR, creating a population of full-length mutagenized *SYC1* genes which overlap the gap in the plasmid with ~170 bp on either side. Approximately 1 μg of mutagenized PCR product was cotransformed into YCC32 with 1 μg of the gapped plasmid and 5 μg of salmon sperm DNA, selecting for leucine prototrophy. The transformants (~7,000) were replica plated twice to 5′-FOA plates lacking leucine, and ~1,500 clones were able to grow. The clones that failed to grow on 5′-FOA plates (75%) were presumed to have lethal mutations, since the degree of mutagenesis is so high (an estimated 11 mutations per PCR product on average). The remaining 1,500 transformants were replica plated in duplicate on yeast extract-peptone-dextrose (YPD) and grown at either 23 or 38°C. Six strains had a ts phenotype, and the mutagenized plasmids were recovered, mapped, and used to retest the ts phenotype at 38°C. The two alleles with the most dramatic growth defects at the restrictive temperature, *syc1-8* and *syc1-16*, were used for further studies and integration into the *SYC1* locus in strain CG379. The integration was carried out as described previously (25) by a two-step procedure. The *syc1* ts alleles were subcloned into an integration plasmid with a *URA3* marker, pBS-URA3. The resulting plasmids were linearized by cutting within the *SYC1* gene with either *Eco*47III or *Pme*I, and 3 μg was used to transform strain CG379, selecting for uracil prototrophy. After the mitotic stability of the *URA3* marker was checked, cells from transformants for each ts allele were resuspended in water and spread onto 5′-FOA plates at 25°C. The clones capable of growing were then screened for thermosensitivity on YPD plates, and their respective plasmid-based ts phenotypes at 25 and 38°C were compared.

***syc1-8* ts suppression and synthetic lethal experiments.** To determine if overexpression of *CBF5* could suppress the *syc1-8* ts mutation, plasmid pYHY18-CBF5 (22) was transformed into the strain that carries the *syc1-8* ts allele (YCC95), selecting on media lacking uracil. Two of the transformants were streaked onto media lacking uracil and incubated at 38°C. Growth was scored after 5 days. To determine if the *syc1-8 cbf5-1* double mutant is synthetic lethal, yeast strain YCC95 and the *cbf5-1* mutant strain (YHY64α1) were mated on YPD plates. The resulting diploid strain was sporulated, and tetrads were dissected and analyzed. Tetrads giving rise to four viable spores were assayed for segregation of the *cbf5-1* or *syc1-8* alleles by streaking cells on YPD plates and incubating at 38°C. Growth was scored after 4 to 5 days.

RESULTS

tRNA synthesis and processing in the *cbf5-1* mutant. The partial homology of Cbf5p to the TruB family of pseudouridine synthases prompted us to investigate tRNA processing in the *cbf5-1* ts mutant by using a [^{3}H]uracil pulse-chase strategy and subsequent gel electrophoresis of labeled tRNA. The *cbf5-1* ts mutant or *CBF5* wild-type cells were shifted to the restrictive temperature (38°C) for 1 h, then pulse-labeled with [^{3}H]uracil for 1 h, and finally chased with a large excess of cold uracil for 1 h. If the mutant cells were defective for tRNA processing, labeled pre-tRNA molecules would accumulate and be visible upon electrophoresis as species larger than tRNA but smaller than 5S rRNA. The experiment indicated that tRNA biosynthesis was normal in the *cbf5-1* ts mutant strain at the nonpermissive temperature (Fig. 1, lane 4). However, an obvious reduction in the amount of ^{3}H-labeled rRNAs in the mutant was seen at the restrictive temperature, relative to the amount seen in the wild-type strain (Fig. 1, lanes 2 and 4). But the amount of labeled 5S rRNA in the *cbf5-1* mutant at the restrictive temperature is not reduced, possibly indicating a pol I-specific problem in *cbf5-1*, since tRNA and 5S rRNA are transcribed by pol III (Fig. 1, lane 4).

rRNA synthesis and processing in the *cbf5-1* mutant. The synthesis, stability, and processing of the various rRNAs were examined by using a pulse-chase labeling strategy similar to that described above, with the difference being that cells were pulse-labeled for only 3 min with [*methyl*-^{3}H]methionine or [^{3}H]uracil and then a large excess of either cold methionine or uracil was added. Samples were collected at 1, 10, 20, and 30

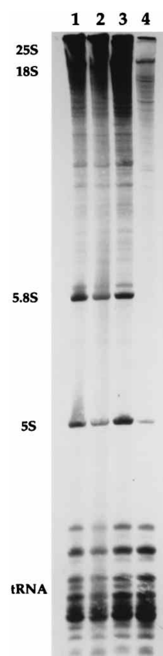

FIG. 1. Autoradiogram of polyacrylamide gel electrophoresis of [^{3}H]uracil-labeled RNA from YPH274 and YWJ64-ts cells. Cells were grown at room temperature (lanes 1 and 3) or shifted to 38°C for 1 h (lanes 2 and 4) prior to the addition of [^{3}H]uracil. After 60 min of incorporation (at room temperature or 38°C), unlabeled uracil was added to the cultures. Sixty minutes later, the cells were collected by centrifugation and the RNA was extracted. Equal amounts of radioactivity were loaded onto a 10% polyacrylamide–7 M urea gel (see Materials and Methods). Lane 1, RNA from YPH274 at 23°C; lane 2, RNA from YPH274 at 38°C; lane 3, RNA from YWJ64-ts at 23°C; lane 4, RNA from YWH64-ts at 38°C.

min after addition of the unlabeled methionine or uracil. RNA was isolated, fractionated by gel electrophoresis, and subjected to autoradiography. This experimental strategy has mainly been used to detect defects in rRNA processing (accumulation of a pre-rRNA species or the absence of a mature rRNA species) but may also show a global decrease in all rRNA species. When the *cbf5-1* ts and *CBF5* strains were pulsed with [*methyl*-^{3}H]methionine at 38°C and approximately equal amounts of total RNA were examined by gel electrophoresis and blotting, we observed significantly less labeled rRNA (approximately ninefold less) in the *cbf5-1* strain than in the *CBF5* strain and there was no appreciable accumulation of any of the pre-rRNA species (Fig. 2A). Using [*methyl*-^{3}H]methionine to visualize the pre-rRNA has the advantage that the blots have a lower background, since [^{3}H]uracil labels both mRNA and nascent rRNA transcripts. However, to examine the possibility that inactivation of Cbf5p causes demethylation or alters the methylation pattern of pre-rRNA, pulse-chase studies were also carried out with [^{3}H]uracil. When cells incubated at 38°C were labeled with [^{3}H]uracil and then chased with a large excess of cold uracil, the *cbf5-1* ts mutant strain again displayed less of the labeled rRNA species (Fig. 2B), although the difference was less extreme (approximately fourfold less). The [^{3}H]uracil experiments also failed to show significant accumulation of any of the species of pre-rRNA in the *cbf5-1* cells. These experiments are suggestive of a defect in the overall synthesis of rRNA in *cbf5-1*, as opposed to a defect in processing. We see no evidence for increased degradation of the mature rRNAs in the *cbf5-1* mutant during the course of the 30-min chase, although the stabilities of the precursor rRNAs are unknown.

Examining the accumulation of newly synthesized rRNA in the *cbf5-1* mutant. rRNA biosynthesis is closely coupled to cell growth rates; thus, mutations that prevent cell growth (or division) would be expected to reduce rRNA biosynthesis (11, 20). When the *cbf5-1* strain is shifted to 38°C, growth continues for several hours before finally leveling off about 4 to 5 h after the temperature shift (Fig. 3A). In fact, the growth rate of the *cbf5-1* strain is quite similar to that of the isogenic *CBF5* wild-type strain for the first 3 to 4 h after the temperature shift. This slow-arrest phenotype may be characteristic of cells containing an rRNA synthesis or ribosome assembly defect, since growth can be maintained for some time supported by the relatively stable ribosomes synthesized before the shift (37). The overall accumulation of newly synthesized rRNA in *cbf5-1*

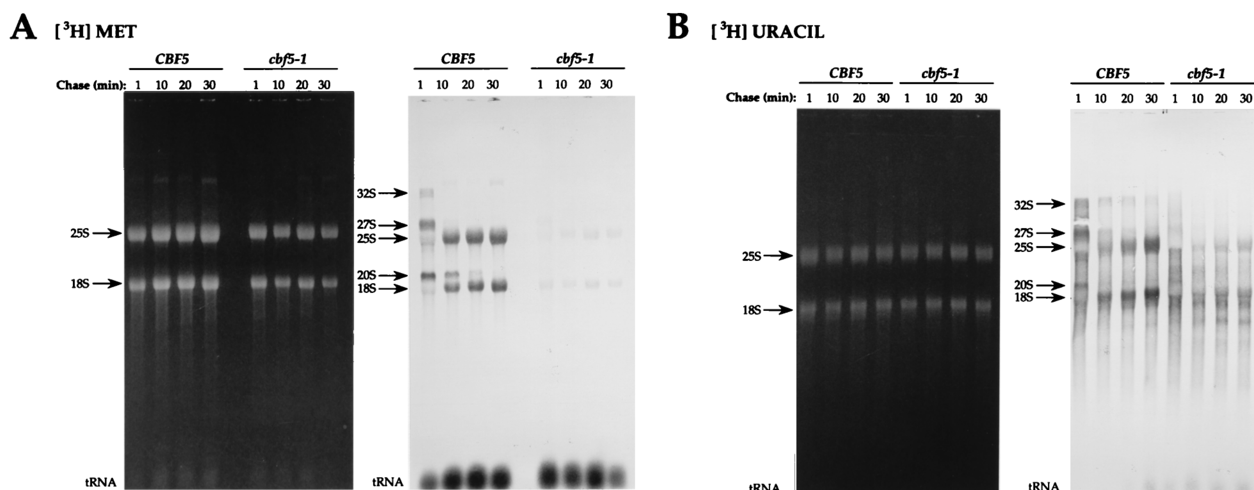

FIG. 2. Pulse-chase labeling of pre-rRNA in YPH274 and YWJ64-ts cells. Pre-rRNA was pulse-labeled with L-[*methyl*-^{3}H]methionine (A) or with [^{3}H]uracil (B) for 3 min at 38°C and chased with a large excess of unlabeled methionine or uracil for 1, 10, 20, or 30 min, as indicated. Labeling was performed after shifting cultures from room temperature to 38°C for 1 h. RNA was isolated, separated on a 1.2% agarose–formaldehyde gel, and visualized by ethidium bromide staining. RNA was then transferred to a nylon membrane and visualized by fluorography (see Materials and Methods). The left side of each panel is the ethidium-stained gel, and the right side is the autoradiogram.

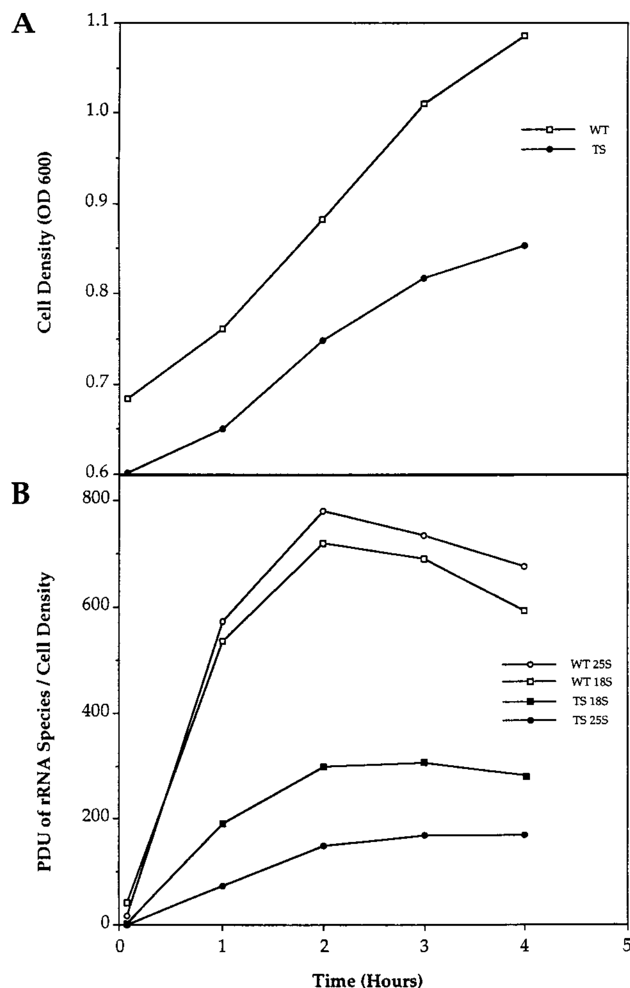

FIG. 3. Long-term incorporation of [^{3}H]uracil into rRNA species in YPH274 (WT) and YWJ64-ts (TS) cells. Cell cultures were grown to an OD_{600} of approximately 0.5 to 0.6 and shifted to 38°C for 1 h prior to the addition of [^{3}H]uracil. At 5 min and at 1, 2, 3, and 4 h after the addition, 1-ml samples of each culture were taken and the cell densities were measured (A). RNA was isolated, and an equal volume of each RNA preparation was fractionated on a 1.2% agarose–formaldehyde gel and visualized by ethidium bromide staining. The incorporation of [^{3}H]uracil in the 18S and 25S rRNA species was as described in Materials and Methods. Incorporation values (pixel density units [PDU]) were divided by the corresponding cell density values to correct for differences in the amounts of RNA loaded onto the gel (B). The zero time on the *x* axis refers to the time of [^{3}H]uracil addition, not the time of the shift to 38°C.

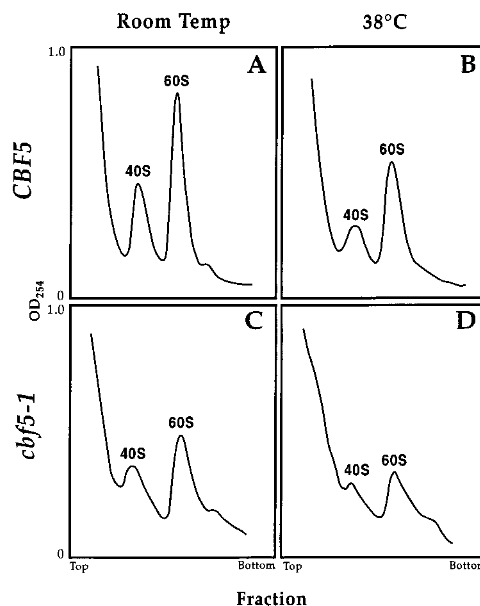

FIG. 4. Ribosomal subunit profiles in cytoplasmic extracts from YPH274 and YWJ64-ts cells. YPH274 (A and B) or YWJ64-ts (C and D) log-phase cell cultures were grown at room temperature (A and C) or shifted to 38°C for 3 h prior to cell harvest (B and D). Cell lysis supernatants corresponding to 20 A_{260} U were layered onto 7 to 47% linear sucrose gradients and sedimented in the ultracentrifuge (see Materials and Methods).

and *CBF5* cells after a temperature shift was determined by a long-term [^{3}H]uracil-labeling experiment (see Materials and Methods). The total amounts of [^{3}H]uracil in the 18S and 25S rRNA species were divided by the cell density value at the corresponding time point to provide a measurement of the accumulation of newly synthesized rRNA per cell (Fig. 3B). During the first 3 to 4 h after the shift, the slope of the cell growth curve of the mutant is very similar to that of the wild type; however, the incorporation of [^{3}H]uracil into the rRNA species is significantly less in the mutant. This experiment, in conjunction with the pulse-labeling studies, indicates that the *cbf5-1* mutant is defective in the biosynthesis of 18S and 25S rRNAs at the restrictive temperature, even under conditions where cell growth is relatively unimpaired.

Ribosome profiles and synthesis studies. A block in rRNA synthesis at restrictive temperatures in *cbf5-1* cells should result in a reduction in assembled cytoplasmic ribosomal subunits. The relative levels of cytoplasmic ribosome subunits in the *cbf5-1* and *CBF5* cells were assayed by examining ribosome profiles formed by sedimentation of cell extracts through sucrose gradients (see Materials and Methods). We found that the *cbf5-1* cells, even at the permissive temperature, contain approximately one-half the wild-type level of both ribosomal subunit peaks (Fig. 4A and C). The ribosome deficiency is even more severe when the *cbf5-1* cells are held at the nonpermissive temperature for 3 h (Fig. 4D). As noted above (Fig. 3A), the ts mutant continues to grow at a rate similar to that of the isogenic wild-type strain at nonpermissive temperatures for several hours after the shift, even though the total ribosome content per cell is significantly less in the mutant. The *CBF5* wild-type strain shows a decrease in ribosome content when shifted to 38°C (Fig. 4B), probably due to known effects of heat shock on protein synthesis (55). The expression of over 300 proteins is affected after a shift from 23 to 36°C, and expression of almost all of the ribosomal proteins is reduced (55). Still, the ribosome profiles show that the mutation in *cbf5-1* has an effect on the production of cytoplasmic ribosomes.

Isolation and characterization of *SYC1*, a multicopy suppressor of *cbf5-1*. The results described above strongly suggest a role for Cbf5p in rRNA biosynthesis. A genetic approach was employed in an attempt to identify gene products that interact directly or indirectly with Cbf5p, with the hope that further confirmation of an rRNA biosynthesis function for Cbf5p could be obtained. A yeast high-copy-number plasmid-based genomic library was screened for dosage suppressors of the *cbf5-1* ts mutation (see Materials and Methods) to find interacting genes. The screen resulted in the isolation of a dosage suppressor gene that we named *SYC1* for suppressor of yeast *cbf5-1* (Fig. 5A). *SYC1* was subcloned to a 2.3-kb DNA fragment (Fig. 6A), mapped, and sequenced. The resulting sequence encoded a 627-amino-acid protein with a predicted

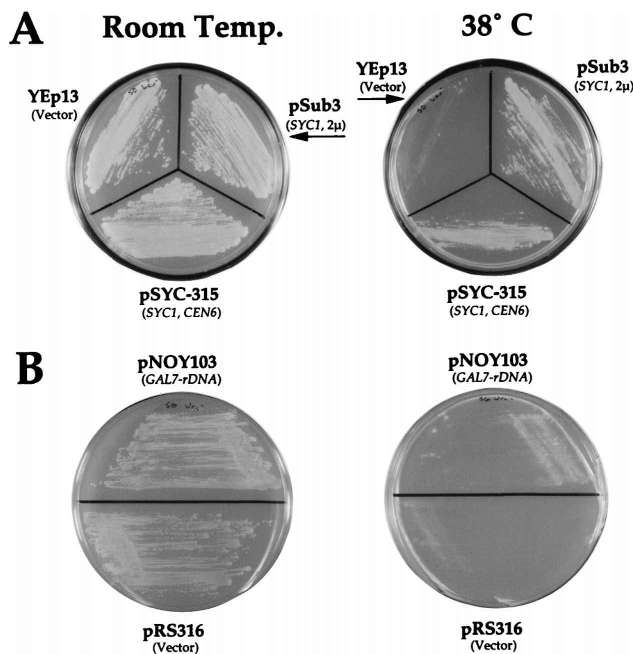

FIG. 5. Suppression of the *cbf5-1* ts mutation. (A) Plasmids YEp13 (2μm *LEU2*), pSub3 (*SYC1* 2μm *LEU2*), and pSYC1-315 (*SYC1 CEN6 LEU2*) were introduced into yeast strain YHY64α1 by transformation. Leu^+ transformants were streaked onto plates lacking leucine and incubated at room temperature or 38°C. (B) Plasmids pNOY103 (*GAL7-rDNA* 2μm *URA3*) and pRS316 (*CEN6 URA3*) were introduced into yeast strain YWJ64-ts by transformation. Ura^+ transformants were streaked onto galactose plates lacking uracil at room temperature. Strains were then restreaked onto galactose plates lacking uracil and incubated either at room temperature or 38°C.

molecular mass of 72 kDa and pI of 4.4. The *SYC1* gene is also capable of suppressing the *cbf5-1* growth defect when expressed on a low-copy-number *CEN* plasmid (Fig. 5A).

To determine the importance of *SYC1* for viability, gene disruption experiments were carried out (see Materials and Methods). The majority of the *SYC1* gene was replaced with a *TRP1* gene (Fig. 6B) in a diploid strain, followed by sporulation and tetrad analysis. *SYC1*, like *CBF5*, is essential for viability. Since the sequence of *SYC1* did not indicate the gene's function and since insight into protein function may often be gained from observing phenotypes of conditional mutants, a *syc1* ts mutant was constructed by using a combination of two previously described protocols (see Materials and Methods). Six plasmid-based *syc1* mutants were isolated on the basis of their impaired growth at 38°C, and the two mutants displaying the most dramatic growth defects at the restrictive temperature (alleles *syc1-8* and *syc1-16*) were used for further studies. In addition, these two alleles were integrated into the yeast genome at the *SYC1* locus, replacing the wild-type *SYC1* allele. To examine the relationship between Syc1p and Cbf5p, a high-copy-number plasmid expressing Cbf5p was transformed into the strain bearing the *syc1-8* mutation to determine if overexpression of *CBF5* could suppress the *syc1-8* ts phenotype (see Materials and Methods). The presence of the *CBF5* plasmid did not relieve the *syc1-8* ts growth defect. In addition, strains bearing either the *syc1-8* or the *cbf5-1* ts mutation were mated and sporulated and tetrads were analyzed to determine if the double mutant is viable (see Materials and Methods). The *cbf5-1 syc1-8* double mutant was readily isolated and grew well at room temperature, showing no synthetic lethality.

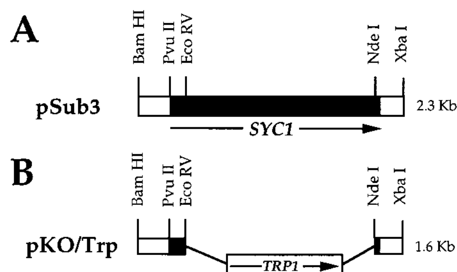

FIG. 6. Genetic disruption of *SYC1*. (A) Restriction map of the 2.3-kb restriction fragment containing the *SYC1* gene. (B) The *syc1::TRP1* allele was constructed by deleting an internal *Eco*RV-*Nde*I *SYC1* fragment and replacing it with a fragment containing the *TRP1* gene.

***SYC1* is identical to *RRN3*.** Subsequent to the isolation and characterization of *SYC1*, it was discovered during a database search that this gene is identical to *RRN3*, a recently described gene which specifies a pol I transcription factor. *RRN3* was isolated in a genetic screen designed to identify genes specifically required only for transcription by pol I (41, 56). In the screen, a copy of the yeast 35S rRNA gene was fused to a *GAL7* promoter on plasmid pNOY103. The rRNA gene can be transcribed by pol II from this fusion, thereby alleviating the need for pol I transcription. When the strain carrying the plasmid is mutagenized, clones that are able to grow on galactose media but not glucose media should harbor mutations in genes specifying proteins involved in pol I-dependent rRNA transcription. Thirteen complementation groups forming a group of genes named *RRN*, for rRNA, were identified (41). We transformed the *syc1-16* ts strain with plasmid pNOY103 to determine if it suppresses our *syc1-16* ts mutation. As expected, pNOY103 was able to suppress this ts mutation at 38°C, confirming that this *syc1* allele has the *rrn* phenotype (data not shown).

pNOY103 fails to suppress the *cbf5*Δ null allele. With any genetic screen, some relevant genes may escape detection. Furthermore, not all of the *RRN* family of genes are completely characterized. Experiments were carried out to determine if a *cbf5*Δ null mutant displays the *rrn* phenotype (suppressed by pNOY103). Although Cbf5p clearly is required for efficient rRNA biosynthesis, it is possible that this protein has multiple essential functions, and thus the *cbf5*Δ null allele might not be suppressed by supplying rRNA transcription from a pol II promoter. Initially, the pNOY103 plasmid was transformed into diploid yeast strain YHY17-8, which is heterozygous for *CBF5* (*CBF5/cbf5::HIS3*). This strain was sporulated, and the tetrads were dissected and allowed to germinate on rich galactose (YPGal) plates. All of the viable spores harbored the wild-type *CBF5* gene. Since this strain has the *trp1-Δ1* mutation that deletes the UAS elements for the adjacent *GAL3* gene, it is also *gal3*. Although *gal3* mutants are capable of growing on galactose after a period of adaptation (51), they are not the best choice for this experiment. Therefore, the *cbf5*Δ*::TRP1* null allele was used to replace one *CBF5* allele in diploid strain NOY397, a more suitable Gal^+ strain (see Materials and Methods). The experiment described above was repeated but yielded the same results. Finally, because germination on galactose media may be difficult for yeast spores, two plasmid shuffle strategies were also attempted (see Materials and Methods). In both cases, we were unable to isolate strains in which the plasmid bearing the 35S rRNA gene suppressed the *cbf5*Δ null allele. Taken together, the results of all approaches strongly indicate that the *cbf5*Δ null allele is not suppressed by pNOY103; thus, *CBF5* does not belong to the *RRN* group of genes. However, pNOY103 was able to partially

suppress the *cbf5-1* ts mutation in strain YWJ64-ts (Fig. 5B). We interpret these results as indicating that Cbf5p is a multifunctional protein, with at least one of the essential functions distinct from rRNA transcription but not completely abrogated in the *cbf5-1* mutant.

DISCUSSION

Many separate lines of evidence point to multiple functions for Cbf5p, including roles in centromere function and microtubule binding (22, 23), pseudouridine synthase activity (27, 42), nucleocytoplasmic transport (33, 34), and, finally, rRNA biosynthesis (this work). Although Cbf5p was originally isolated as a low-affinity *CEN* DNA binding protein (23), this alone is not compelling evidence for an involvement in centromere structure and function. Cbf5p is highly charged toward its C terminus (overall pI 9.5) and might be expected to bind nonspecifically to DNA affinity columns. Cbf5p does bind to bovine microtubules in vitro, an observation supportive of a centromere-kinetochore function (23), but the presence of a highly charged basic region in Cbf5p might lead to nonspecific microtubule binding in vitro. However, genetic interactions with both *MCK1* and *CBF2/NDC10* support the idea of a centromere-chromosome segregation role for Cbf5p. Overexpression of *MCK1* suppresses (i) a partially disabling mutation in CDEIII, an important protein binding site in the yeast *CEN* DNA locus (50), (ii) a ts mutation (*ndc10-1*) in the gene specifying the 110-kDa subunit of the multisubunit protein complex (CBF3) binding specifically to CDEIII (22), and (iii) the *cbf5-1* ts mutation (22). The *MCK1* gene specifies a nonessential phosphotyrosyl protein with protein kinase activity (29, 39). In addition, overexpression of Cbf5p suppresses the *ndc10-1* ts growth defect in certain genetic backgrounds (23). Finally, complementation of a *cbf5*Δ null allele by a C-terminal truncated *CBF5* gene causes cells to delay in the process of DNA transfer during mitosis; this supports the idea of a role for Cbf5p in chromosome segregation (23). The predominantly nucleolar location of Cbf5p does not preclude a functional role for this protein in chromosome segregation, since it has been found that various proteins that localize to the centromere regions of condensed chromosomes in mammalian cells are found in the nucleolus during interphase (43).

Cbf5p shows considerable sequence homology to the *E. coli* TruB protein, which functions as a pseudouridine Ψ synthase for tRNA at position 55 (42). TruB was found to be a member of a family of Ψ synthases as the sequences of more bacterial homologs became available (27). The region of TruB believed to be the conserved U-binding domain is the area showing the greatest homology to Cbf5p (27). These findings and the fact that Cbf5p is nucleolar point to a role for Cbf5p in Ψ formation in tRNA or, more likely, in rRNA. There is one example of a Ψ synthase functioning on both tRNA and rRNA (42); however, most enzymes of this type appear to be more substrate specific. The biological role of Ψ residues is not clear, but in rRNAs, Ψ residues are clustered near the peptidyltransferase center of the ribosomes and may have a role in peptide bond formation (4, 5). We have no direct evidence bearing on a presumptive Ψ synthase role for Cbf5p. The processing of larger pre-rRNA species appears normal in the *cbf5-1* strain at restrictive temperatures, but it is unknown whether the lack of Ψ modifications would prevent normal cleavage of the pre-rRNAs.

The high degree of sequence conservation in the Cbf5p-NAP57 family of proteins (71% identity and 85% homology between rat NAP57 and yeast Cbf5p [33]) suggests a structural role for these proteins. In general, structural proteins such as tubulin, actin, and ribosomal proteins show high levels of sequence conservation (15, 26, 38). In fact, functionality within the Cbf5p-NAP57 family is conserved between widely divergent species; e.g., the *Drosophila CBF5* homolog cDNA complements the *cbf5*Δ null allele when introduced into yeast on a plasmid and expressed under the control of the yeast *ADH1* promoter (8). Although Cbf5p is apparently not one of the protein components of mature ribosomal subunits, it might well be a component of newly assembled preribosomal particles in the nucleolus and might function in some aspect of subunit assembly and/or transport. The putative U-binding domain in Cbf5p could play a key structural role in terms of ensuring tight binding of the protein either to rRNAs or small nucleolar RNAs.

Studies of NAP57, the Cbf5p homolog in rats, are suggestive of a nucleolar-cytoplasmic transport function (33). NAP57 was isolated by coimmunoprecipitation with Nopp140. This nuclear localization signal-binding protein was found to shuttle on tracks between the nucleolus and the cytoplasm (34), and NAP57 was found to colocalize with Nopp140 (33). The authors were unable to demonstrate that NAP57 can also shuttle due to difficulties with the antibody used, but a role for NAP57 in transport seems likely. The subnucleolar location of NAP57 to the DFC could indicate a role in ribosome assembly or perhaps transport of a component(s) necessary for assembly. Recently, it has been shown that Nopp140 is a pol II transcription factor and also modulates the interaction between two other transcription factors (36). In addition, the yeast homolog of Nopp140 has recently been identified as *SRP40* (32). Interestingly, *SRP40* was identified as a weak multicopy suppressor of a ts mutation in AC40, one of the shared subunits of pol I and pol III (28). Thus, both *SRP40* and Cbf5p may function directly or indirectly in some aspect of rRNA biosynthesis.

Our studies with the *cbf5-1* ts mutant indicate a pronounced defect in 18S and 25S rRNA synthesis in this strain at the nonpermissive temperature. The processing of pre-rRNAs (cleavage) appears to be relatively normal in mutant cells, although defects in base or ribose modifications would not have been detected in our experiments. These findings suggest a pol I-specific problem in the mutant, since tRNA and 5S rRNA synthesis appears relatively normal. The identification of *SYC1/RRN3* as a strong suppressor of *cbf5-1* also strongly implicates Cbf5p in some aspect of pol I-directed rRNA transcription, since Rrn3p is necessary for rRNA transcription both in vivo and in vitro (56). Inactivating mutations in any of the yeast *RRN* family of genes are rescued by the presence of a plasmid (pNOY103) containing the 35S rRNA gene driven by a pol II-specific promoter (41). Since pNOY103 cannot suppress a *cbf5*Δ null allele, we conclude that Cbf5p must have an essential function(s) in addition to its role in rRNA synthesis. The plasmid does, however, weakly suppress the ts growth phenotype of *cbf5-1* and, as expected, strongly suppresses the ts growth phenotype of *syc1-16* (an *rrn3* mutant allele). Apparently, all functions of Cbf5p are not lost in the *cbf5-1* mutant at the restrictive temperature. At this point, the exact functional relationship between Cbf5p and Syc1p/Rrn3p is unclear. Overexpression of Cbf5p does not suppress the *syc1-8* mutation, and *cbf5-1* and *syc1-8* are not synthetically lethal. In vitro data suggests that Syc1p/Rrn3p may serve to "activate" pol I (56), and it is possible that Cbf5p functions upstream of Syc1p/Rrn3p in an rRNA transcriptional-control pathway. The availability of a well-defined in vitro pol I-dependent transcription system in yeast (46, 56) should eventually lead to a definition of the relationship between these proteins, as well as of the molecular role of Cbf5p in rRNA synthesis.

Our work establishes a biochemical link between Cbf5p and

rRNA synthesis in yeast and expands upon the previously described link between centromere proteins and nucleoli (43). Recently, another centromere protein (human CENP-C) was found to be nucleolus associated and biochemically linked with rRNA synthesis (44). CENP-C was shown to interact specifically with nucleolar pol I transcription factors UBF1 and UBF2 (upstream binding factors 1 and 2) in affinity chromatography experiments (44). CENP-C was originally identified as a centromeric autoantigen in scleroderma patients, and evidence for a functional role in centromere structure and function has been accumulating (13, 47, 53). At this point, evidence for an association of Cbf5p with the yeast centromere is largely indirect. The significance of a dual association with the nucleolus and the centromere is not clear, but one intriguing possibility is a role in the down-regulation of rRNA synthesis during mitosis. Pol I-driven transcription of the rRNA genes ceases during prometaphase concomitant with the disintegration of the nucleolus and does not resume until telophase (54). A hypothesis that supports the idea that Cbf5p is multifunctional is that the function of Cbf5p and/or its location varies depending upon the stage of the cell cycle.

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