Nop5p Is a Small Nucleolar Ribonucleoprotein Component Required for Pre-18 S rRNA Processing in Yeast*

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We have identified a novel nucleolar protein, Nop5p, that is essential for growth in Saccharomyces cerevisiae. Monoclonal antibodies B47 and 37C12 recognize Nop5p, which has a predicted size of 57 kDa and possesses a KXX repeat motif at its carboxyl terminus. Truncations that removed the KXX motif were functional and localized to the nucleolus, but conferred slow growth at 37 °C. Nop5p shows significant sequence homology with yeast Sik1p/Nop56p, and putative homologues in archaea, plants, and human. Depletion of Nop5p in a GAL-NOP5 strain lengthened the doubling time about 5-fold, and selectively reduced steady-state levels of 40 S ribosomal subunits and 18 S rRNA relative to levels of free 60 S subunits and 25 S rRNA. Northern blotting and primer extension analyses showed that Nop5p depletion impairs processing of 35 S pre-rRNA at the A₀ and A₂ cleavage sites. Nop5p is associated with the small nucleolar RNAs U₃, snR₁₃, U₁₄, and U₁₈. Depletion of Nop5p caused the nucleolar protein Nop1p (yeast fibrillarin) to be localized to the nucleus and cytosol. Also, 37C12 co-immunoprecipitated Nop1p. These results suggest that Nop5p functions with Nop1p in the execution of early pre-rRNA processing steps that lead to formation of 18 S rRNA.

Most of the steps of ribosome biogenesis in eukaryotic cells take place in the nucleolus. In the yeast Saccharomyces cerevisiae, a single long 35 S pre-rRNA is transcribed by RNA polymerase I and processed to 18 S, 5.8 S, and 25 S rRNAs through a series of co- and post-transcriptional steps. Ribosomal proteins imported from the cytoplasm are assembled with pre-rRNAs to form the small 40 S subunit and the large 60 S subunit. The 5 S rRNA is transcribed by RNA polymerase III from a separate transcription unit and is incorporated into the large subunit along with the 5.8 S and 25 S rRNAs, while 18 S rRNA is incorporated into the small subunit. During transcription and processing of pre-rRNA, a number of nucleotides are modified, primarily by the addition of 2′-O-methyl groups or by the formation of pseudouridine residues. The processing and modification of pre-rRNAs require non-nucleolar nucleolar proteins, many of which are associated with small nucleolar RNAs (snoRNAs) in the form of small nucleolar ribonucleoprotein (snoRNP) complexes (reviewed in Refs. 1 and 2).

The earliest processing events are those involved in the removal of the promoter proximal 5′-externally transcribed spacer (5′-ETS). Cleavage occurs at two sites within the 5′-ETS: at A₀, in the middle region of the 5′-ETS; and at A₁, which results in the formation of the 5′-end of the mature 18 S rRNA (reviewed in Ref. 3). Formation of 18 S requires processing to form its 3′-end, which involves processing at site A₂ in the first internally transcribed spacer (ITS₁) followed by processing at site D, which yields the 3′-end (see Fig. 9). In yeast, many gene products are required for, or participate in, cleavage at sites A₀, A₁, and A₂, attesting to the complex nature of this process. The yeast RNase III encoded by RNT1 is involved in endonucleolytic cleavage at the A₀ site, and can function in vitro in the absence of other factors (4). Genetic depletion of the snoRNAs U₁₄, snR₁₀, snR₃₀, and depletion of the snoRNP proteins Nop1p, Rok1p, Rrp5p, Sof1p, and Gar1p impair cleavage at A₀, A₁ and A₂ (5–14). These depletion experiments give rise to a similar phenotype: accumulation of 35 S pre-rRNA and reduction of 18 S rRNA levels. However, different underlying mechanisms are responsible for the reduction in 18 S rRNA levels. For example, the C/D box snoRNAs U₃ and U₁₄ are required for processing and 2′-O-methylation, and are associated with Nop1p (15). The H box/ACA snoRNA, snR₃₀, is required for conversion of uridine to pseudouridine and is associated with Gar1p, which has been shown to be involved in pseudouridine formation (16–20).

Nop1p is an essential and conserved nucleolar protein that is part of the U₃ snoRNP complex, which is required for early processing steps (9, 14, 15, 21). The U₃ snoRNP complex and the Nop1p homologue fibrillarin have been investigated in a number of different organisms (reviewed in Refs. 22 and 23). Nop1p is associated with multiple snoRNAs, indicating that it associates with more than one snoRNP complex and is not unique to the U₃ snoRNP (9, 14). This is consistent with the fact that Nop1p is multifunctional and participates in different aspects of ribosome biogenesis, including pre-rRNA modification, processing, and ribosome subunit assembly (15). On the other hand, the essential nucleolar protein Mpp₁₀p is required for processing at sites A₀, A₁, and A₂, and is predominantly associated with U₃, indicating that it is a specific U₃ snoRNP component (24). The only other known protein in yeast that is specific for the U₃ snoRNP is Sof1p, which plays an essential role in pre-18 S rRNA processing as well (10). Thus, although the U₃ snoRNP is one of the best understood snoRNPs, knowledge of its composition and function remains incomplete.

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1 The abbreviations used are: snoRNA, small nucleolar RNA; ETS, externally transcribed spacer; ITS, internally transcribed spacer; mAb, monoclonal antibody; snoRNP, small nucleolar ribonucleoprotein; PAGE, polyacrylamide gel electrophoresis; kb, kilobase(s); 5-FOA, 5-fluoroorotic acid; IP, immunoprecipitation; IF, immunofluorescence.
To better understand the function of snoRNPs involved in early pre-rRNA processing steps and 18 S rRNA synthesis, it is necessary to identify and functionally characterize novel snoRNA components, especially those that interact with Nop1p and/or U3. Monoclonal antibodies generated against nuclear antigens have been useful in this regard, and have allowed us to identify novel nucleolar proteins in yeast. The studies reported herein center on a gene we term NOP5. Our studies show that NOP5 is essential for cell growth, is required for synthesis of the small 40 S subunit, and is involved in processing of pre-18 S rRNA. Genetic depletion of NOP5 impairs cleavage at sites A0 and A2. NOP5 has been conserved during evolution. We present evidence that NOP5 is associated with certain snoRNAs, including U3, and with Nop1p, suggesting that NOP5 functions together with Nop1p in snoRNP complexes required for 18 S rRNA synthesis.

EXPERIMENTAL PROCEDURES

Microbiological and Molecular Biological Techniques—The S. cerevisiae strains and plasmids used in this study are described in Table I. Growth of yeast, yeast transformation, sporulation, microdissection, tetrad analysis, and plasmid shuffling, were done according to standard procedures as described previously (25, 26). For genetic depletion of NOP5, YPW48 was grown in liquid medium to mid-log phase (OD600 = 0.25–0.5), washed with sterile water, and transferred to fresh medium. Rich media (YPD or YPGal) or synthetic media (SD or SGal) plus 0.25–0.5, washed with sterile water, and transferred to fresh medium. Rich media (YPD or YPGal) or synthetic media (SD or SGal) plus 0.25–0.5, washed with sterile water, and transferred to fresh medium. Rich media (YPD or YPGal) or synthetic media (SD or SGal) plus 0.25–0.5, washed with sterile water, and transferred to fresh medium. Rich media (YPD or YPGal) or synthetic media (SD or SGal) plus 0.25–0.5, washed with sterile water, and transferred to fresh medium.

Immunofluorescence Localization—

Preparation of nuclear extracts and immunoprecipitations were done according to standard methods (25, 26). The Molecular Analyst (Bio-Rad) software package was used for quantitative comparison of relative band intensities on films.

Polyacrylamide Gel Electrophoresis—Gel electrophoresis and immunoblotting were done according to standard methods (26). Labeled yeast cells were grown in YPD at 30 °C and analyzed according to Hong et al. (26). Labelling with [methyl-3H]methionine or [3H]uracil was done with cells collected after 0, 4, 8, and 12 h of growth in dextrose-containing media as described previously (26). Primer extension was done using template equivalent amounts of total RNA extracted from W303–1a or YPW48 grown in YPGal and transferred to YPD for 0, 2, 4, 12, or 24 h. The sequences used for these studies because previous experiments indicated that monoclonal antibodies against nuclear antigens (i.e. mAb A66 against Nop1p and mAb 3F2 against Nab2p) immunoprecipitated the predicted protein band with nuclear extracts, but not with whole cell extracts prepared under comparable conditions. Labeled yeast cells were collected, washed, and sonicated, and the sonicate was diluted 1/10 in 40 mM of 

Centrifugation in an SW50.1 rotor for 20 min at 22,000 rpm (58,165 

Gels were prepared under comparable conditions. 

Labeled yeast cells were collected, washed, and sonicated, and the sonicate was diluted 1/10 in 40 mM of 

Vehicles were completely removed, and the pellet was quickly frozen and stored at −80 °C. The frozen nuclear pellet was thawed in immunoprecipitation (IP) buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 2 mM EDTA) containing 0.1% (w/v) Nonidet P-40, and bath sonicated on ice. 

The immunoprecipitates were washed 5 × 2 min at −25 °C with IP buffer followed by Nonidet P-40, and were washed 5 times with IP buffer alone. Immunoprecipitates were boiled for 5 min in sample buffer, and analyzed by SDS-PAGE. All buffers after the digestion step contained protease inhibitor mixtures (28). For immunoprecipitation, two immunoprecipitations were pooled, solubilized with 2% SDS, 20 mg dithiothreitol, 25 mM Tris-HCl, pH 6.5, for 10 min at 65 °C, and treated with 40 μM N-ethylmaleimide following the protocol of Furukawa and Aviv. After this treatment, IP buffer alone or IP buffer plus 1% Nonidet P-40 and protease inhibitor mixture were used, and the sample was microcentrifuged at top speed for 5 min. The supernatant

1. Buber T. and J. P. Aris, unpublished results.

2. T. Buber and J. P. Aris, unpublished results.
was used for a second round of IP conducted essentially the same as the first round.

For RNA immunoprecipitations, nuclei isolated from BJ2168 using 2 Ficoll step gradients (28) were diluted in 20 mM KF, pH 6.5, 1 mM MgCl₂ (PM buffer), centrifuged, and the pellet resuspended in RNA IP buffer: 50 mM Tris-HCl, pH 8, 150 mM NaCl, 100 mM KCl, 5 mM MgCl₂, 0.1% Nonidet P-40, protease inhibitor mixtures, 8 mM vanadyl ribonucleoside complex (Life Technologies, Inc.). To dissolve vanadyl ribonucleoside complex, the buffer was heated to ~50 °C and tip sonicated for 10 min. The nuclear pellet was dissolved in RNA IP buffer, and tip sonicated 3 x 20 s, with intermittent chilling on ice. The lysate was precleared with rabbit antimouse protein A-Sepharose that had been washed with RNA IP buffer, and immunoprecipitates were prepared as described above using RNA IP buffer. The immunoprecipitate was treated for 10 min at 37 °C with 25 µg of proteinase K in 5 mM Tris-HCl, pH 8, 2 mM EDTA, 0.2% SDS containing 10 µg of glycerol, followed by extraction with phenol, phenol:chloroform, and chloroform, and precipitation and washing with ethanol. RNAs were 3'-end labeled with RNA ligase (New England Biolabs) using a standard method (34), purified, and electrophoresed on a 6% denaturing polyacrylamide gel. The “total” labeling sample mixture consisted of a portion of the supernatant fraction from the control IP treated, extracted, precipitated, and labeled as described above.

RESULTS

Monoclonal Antibodies 37C12 and B47 Are Directed Against Nucleolar Proteins—Monoclonal antibodies raised against nucleolar antigens were evaluated by immunofluorescence (IF) staining. Monoclonal antibody 37C12 produced a bright and specific intranuclear IF pattern that substantially overlapped the distribution of the nucleolar protein Nop2p (Fig. 1, A-D). The mAb B47 also gave an IF staining pattern that coincided with the distribution of Nop2p (Fig. 1, E-H). The IF pattern in both cases was offset from the distribution of chromatin in many cells, depending on the orientation of the nucleus, which resulted in the appearance of a crescent shape that is characteristic of the nucleolus in yeast (Fig. 1, arrows). Thus, 37C12 and B47 recognized nucleolar antigens in yeast.

To identify the antigen(s) recognized by 37C12, immunoprecipitations were done with 35S-labeled nuclear extracts. 37C12 immunoprecipitated two proteins of approximately 67 and 38 kDa, which were not observed in the absence of primary antibody (Fig. 2). 37C12 immunoprecipitates washed with IP buffer containing 0.5 mM NaCl, 2 mM urea, or 0.2% SDS, 1% Nonidet P-40 also contained the 67-kDa protein, but with lower relative amounts of the 38-kDa band (data not shown). This suggested that 37C12 recognized a 67-kDa protein in the nucleolar fraction. Considering that Nop1p is known to migrate on SDS gels at 38 kDa (30), we tested the possibility that this protein was Nop1p. The immunoprecipitate obtained with 37C12 was solubilized with SDS, diluted with non-ionic detergent, and re-immuno-precipitated with mAb A66, which is specific for Nop1p (30). A66 quantitatively immunoprecipitated the 38-kDa protein, proving that it is Nop1p (Fig. 2).

Monoclonal 37C12 did not immunoblot yeast nuclear protein extracts, despite the use of protocols to renature proteins prior to transfer or after transfer. On the other hand, B47 was not very effective in protein immunoprecipitation experiments, but produced a specific signal on immunoblots (see Figs. 5B and 14B).

NOP5 Encodes A Novel Protein That Is Localized to the Nucleolus—To molecularly clone the gene encoding the 67-kDa protein, a yeast expression library was screened with 37C12. Eight positives fell into two classes of overlapping clones, the largest one of which contained an insert that encoded approximately 70% (amino acids 71–436) of the YOR310C open reading frame present on chromosome XV (35). A lacZ gene fusion from a positive clone was expressed in a λ-lysogen and yielded a protein of the expected size that was recognized by 37C12 on an immunoblot (Fig. 3A). Thus, although full-length protein from a yeast nuclear extract did not react on immunoblots probed with 37C12 (see above), a fusion protein produced in E. coli was recognized by 37C12. We refer to this gene as NOP5 (nucleolar protein 5). NOP5 encodes a 511-amino acid protein of predicted molecular weight 56,953, with a predicted pI of 9.4, whose most notable sequence characteristic is a highly hydrophilic and charged KKKxK repeat motif at the carboxyl terminus (Fig. 3B). The predicted molecular mass of 57.0 kDa is smaller than the size observed on SDS gels. This difference is likely due to the highly charged COOH terminus (see below). Other nucleolar proteins with clusters of charged amino acids also migrate anomalously on SDS gels (e.g. Ref. 31). During the course of our studies, Gautier et al. (36) also identified this gene in a screen for genes that are synthetically lethal with the nop1–3 allele and termed it NOP58.

To confirm via an independent means that NOP5 encoded a nucleolar protein, Nop5p containing a carboxyl-terminal hemagglutinin antigen (HA-1) epitope tag was expressed under control of the GAL promoter in plasmid pPW73 in YPW38.

4 S. Chen and J. P. Aris, unpublished results.
Fig. 3. **Nop5p functions.** A, an immunoblot of protein extracts from a 37C12 positive λ lysogen (PL) or a control λ lysogen (CL) induced with isopropyl-1-thio-D-galactopyranoside (+) or not induced (−) was probed with mAb 37C12. The apparent size of the inducible immunoreactive protein (−−) is ~140 kDa. A protein of ~67 kDa (*) reacted nonspecifically. B, predicted sequence of Nop5p.

**TABLE I**

| Strain or Plasmid | Description |
|-------------------|-------------|
| **Strains**       |             |
| W303–1α          | MATa, ade2–1, can1–100, his3–11, 15, leu2–3, 112, trp1–1, ura3–1 (from C. A. Styles and G. R. Fink) |
| W303–1α          | Caspase-3, ade2–1, can1–100, his3–11, 15, leu2–3, 112, trp1–1, ura3–1 (from C. A. Styles and G. R. Fink) |
| YSB25            | Micromanipulated zygote from W303–1α × W303–1α |
| YPW38            | YSB25 pPW73 (GAL-NOP5-HA tag, URA3, CEN6) |
| YPW42            | YSB25 nop5::TRP1 |
| YPW43            | YSB25 nop5::TRP1 |
| YPW45            | YSB25 nop5::TRP1 pPW80 (NOP5, URA3, CEN6) (meiotic segregant from YPW42 carrying pPW80) |
| YPW48            | YSB25 nop5::TRP1 pPW83 (GAL-NOP5, LEU2, CEN6) |
| YPW51            | YSB25 nop5::TRP1 pPW92 (nop5Δ1, LEU2, CEN6) |
| YPW52            | YSB25 nop5::TRP1 pPW88 (nop5Δ2, LEU2, CEN6) |
| YPW53            | YSB25 nop5::TRP1 pPW91 (NOP5, LEU2, CEN6) |
| **Plasmids**     |             |
| pPW69            | PCR product carrying NOP5 (primers 4 and 5) cloned between BanHI and XhoI sites of pRS314 (TRP1, CEN6) (37). |
| pPW73            | PCR product carrying HA-epitope tagged NOP5 (primers 1 and 2) cloned between BanHI and Clal sites of pRD53 (GAL1/10 promoter, URA3) (26). |
| pPW80            | PCR product carrying NOP5 (primers 4 and 5) cloned between BanHI and Xhol sites of pRS316 (URA3, CEN6) (37). |
| pPW81            | PCR product carrying NOP5 (primers 1 and 3) cloned between BanHI and Xhol sites of pRD53 (GAL1/10 promoter, URA3) (26). |
| pPW83            | SacI-XhoI fragment carrying GAL-NOP5 cloned between same sites of pRS315 (LEU2, CEN6) (37). |
| pPW84            | EcoRI-NcoI fragment of pJJ280 (32) carrying TRP1 cloned into EcoRI and PstI sites in NOP5 in pPW69. |
| pPW85            | BanHI-XhoI fragment carrying nop5::TRP1 from pPW84 cloned between same sites in pBluescript SK+. |
| pPW92            | A derivative of pPW69 that carries nop5Δ1 (removes the COOH-terminal 38 amino acids) was constructed using inverse PCR (primers 6 and 7), and subcloned between BanHI and Xhol sites in pRS315 (LEU2, CEN6) (37). |
| pPW88            | A derivative of pPW69 that carries nop5Δ2 (removes the COOH-terminal 61 amino acids) was constructed using inverse PCR (primers 6 and 7), and subcloned between BanHI and Xhol sites in pRS315 (LEU2, CEN6) (37). |
| pPW91            | NOP5, LEU2, CEN6. A BanHI and Xhol fragment from pPW69 carrying NOP5 was cloned into the same sites in pRS315 (LEU2, CEN6) (37). |

(Table I). Plasmid pPW73 complemented a nop5 null allele (data not shown), indicating that the epitope tag does not interfere with Nop5p function. Growth of YPW38 on galactose-induced expression of epitope-tagged Nop5p (Fig. 4A). The protein band induced in the presence of galactose also reacted with mAb B47 (data not shown). IF analysis revealed a range of signal intensities (Fig. 4B). A range of signal intensities has been observed in other experiments with YPW38 (data not shown), even though pPW73 carries CEN6, which should limit plasmid copy number to 2–5 (37). This range may be due to variation in plasmid copy number coupled with the effects of a strong GAL10 promoter. Cells expressing low to moderate levels of epitope-tagged Nop5p showed colocalization with the nucleolus (protein Nop5p and a typical crescent-shaped nucleolar pattern (Fig. 4B, arrows). Cells overexpressing Nop5p showed signal distributed throughout the nucleus, including the nucleolus. Because overexpression of a protein can lead to an anomalous intracellular distribution, cells expressing low to moderate levels are the most reliable indicator of localization. YPW38 grown on glucose, and a control strain lacking pPW73, do not produce an IF signal with mAb 12CA5 (data not shown). Thus, we conclude that epitope-tagged Nop5p is localized to the nucleolus.

The COOH-terminal KXX Repeat Motif Is Not Required for Cell Growth—Nop5p contains a KXX repeat motif at its carboxyl terminus. Similar motifs are present in Cbu5p (at the COOH terminus; Ref. 38) and Dbp5p (near the NH₂ terminus; Ref. 39). To examine the functional significance of the KXX motif in Nop5p, two COOH-terminal truncations were made: nop5Δ1 and nop5Δ2 (see Table I and Fig. 5A). Western blotting with B47 and immunoprecipitation with 37C12 detected truncated proteins of apparent molecular masses 54 and 51 kDa from strains bearing nop5Δ1 and nop5Δ2, respectively (Fig. 5, B and C). The truncated forms of Nop5p corresponded more closely to the predicted molecular masses 52.1 and 49.3 kDa, respectively. This indicates that the highly charged COOH terminus of Nop5p is responsible for the larger than predicted size observed in SDS gels.

It is important to note that 37C12 immunoprecipitated a protein from YPW51 and YPW52 that comigrated with the...
67-kDa band from YPW58 (Fig. 5C). Thus, 37°C12 recognized, or co-immunoprecipitated, an additional protein identical in size to Nop5p on SDS gels. Considering that Sik1p/Nop56p (36, 40) is 504 amino acids in length with a predicted size of 56.9 kDa, and is 43% identical to Nop5p, it is likely that Sik1p/Nop56p is the additional protein. In contrast, mAb B47 did not recognize a 67-kDa band in extracts from strains YPW51 and YPW52, and specifically recognized Nop5p (Fig. 5B).

Both of the truncated alleles nop5Δ1 and nop5Δ2 complemented the nop5 disruption (Fig. 5D). Growth rates of YPW51 (nop5Δ1), YPW52 (nop5Δ2), and YPW53 (nOP5) on different media and at different temperatures were compared. Growth of the COOH-terminal truncations was normal at 14 and 25 °C (Fig. 5D). Measurements of doubling times on minimal and rich liquid medium at 30 °C did not reveal significant differences between YPW51, YPW52, and YPW53 (data not shown). However, growth of YPW51 and YPW52 was impaired at 37 °C (Fig. 5D), implying a function for the KXX sequence. IF localization of Nop5p in YPW51, YPW52, and YPW53 grown at 25 °C revealed that the truncated forms of Nop5p were localized to the nucleolus in a manner indistinguishable from wild type (data not shown).

Nop5p is a Member of an Evolutionarily Conserved Protein Family—Data base searches revealed that Nop5p is related to the yeast protein encoded by SKI1/NOP56 (36, 40), and proteins in Methanococcus jannaschii, Arabidopsis thaliana, Caenorhabditis elegans, and human (Fig. 6). Sik1p/Nop56p is 43% identical to Nop5p (pairwise Lipman-Pearson alignment). Two proteins of unknown function from A. thaliana are 52 and 47% identical to Nop5p. A. C. elegans protein is 39% identical to Nop5p and a M. jannaschii protein is 35% identical to Nop5p. Six tentative human consensus sequences shared sequence similarity to Nop5p and may be grouped into two classes. One group aligns with a human homologue of Sik1p/Nop56p (hNop56; Ref. 36), which is more similar to Sik1p/Nop56p (51% identity) than to Nop5p (38% identity). A putative human protein is encoded by a second grouping of three tentative human consensus (see Fig. 6). We refer to this putative human protein as hNop5p. The putative hNop5p is 48% identical to

Nop5p and is 38% identical to Sik1p/Nop56p.

Nop5p is an Essential Gene—To determine if NOP5 is essential, ~90% of one copy of NOP5 in a diploid strain was replaced by TRP1 (Fig. 7A; Table I). Southern blotting confirmed the transplacement of NOP5 with TRP1, and produced the predicted results: ClaI digestion of genomic DNA gives a 6.4 kb band corresponding to wild type NOP5, and an additional 5.7-kb band in YPW42 and YPW43; XbaI gives a 3.8-kb band from wild type, and an additional 2.95-kb band from the disrupted locus (Fig. 7B). YPW42 was transformed with pFW80, sporulated, dissected, and a 5-FOA sensitive strain (YPW45) was obtained. Plasmid shuffling was used to replace pFW80 in YPW45 with pFW83, which carried NOP5 under GAL promoter control, to yield YPW48. YPW48 was viable when grown on galactose containing medium, but not in the presence of glucose, whereas YPW45 was viable on both carbon sources, but was inviable on 5-FOA containing medium (Fig. 7C). This demonstrated that NOP5 is an essential gene.

YPW48 was used to genetically delete Nop5p in vivo by shifting from YPGal to YPD medium. During the first 10 h in YPD, YPW48 grew slightly faster than cells in YPGal (Fig. 7D). However, after approximately 10 h in YPD, cell growth was inhibited and the doubling time increased about 5-fold from ~2.0 to ~10.5 h. Northern blotting showed that NOP5 mRNA levels became undetectable after 1 h of depletion, whereas actin (ACT1) mRNA levels remained unchanged over the time course.
Depletion of Nop5p Leads to Reduced Levels of 40 S Subunits—Since Nop5p is localized in the nucleolus and its depletion leads to a reduction in growth rate, we reasoned that Nop5p was likely to play a role in ribosome synthesis. To test this, polysomes, ribosomes, and ribosomal subunits from cells depleted of Nop5p were analyzed on sucrose density gradients. YPW48 was grown in YPGal, shifted to YPD, and grown for 4, 8, or 12 h. The wild type haploid strain W303-1a grown in both YPGal and YPD showed typical levels of 40 S and 60 S subunits, 80 S monosomes, and polysomes corresponding to 2 to 10 ribosomes (Fig. 8, A and F). YPW48 showed reductions in 40 S, 80 S, and polyome peaks after 8 and 12 h in YPD, but the effect was not dramatic after 4 h in YPD (Fig. 8, B-E). The increase in the 60 S peak reflected an increase in the cytoplasmic pool of free subunits. Because the reductions in peak heights observed at 4 and 8 h preceede the reduction in growth rate at about 10 h, these results cannot be attributed to a secondary effect of reduced growth rate.

Depletion of Nop5p Impairs Synthesis of 18 S rRNA and Processing of Pre-rRNA—The 18 S rRNA is synthesized by the pathway diagrammed in Fig. 9. To explore 18 S rRNA synthesis, the levels of 18 S and 25 S rRNAs from YPW48 grown in YPGal or shifted to YPD for 24 h were compared (Fig. 10A). After growth in YPD for 24 h, the abundance of the 18 S rRNA was reduced by approximately 50%, whereas the abundance of the 25 S rRNA was unaffected (Fig. 10A). This indicated that Nop5p depletion leads to a specific reduction of 18 S rRNA, which could either be at the level of reduced synthesis or stability, or both.

To investigate a role for Nop5p in pre-rRNA processing, YPW48 was analyzed by pulse-chase labeling with [methyl-3H]methionine (Fig. 10B). In SGal, after 2 min of chase, there was little or no accumulation of 35 S pre-rRNA and levels of 27 S and 20 S intermediates were normal. By 8 min of chase, most of the 27 S and 20 S intermediates were processed to mature 25 S and 18 S rRNAs. At 12 min of chase only mature rRNAs were detected. After 4 h of growth in SD, accumulation of 35 S pre-rRNA became visible. Levels of 20 S and 18 S rRNAs were reduced as compared with 27 S and 25 S rRNAs. After 8 and 12 h, the 35 S pre-rRNA accumulation became more prominent, 20 S rRNA levels were reduced substantially, and 18 S rRNA levels decreased to very low levels. On the contrary, processing from 27 S to 25 S rRNA remained similar to results obtained with cells grown in SGal.

To ensure that these results were not due to a change in the methylation pattern of pre-rRNAs, pulse-chase labeling was repeated with [methyl-3H]uracil. YPW48 cells grown in SGal were shifted to SD for 12 h. The results were essentially the same as observed with [methyl-3H]methionine pulse-chase labeling: the 35 S pre-rRNA and levels of 32 S, 20 S, and 18 S rRNAs. After growth in SGal, after 2 min of chase, there was little or no accumulation of 35 S pre-rRNA and levels of 27 S and 20 S intermediates were normal. By 8 min of chase, most of the 27 S and 20 S intermediates were processed to mature 25 S and 18 S rRNAs. At 12 min of chase only mature rRNAs were detected. After 4 h of growth in SD, accumulation of 35 S pre-rRNA became visible. Levels of 20 S and 18 S rRNAs were reduced as compared with 27 S and 25 S rRNAs. After 8 and 12 h, the 35 S pre-rRNA accumulation became more prominent, 20 S rRNA levels were reduced substantially, and 18 S rRNA levels decreased to very low levels. On the contrary, processing from 27 S to 25 S rRNA remained similar to results obtained with cells grown in SGal.

Although processing from 27 S to 25 S rRNA does not seem to be affected by Nop5p depletion, 5.8 S rRNA processing could be affected nevertheless (e.g. 13). Thus, we analyzed the synthesis of 5.8 S rRNA by [3H]uracil pulse-chase labeling. At the different chase times examined (2, 8, 16, and 32 min) there was no observable decrease in 5.8 S rRNA levels relative to the control (data not shown).

Depletion of Nop5p Affects Processing of the 5'-Externally Transcribed Spacer—The defect in production of 18 S rRNA suggested an early defect in pre-rRNA processing. To determine the steady-state levels of pre-rRNAs and rRNAs in Nop5p-depleted cells, Northern blotting analysis was done (see Fig. 9 for oligonucleotide positions). YPW48 cells depleted for 2,
4, 8, and 12 h showed an accumulation of 35 S pre-rRNA and a decrease in levels of 32 S, 20 S, and 18 S rRNAs (Fig. 11). The 23 S intermediate is usually present at very low levels in wild type cells and corresponds to an intermediate in which cleavage at A₀, A₁, and A₂ has failed to take place (5, 8, 12, 14). Levels of the 27 S intermediate decreased by approximately 2.5-fold after 2 h of growth in glucose, but did not decrease dramatically at longer times in SD medium (Fig. 11). Levels of 18 S and 25 S rRNAs decreased only a small amount during the depletion time course (Fig. 11), and after 12 h of depletion were 60 and 68%, respectively, of the levels at the 0-h time point. Taken together, the Northern blotting results suggested a defect in early processing steps in the 5'-ETS and ITS1.

To examine processing at sites in the 5'-ETS and ITS1, primer extension analysis was done (see Fig. 9 for oligonucleotide positions). At 12 and 24 h of Nop5p depletion, processing at site A₀ was progressively impaired (Fig. 12A). At 24 h, the band corresponding to processing at A₀ was decreased in intensity by 76% compared with the band at 0 h. In addition, a number of longer primer extension products were observed (Fig. 12A, lanes 12 and 24), which was consistent with the accumulation of unprocessed 35 S pre-rRNA. Similarly, processing in ITS1 at site A₂ was impaired (Fig. 12B). At 24 h, the band corresponding to processing at A₂ was decreased in intensity by 86%.

**Fig. 7. NOP5 is an essential gene.** A, map of a portion of chromosome XV illustrating the replacement of NOP5 with TRP1. The positions of primers 1–5 are indicated. B, Southern blot of genomic DNA from YSB25, YPW42, or YPW43 digested with ClaI (lanes 1–3) or XbaI (lanes 5 and 6). Sizes in kb. C, replica platings on media with and without 5-FOA. Serial dilutions (10-fold) were grown at 30 °C for 3 (SD) or 5 (SGal) days. D, growth after shift to YPD or YPGal media. Cultures were diluted to maintain OD₆₀₀ below 0.5.

**Fig. 8. Nop5p is required for 40 S subunit synthesis.** W303–1a or YPW48 were grown in YPGal (Gal) or in YPD (Glu) for 4, 8, or 12 h. Cell extracts were separated on linear 10–50% sucrose gradients and analyzed by absorbance measurement at 254 nm (shown in arbitrary units). The positions of the 40 S, 60 S, and 80 S peaks are indicated ( ). Peaks corresponding to polysomes are distributed between the 80 S peak and the bottom of the gradient.
compared with the band at 0 h. To control for variables such as RNA yield, the relative abundance of the 18 S rRNA was determined. Bands corresponding to the 5'-end of 18 S rRNA (processing site A1) showed only small variations in intensities (Fig. 12C). At 24 h, the band corresponding to processing at A1 was decreased in intensity by only 10% compared with the band at 0 h. We note that the primer extension method we use does not allow us to address processing at A1 during Nop5p depletion (see “Experimental Procedures”). To rule out the possibility that reductions in A0 and A2 band intensities could be attributed to reduced transcription of the 35 S precursor, the relative amounts of the 5'-end were determined (Fig. 12D). At 24 h, the band corresponding to the 5'-end was decreased in intensity by only 4% compared with the band at 0 h. Thus, reductions in processing at sites A0 and A2 during Nop5p depletion cannot be accounted for by alterations in 35 S transcription.

Nop5p Is Associated with Small Nucleolar RNAs—Given the effects of Nop5p depletion on pre-rRNA processing and the likely interaction between Nop5p and Nop1p, we tested whether Nop5p was associated with snoRNAs. RNAs immunoprecipitated by B47 and 37C12 were 3'-end-labeled and analyzed by denaturing PAGE. Identification of snoRNAs was based on RNA lengths determined by comparison with a DNA sequencing ladder (data not shown). B47 immunoprecipitated snoRNAs that migrated at positions corresponding to U3, U14, snR13, and U18 (Fig. 13). 37C12 immunoprecipitated snR13 and U18 strongly, but immunoprecipitated U3 only weakly, and immunoprecipitated only one of the U14 isoforms. Minor bands were also immunoprecipitated by the mAbs, especially by 37C12, and may be snoRNAs more loosely associated with Nop5p, or snoRNAs that were nonspecifically associated with Nop5p. Small amounts of 5.8 S and 5 S rRNAs were immunoprecipitated nonspecifically, and were also observed in a control immunoprecipitate (Fig. 13). These findings indicated that Nop5p is associated, either directly or indirectly, with the snoRNAs U3, U14, snR13, and U18.
As mentioned above, B47 did not immunoprecipitate Nop5p from yeast nuclear extracts. To investigate this discrepancy, we compared immunoprecipitates obtained with B47 and 37C12 using the two different methods for protein and RNA immunoprecipitation (see “Experimental Procedures”). We found that B47 immunoprecipitated a 67-kDa band of moderate intensity with the RNA method, but not with the protein method (data not shown). Conversely, the band observed in 37C12 immunoprecipitates is considerably weaker with the RNA method compared with the protein method (data not shown). Thus, the difference in RNA and protein immunoprecipitation buffers was an important factor in the binding of antigens by B47 and 37C12, perhaps as a consequence of epitope conformation. In addition, the 67-kDa band immunoprecipitated by B47 comigrated with the band immunoprecipitated by 37C12, indicating that the 67-kDa band recognized in immunoprecipitations was the same as the 65-kDa band recognized by B47 on immunoblots. Immunoprecipitates contain a large amount of IgG heavy chain, which could influence the mobility of Nop5p during SDS-PAGE and result in a small difference in apparent size in immunoblotting and immunoprecipitation experiments.

Nop5p Is Required for Localization of Nop1p to the Nucleolus—

Of interest to us are the mechanisms by which nucleolar proteins are localized to, and interact within, the nucleolus. Immunofluorescence and cell fractionation approaches were used to determine the extent to which the nucleolar localization of Nop5p and Nop1p was interdependent.

Strikingly, Nop5p depletion affected the localization of Nop1p, and caused Nop1p to become distributed in the nucleus and cytoplasm (Fig. 14A). After growth of YPW48 for 4 h in glucose, Nop5p was only faintly detected by mAb B47. Staining with mAb 37C12 also showed a decrease in intensity after 4 and 8 h, but faint staining remained even after 12 h in glucose medium. We attribute this residual staining to the recognition of Sik1p/Nop56p, whose levels may have decreased during Nop5p depletion. The effect on distribution was specific to Nop1p because the localization of Nsr1p was not affected by Nop5p depletion (Fig. 14A). The distribution of the nucleolar protein Nop2p, the nuclear protein homocitrate synthase, and the nuclear pore complex protein Nsp1p were not affected by Nop5p depletion (data not shown). In addition, the localization of Nop1p was strictly nucleolar in strains carrying the nop51 and nop52 COOH-terminal truncation alleles grown at either 30 or 37 °C (data not shown).

To confirm the immunofluorescence results, immunoblotting was done using crude nuclear and cytoplasmic fractions (see “Experimental Procedures”). Depletion was rapid and Nop5p was barely detectable after only 4 h of growth on glucose (Fig. 14B). During depletion, nuclear levels of Nop1p decreased, while cytoplasmic levels increased (Fig. 14B). The level of nuclear Nop1p increased by 15% after 4 h on glucose, but Nop1p levels decreased by 25 and 31% at 8 and 12 h, respectively. The level of cytoplasmic Nop1p increased steadily between the 4 and 12 h time points, and at 12 h reached a level equal to 219%
of the level at zero time. Thus, Western blotting results confirmed that efficient localization of Nop1p to the nucleolus requires normal levels of Nop5p.

**DISCUSSION**

In this report, two monoclonal antibodies have been used to identify and characterize a novel, essential nucleolar protein, Nop5p, that is required for processing of pre-18 S rRNA. Our findings suggest that Nop5p functions in concert with Nop1p, which is known to be involved in pre-rRNA processing and ribosome assembly (9, 15). During the course of these studies, Gautier et al. (36) also identified this nucleolar protein and termed it Nop58p, but did not investigate its function in pre-rRNA processing or ribosome synthesis.

Nop5p contains a carboxyl-terminal KKX repeat motif (X is not basic and is usually Glu or Asp), which has been found in the nucleolar proteins Chb5p and Dbp3p (38, 39). Nop5p contains 16 repeats, whereas Chb5p and Dbp3p both possess 10 repeats. Chb5p was originally identified as a centromere binding factor (38), but subsequent studies have shown that Chb5p is a nucleolar protein required for rRNA synthesis (41). Dbp3p is a DEAD-box helicase required for normal rates of synthesis of 25 S rRNA (39). Deletion of the KKX repeats in Chb5p results in no detectable growth phenotype (38), whereas deletion of the KKX motif in Dbp3p caused Dbp3pΔKKX to be distributed throughout the cell (39). In Nop5p, removal of either 12 or all 16 of the KKX repeats is dispensable for growth at 25 and 30 °C, and has no visible effect on localization of Nop5p or Nop1p to the nucleolus. However, we observe that strains expressing Nop5p without KKX repeats grow substantially slower at 37 °C. Immunofluorescence and immunoblotting studies show that COOH-terminal truncations of Nop5p render the protein more labile at 37 °C, which suggests a function for this motif in maintaining the stability of Nop5p. In vivo genetic depletion with a conditional GAL-NOP5 allele indicates a role for Nop5p in pre-rRNA processing and ribosome synthesis. Specifically, Nop5p is required for early steps in the processing of the 35 S precursor rRNA at sites A0 and A2. Other gene products required for processing at A0 and A2 are also required for processing at A1 (5–13). Therefore, it is likely that Nop5p is also required for processing at A1, although our studies do not address processing at this site. Depletion of Nop5p leads to impaired synthesis of the 18 S rRNA and leads to reduced levels of the small 40 S ribosomal subunit. Processing at sites within ITS2 or within the 3’-ETS is not significantly affected, which results in levels of 25 S and 5.8 S rRNAs that are only slightly reduced compared with wild type cells. Electron microscopic analysis of cells arrested after 12 h of Nop5p depletion reveals a failure of nuclei to orient and migrate toward the bud neck during mitosis in a significant percentage of cells. This growth arrest phenotype could, however, be a secondary effect due to failure to synthesize a protein required for normal progression through mitosis.

Consistent with its role in rRNA processing, we find that Nop5p is associated, either directly or indirectly, with the snoRNAs U3, snR13, U14, and U18, which indicates that Nop5p is a component of a snoRNP complex. It is likely that Nop5p interacts with Nop1p in this snoRNP complex, based on our immunoprecipitation studies. In support of this view, Gautier et al. (36) have used Protein A fusions to study interactions between Nop58p (Nop5p), Nop56p, and Nop1p, and present evidence for the existence of a complex containing all three nucleolar proteins. U3, U14, snR30, and a number of other snoRNAs have previously been shown to be associated with

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5 P. Wei and J. P. Aris, unpublished results.
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Nop1p (9). The Nop1p homologue in vertebrates, fibrillarin, is a component of the U3 snoRNP complex (42, 43). U3, snR13, U14, and U18 are members of the C/D class of snoRNAs, which designate sites within pre-rRNAs for processing and modification (18, 20). However, Nop5p does not appear to be unique to the U3 snoRNP, which distinguishes it from snoRNP components such as Mpp1p, which is primarily associated with one snoRNA, U3 (24).

Depletion of Nop5p leads to mislocalization of the nucleolar protein Nop1p to the nucleoplasm and cytoplasm, while the nucleolar proteins Nop2p and Nsr1p are not affected. This provides evidence for a functional interaction between Nop5p and Nop1p, and raises the question of mechanism for Nop1p mislocalization during Nop5p depletion. Studies in Xenopus indicate that post-transcriptional maturation of U3, U8, and U14, and incorporation into snoRNP complexes takes place within the nucleolus (44, 45). Thus, Nop5p could be responsible for, or contribute to, binding interactions that retain Nop1p in the nucleolus. Alternatively, the absence of Nop5p may retard nuclear import of a Nop1p-containing snoRNP complex that shuttles between cytosolic and nuclear compartments. A Nop5p-containing snoRNP complex is likely to contain additional proteins. The mAb 28C4 immunoprecipitates a protein of apparent molecular mass 120 kDa in addition to bands that comigrate with Nop5p and Nop1p.6

Nop5p is a member of a protein family that has been conserved through evolution. The existence of a human counterpart of Nop5p, hNop5p, is supported by sequence data that have emerged from the human genome project. Also, Nop5p is similar to the yeast protein Sik1p/Nop56p, which shares significant sequence homology with a related human protein hNop5p (40). Construction of an evolutionary tree based on sequence comparisons reveals a grouping of Nop5p with hNop5p on one branch and a grouping of Sik1p/Nop56p and hNop56p on another branch. A. thaliana also possesses a pair of closely related Nop5p-like gene products, but only one putative homologue has been identified in C. elegans and M. jannaschii. The Nop5p-like proteins in human, A. thaliana, and C. elegans do not possess the KK repeat motif, but possess basic COOH termini. In M. jannaschii the Nop5p-like protein possesses eight COOH-terminal KXX repeats and is on an evolutionary branch separate from the branch that gives rise to the other Nop5p-like family members. It is interesting that a pair of related proteins exists in yeast, human, and plant. The fact that a nop5 null allele is lethal demonstrates that the functions of Nop5p and Sik1p/Nop56p do not overlap. Furthermore, our studies show that Nop5p is required for synthesis of the small 40 S ribosomal subunit, whereas Nop56p has been shown to be primarily involved in synthesis of the large 60 S ribosomal subunit (36). One possibility is that each protein executes a similar function, but at different points in the rRNA processing pathway. It will be interesting to see whether the presence of a pair of related proteins is the rule for most species, and to define the functions of these nucleolar proteins to ascertain their shared and distinctive attributes.

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