The yeast *NOP4* gene product is an essential nucleolar protein required for pre-rRNA processing and accumulation of 60S ribosomal subunits

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The *Saccharomyces cerevisiae* *NOP4* gene was isolated by screening a λ gt11 yeast genomic DNA library with a monoclonal antibody against a yeast nucleolar protein. *NOP4* encodes a 78 kDa protein that contains two prototypical RNA recognition motifs (RRMs) flanking an imperfect RRM lacking characteristic RNP1 and RNP2 motifs. In addition, there is a fourth incomplete RRM. *NOP4* is a single copy essential gene present on chromosome XVI, between *RAD1* and *PEP4*. To examine the function of Nop4p, we constructed a conditional null allele of *NOP4* by placing this gene under the control of the glucose-repressible *GAL1* promoter. When cells are shifted from galactose-containing medium to glucose-containing medium, *NOP4* transcription is terminated, Nop4 protein is depleted and cell growth is impaired. Nop4 protein depletion results in diminished accumulation of 60S ribosomal subunits, assignable to a defect in ribosome biogenesis arising from a lack of production of mature 25S rRNA from 27S precursor rRNA.

Key words: pre-rRNA processing/ribosome assembly/RNA recognition motifs/yeast nucleolar protein

Introduction

Most steps of eukaryotic ribosome biogenesis occur in the nucleolus, a subnuclear compartment defined by the location of actively transcribed rDNA (Karpen *et al.*, 1988; reviewed in Hadjiolov, 1985; Sommerville, 1986; Nigg, 1988; Scheer and Benavente, 1990; Warner, 1990; Hernandez-Verdun, 1991; Scheer *et al.*, 1993). Nucleolar functions necessary for ribosome biogenesis include transcription of rDNA, post-transcriptional modification and processing of pre-rRNA, transport of molecules to and from the nucleolus, and assembly of ribosomal macromolecules in a coordinated fashion. These processes require the function of a large number of factors, both ribosomal and non-ribosomal, including RNAs, enzymes and other proteins.

Factors required for transcription of rDNA, including RNA polymerase I and associated transcription factors and topoisomerases, have been identified and characterized in some detail (Ross *et al.*, 1988; reviewed in Reeder, 1990; Sollner-Webb and Mougey, 1991). However, the functions of only a small number of other non-ribosomal nucleolar molecules that participate in ribosome synthesis have been investigated.

At least six different small RNA molecules are specifically localized to metazoan nucleoli. By virtue of their sequence complementarity with pre-rRNA or their demonstrated association with pre-rRNA or pre-ribosomal particles, these small nucleolar RNAs (snoRNAs) are hypothesized to be involved in ribosome assembly (reviewed in Fournier and Maxwell, 1993; Mattaj *et al.*, 1993). The U3 and U8 snoRNAs have been shown to be necessary for pre-rRNA processing (Kass *et al.*, 1990; Savino and Gerbi, 1990; Peculis and Steitz, 1993).

Several non-ribosomal nucleolar proteins are known to be required for ribosomal subunit synthesis. Fibrillarin is associated with the U3, U8 and U13 snoRNAs, and is required for early steps of rRNA processing (Tyc and Steitz, 1989; Kass *et al.*, 1990). Nucleolin binds to both rDNA and pre-rRNA and shuttles between the nucleolus and cytoplasm. These findings suggest roles for nucleolin in rRNA transcription and assembly, as well as transport of nucleolar molecules (Herrera and Olson, 1986; Bugler *et al.*, 1987; Lapeyre *et al.*, 1987; Borer *et al.*, 1989; Caizergues-Ferrer *et al.*, 1989). B23, also called NO38, numatrin or ribocharin, is associated with preribosomal particles and may facilitate ribosome assembly (Spector *et al.*, 1984; Hügler *et al.*, 1985; Schmidt-Zachmann and Franke, 1988; Zhang *et al.*, 1989). Like nucleolin, B23 shuttles between the nucleolus and cytoplasm and may be involved in transport of nucleolar components (Borer *et al.*, 1989).

The mechanisms of pre-rRNA processing and ribosome assembly have been investigated in the yeast *Saccharomyces cerevisiae*. Mutants defective in pre-rRNA processing or ribosome assembly and genes encoding snoRNAs or nucleolar proteins have been identified and manipulated to study the functions of nucleolar molecules (reviewed in Warner, 1990; Woolford, 1991; Woolford and Warner, 1991; Fournier and Maxwell, 1993). Four of the 12 yeast snoRNAs, U3, U14, snR10 and snR30, have been shown to be necessary or important for pre-rRNA processing (reviewed in Tollervey and Hurt, 1990; Fournier and Maxwell, 1993). Nop1p, the yeast homologue of fibrillarin, is associated with all of the known yeast snoRNAs and is required for methylation and processing of pre-rRNA (Schmang *et al.*, 1989; Tollervey *et al.*, 1991, 1993). Ssb1p, Gar1p and Sof1p are associated with a subset of snoRNAs and are important or necessary for one or more steps of pre-rRNA processing (Clark *et al.*, 1990; Girard *et al.*, 1992; Jansen *et al.*, 1993). Nop3p (Npl3p) and Nsr1p have not been shown to be associated with snoRNAs but are necessary for ribosome biogenesis (Bossie *et al.*, 1992; Kondo and Inouye, 1992; Lee *et al.*, 1992; Russell and Tollervey, 1992). Spb4p, Drs1p, Dbp3p and CA9 are putative RNA dependent helicases required for assembly of either 40S or 60S ribosomal subunits (Sachs and Davis, 1990; Ripmaster *et al.*, 1992; P. Weaver

and T.H.Chang, personal communication; C.O'Day and J.Abelson, personal communication). *RRP2* (*NME1*) encodes the RNA moiety of RNase MRP that is necessary for processing of pre-5.8S rRNA (Shuai and Warner, 1991; Lindahl *et al.*, 1992; Schmitt and Clayton, 1993; Chu *et al.*, 1994).

To investigate further the role of nucleolar proteins in ribosome biogenesis, we have isolated a new yeast nucleolar protein gene, *NOP4*. We utilized a monoclonal antibody against a novel nucleolar antigen to screen a λ gt11 yeast genomic DNA expression library. Nop4p is an essential nucleolar protein containing several RNA recognition motifs. Depletion of Nop4p impairs pre-rRNA processing, particularly the steps leading to the production of 25S rRNA. Consequently, assembly of 60S ribosomal subunits is disrupted. *NOP4/NOP77* was also identified in a screen for synthetic lethal mutation with a mutation in the *NOP1* gene (Berges *et al.*, 1994).

Results

Cloning of *NOP4*

Mouse monoclonal antibody 28.3a specifically stains the yeast nucleolus (Figure 1A and D), identifiable as a crescent-shaped structure that occupies approximately one-third of the nucleus adjacent to the nuclear envelope (Sillevis-Smitt *et al.*, 1973). Identical immunofluorescence staining patterns were obtained with antisera against the bona fide yeast nucleolar proteins Nop1p or Nsr1p (data not shown). Western immunoblot analysis indicated that antibody 28.3a recognizes a yeast polypeptide that migrates on SDS-polyacrylamide gels with an apparent molecular size of 100 kDa and is present in subcellular fractions

enriched for nuclei (data not shown). Because the apparent size of this protein is different from any previously identified yeast nucleolar protein, we used the 28.3a antibody to screen a λ gt11 yeast genomic DNA expression library to identify a novel yeast nucleolar protein gene.

One positive clone was identified by screening 8×10^5 plaques. Western immunoblotting of a lysate from *Escherichia coli* transfected with this phage identified a 180 kDa protein that cross-reacted with both antibody 28.3a and anti- β -galactosidase antibody (data not shown). The complete gene was recovered by probing a library of yeast genomic DNA cloned in YEp13 with yeast DNA amplified by PCR from the phage clone. We verified that the single plasmid identified from the YEp13 library, YEp13NOP4, contains a gene encoding the yeast nucleolar antigen by the following criteria. (i) The intensity of nucleolar staining by antibody 28.3a was increased in cells containing the high copy plasmid YEp13NOP4 relative to wild type cells (Figure 1, compare A with D). (ii) A significantly greater amount of the 100 kDa antigen could be detected by immunoblot analysis of extracts from cells transformed with YEp13NOP4, compared with wild type cells or YEp13 vector transformed cells (data not shown). We have named this gene *NOP4*, for nucleolar protein 4. *NOP4* was localized within the 10 kb yeast DNA insert of YEp13NOP4, as determined by comparison of the restriction map of this DNA with that of the original λ gt11 clone (Figure 2) and by Southern hybridization.

Nop4p contains multiple RNA recognition motifs

The nucleotide sequence of *NOP4* and deduced amino acid sequence of Nop4p are shown in Figure 3. The sequence revealed a single open reading frame that could

Fig. 1. Yeast Nop4p detected by monoclonal antibody 28.3a is localized in the nucleolus. SC252 (wild type) cells (A–C) and JWY4905 cells (containing *NOP4* on a high copy plasmid) (D–F) were grown in YEPGlu at 30°C to a density of 1×10^8 cells per ml, and fixed. (A and D) Cells incubated with mouse monoclonal antibody 28.3a followed by rhodamine-conjugated anti-mouse IgG antibody to detect Nop4p antigen. (B and E) Cells treated with 4',6 diamidino-2-phenylindole (DAPI) to visualize nuclear and mitochondrial DNA. (C and F) Cells viewed by Nomarski DIC optics. The exposure time for rhodamine fluorescence images of SC252 cells (A) was 5-fold longer than that for JWY4905 cells (D), in order to obtain comparable signal intensity.

encode a 685 amino acid protein with a predicted molecular mass of 77 824 Da and an isoelectric point of 9.8. Nop4p contains four RNA recognition motifs (RRMs, also called RBDs or RNP-80). This motif has been found in many RNA binding proteins, including a number of nucleolar proteins (reviewed in Bandziulis *et al.*, 1989; Mattaj, 1989; Scherly *et al.*, 1989; Keene and Query, 1991; Kenan *et al.*, 1991; Dreyfuss *et al.*, 1993). Three tandem RRM are present in the amino-terminal half of Nop4p, between amino acids 28 and 358. A fourth incomplete RRM is present between amino acids 533 and 574. A short acidic domain (14/18 amino acids are aspartate or glutamate residues) is present in the region between the second and third RRM, from amino acids 250 to 267. The carboxy-terminal domain of Nop4p contains many charged residues; 24 of the last 68 amino acids are arginines or lysines, and 13 are glutamate or aspartate residues. A diagram of motifs in Nop4p is shown in Figure 4.

The discrepancy between the apparent molecular size of Nop4p as determined by SDS-PAGE (100 kDa) and the predicted size (78 kDa) can be accounted for by the presence of the RRM. Many proteins containing RRM migrate more slowly than predicted from their sizes (Wilk *et al.*, 1985; Adam *et al.*, 1986; Sachs *et al.*, 1986; Jong *et al.*, 1987; Lapeyre *et al.*, 1987; Bossie *et al.*, 1992; Lee *et al.*, 1992; Russell and Tollervey, 1992).

***NOP4* is a single copy essential gene**

Genomic Southern blot analysis indicated that *NOP4* is a single copy gene. A 2.2 kb *EcoRI* fragment containing most of the *NOP4* coding sequence hybridized to a single *PstI* genomic DNA fragment, two *ClaI* genomic DNA fragments and three *BglIII* genomic DNA fragments,

Fig. 2. Restriction map of yeast genomic DNA containing *NOP4* and map of the *nop4::TRP1* disruption allele. (A) Restriction map of the 6.6 kb *PstI* fragment of yeast genomic DNA cloned in plasmid YEp352NOP4. (B) *nop4::TRP1* null allele. A 1009 bp *SpeI*-*ClaI* fragment containing the 5' half of the *NOP4* gene was replaced with the *TRP1* gene, as shown. The direction of transcription of *NOP4* is indicated by the arrowhead. B, *BglIII*; C, *ClaI*; P, *PstI*; R, *EcoRI*; Sa, *SacI*; Sc, *Scal*; Sp, *SpeI*; Ss, *SspI*.

respectively (data not shown), as predicted from the restriction map of YEp352NOP4 (Figure 2). *NOP4* was localized to the left arm of chromosome XVI, between *PEP4* and *RAD1*, by hybridization of the *NOP4* DNA first to a blot of electrophoretically separated yeast chromosomes and subsequently to a grid of cloned overlapping genomic restriction fragments (data not shown) (Link and Olson, 1991; Riles *et al.*, 1993).

A null allele of *NOP4* was created by removal of a 1 kb *SpeI*-*ClaI* fragment containing the 5' 323 codons of the *NOP4* open reading frame and replacement of this fragment with the yeast *TRP1* gene (Figure 2B). One copy of *NOP4* in diploid JM749 was replaced with the *nop4::TRP1* allele by transformation and homologous recombination (Rothstein, 1983). The expected gene replacement was confirmed by genomic Southern hybridization (data not shown). The resulting heterozygote, JWY4914, of *NOP4/nop4::TRP1* genotype, was sporulated and 40 tetrads were dissected. Thirty tetrads gave two viable spores per ascus, while 10 gave only one viable spore. All of these viable spores were *Trp*⁻. To eliminate the possibility that *NOP4* is essential only for spore

Fig. 3. Nucleotide sequence of *NOP4* and predicted amino acid sequence of Nop4p. The nucleotide sequence is shown on the top line, and the deduced amino acid sequence in single-letter code is shown on the bottom line. The RNA recognition motifs (RRMs) are boxed, the conserved octamer sequence RNPI and hexamer sequence RNP2 are shaded, and the potential nuclear localization sequences are underlined. The GenBank accession number for *NOP4* is U02598.

Fig. 4. Schematic of motifs in Nop4p. The Nop4 protein contains two RRM s containing RNP1 (■) and RNP2 (▨), flanking an imperfect RRM, and a partial RRM containing only the RNP1 octamer sequence. There is a short acidic domain (▤) between RRM2 and RRM3, and a basic domain (⋯) near the carboxy-terminus of Nop4p.

germination, we also constructed haploid strain JWY4917, which contains *nop4::TRP1* on chromosome XVI and *NOP4* plus *URA3* on plasmid pRS316NOP4. This strain was unable to grow on medium containing 5-fluoroorotic acid (5-FOA). Taken together, these results indicate that *NOP4* is essential for vegetative growth of yeast.

Construction of a conditional lethal null allele of *NOP4*

In order to examine the function of Nop4p, strain JWY4919 was constructed, in which the only functional copy of *NOP4* is a plasmid-borne *GALI-NOP4* promoter fusion that is expressed in galactose-containing medium (YEP-Gal) but not in glucose-containing medium (YEPGlu). JWY4919 grows with a doubling time of 3.2 h in YEPGal, similar to wild type *NOP4* strains (Figure 5A and data not shown), indicating that the *GALI-NOP4* construct is fully functional. Within 30 min after shifting JWY4919 from YEPGal to YEPGlu, no *NOP4* mRNA was detected by Northern analysis (data not shown). Western immunoblot analysis indicated that Nop4p decreased after the shift to YEPGlu; by 15 h no Nop4p could be detected (Figure 5B). JWY4919 divided about every 2 h during the first 15 h after shifting from YEPGal to YEPGlu. Fifteen hours after the medium shift, the doubling time of JWY4919 began to increase significantly (Figure 5A). This diminished growth upon termination of *NOP4* expression was reversible; ~80% of the JWY4919 cells grown in YEPGlu for 40 h were able to form colonies if plated on YEPGal medium, where *GALI-NOP4* is expressed. No colonies formed when cells were plated on YEPGlu medium. Thus, the cells that have stopped dividing are not dead but can resume growth once Nop4p is provided.

Depletion of Nop4p results in a deficit of 60S ribosomal subunits

To determine whether Nop4p is essential for production of ribosomes, the amounts of 40S and 60S ribosomal subunits, 80S monoribosomes, and polyribosomes were assayed upon depletion of Nop4p. JWY4917 containing the low copy plasmid pRS316 bearing *NOP4* under the control of its own promoter was used as a positive control. Extracts prepared from JWY4917 and JWY4919 grown in YEPGal and shifted to YEPGlu for 4 h or 12 h were fractionated by sucrose velocity gradient centrifugation. Assaying amounts of free 40S and 60S ribosomal subunits by this means has proven to be a sensitive measure of defects in ribosome assembly (Moritz *et al.*, 1990, 1991; Ripmaster *et al.*, 1992, 1993; Deshmukh *et al.*, 1993). When expression of *NOP4* was terminated in JWY4919, fewer free 60S ribosomal subunits accumulated relative to free 40S subunits (Figure 6C and D). The relative amounts of 40S and 60S subunits were unchanged in JWY4917, in which Nop4p continued to be synthesized in wild type amounts (Figure 6, A and B). Additional

Fig. 5. Yeast cells stop dividing upon depletion of Nop4p. (A) Growth curve of JWY4919 (*GALI-NOP4*) grown in YEPGal medium and after shift to YEPGlu medium. Data are represented as $\log OD_t/OD_0$, where t is the time in hours after shifting medium. (B) Depletion of Nop4p after switching cells to YEPGlu medium. Cell extracts prepared from the same culture used for the growth curve were assayed by Western immunoblot analysis using monoclonal antibody 28.3a. Equal amounts of protein were loaded in each lane, as judged by immunodetection of ribosomal protein L1 (data not shown). The second, more rapidly migrating band detected by antibody 28.3a probably is a degradation product of the 100 kDa Nop4 polypeptide; it is absent in extracts prepared from the proteinase deficient yeast strains BJ2168 and BJ3501 (data not shown).

peaks sedimenting more quickly than 80S monoribosomes or polyribosomes were evident in the extracts of JWY4919 12 h after the shift from YEPGal to YEPGlu (Figure 6D, arrows). These peaks represent halfmer polyribosomes that contain mRNAs associated with an integral number of ribosomes plus a stalled 48S preinitiation complex. Such halfmers accumulate when 60S subunit assembly is aborted (Rotenberg *et al.*, 1988; Ripmaster *et al.*, 1992; Deshmukh *et al.*, 1993; Goyer *et al.*, 1993). The deficit of 60S ribosomal subunits in JWY4919 was more pronounced 12 h after the shift than at 4 h, in parallel with the decrease in the amount of Nop4p (Figure 5B). These effects on amounts of 60S ribosomal subunits are probably a direct result of depletion of Nop4p, rather than being an indirect result of impaired growth rate, since 60S ribosomal subunits were diminished before any noticeable decrease in the rate of division of the JWY4919 cells.

Nop4p is required for processing of pre-rRNA

We investigated whether the deficit of 60S ribosomal subunits observed upon depletion of Nop4p resulted from

Fig. 6. Depletion of Nop4p results in a deficit of 60S ribosomal subunits. Polyribosome profiles are shown for JWY4917 (*NOP4*) and JWY4919 (*GAL1-NOP4*). Cells were shifted from YEPGal to YEPGlu for 4 h (A and C) and 12 h (B and D). Free ribosomal subunits and polyribosomes in cell extracts were separated on 7–47% sucrose gradients. Peaks representing 40S and 60S ribosomal subunits and 80S monoribosomes are labelled and halfmer polyribosomes are indicated by vertical arrows.

defects in synthesis or processing of pre-rRNA. JWY4919 cells were grown in YEPGal and shifted to YEPGlu for 4 h or 12 h, then pulse-labelled for 3 min with L-([³H]methyl)methionine, and chased with a 2000-fold excess of cold methionine for 3 or 10 min. Synthesis and processing of rRNA in JWY4919 grown in YEPGal or in the *NOP4* strain JWY4917 grown in YEPGlu were identical to previously observed wild type patterns (Figure 7). Four hours after shifting from YEPGal to YEPGlu, synthesis and processing of pre-rRNAs in JWY4919 were similar to the patterns observed for these above-mentioned wild type controls (Figure 7). The 35S rRNA primary transcript, the 32S pre-rRNA processing intermediate, the 27SA and 27SB precursors to mature 25S rRNA and the 20S precursor to 18S mature rRNA were detected in the 3 min pulse, as were small amounts of mature 25S and 18S rRNAs. After the 3 min or 10 min chase, the majority of rRNAs detected were mature 25S and 18S rRNA. Only small amounts of 27S and 20S processing intermediates were evident after the 10 min chase. In other experiments, no processing intermediates were detected after a 20 or 30 min chase (data not shown). In contrast, 12 h after shifting JWY4919 to YEPGlu, rRNA processing was severely perturbed (Figure 7). The 35S pre-rRNA and the 32S, 27SA and 20S processing

Fig. 7. Depletion of Nop4p impairs pre-rRNA processing and blocks accumulation of 25S rRNA. Synthesis and accumulation of pre-rRNA were assayed in JWY4919 grown in YEPGal and 4 h and 12 h after shifting from YEPGal to YEPGlu medium, as well as in JWY4917 grown in YEPGlu medium. Cells were pulse-labelled for 3 min with L-([³H]methyl)methionine, and chased for 3 and 10 min with a 2000-fold excess of unlabelled methionine. Data for JWY4919 grown in YEPGal medium were obtained in a separate experiment. The rRNA precursor, processing intermediates and mature rRNAs are labelled.

Fig. 8. Nop4p is required for pre-rRNA processing and accumulation of 25S rRNA. Cells of JWY4919 grown in YEPGal and 4 h and 12 h after shifting to YEPGlu medium were pulse-labelled for 2 min with [3 H]uracil, and chased for 3, 10 and 30 min with excess unlabelled uracil. The rRNA precursor, processing intermediates and mature rRNAs are labelled.

intermediates, as well as a small amount of mature 18S rRNA, were detected in the 3 min pulse, but no 27SB rRNA precursor was detected. After the 10 min chase, no precursors or intermediates were detected, and very little mature 25S rRNA accumulated. However, substantial amounts of 18S rRNA were produced. The ratio of newly synthesized 25S rRNA to 18S rRNA detected after a 10 min chase in cells shifted for 12 h to YEPGlu was 15-fold lower than in cells grown in YEPGal or shifted to YEPGlu for 4 h.

To exclude the possibility that impaired production of 25S rRNA resulted from the effects of methylation of rRNA, pulse-chase experiments were also performed using [3 H]uracil. Identical results were obtained; the amounts of 25S rRNA were significantly diminished relative to 18S rRNA when Nop4 protein was depleted (Figure 8). Thus the deficit of 60S ribosomal subunits that we observed upon depletion of Nop4p resulted from impaired processing of pre-rRNA and reduced production of mature 25S rRNA.

Discussion

We have identified and characterized a novel yeast nucleolar protein gene, *NOP4*. Nop4p is an essential protein necessary for the production of 25S rRNA and 60S ribosomal subunits.

Nop4p contains several different structural modules, as observed for many nucleolar proteins. There is an acidic domain between amino acids 250 and 267 and a basic domain in the carboxy-terminus, both of which might function as surfaces for interaction with other molecules. Unlike many other nucleolar proteins, Nop4p does not contain an extended glycine- and arginine-rich (GAR) domain (summarized in Girard *et al.*, 1992). Embedded within the basic carboxy-terminal domain of Nop4p are the sequences KRRR (618–621), KRTR (630–633), KKPCK (648–652) and KRKRK (679–683), which resemble

nuclear localization sequences (NLSs) required for entry of proteins into the nucleus (reviewed in Garcia-Bustos *et al.*, 1991). The sequence KRreqqlqartKRTR (618–633) resembles a bipartite nuclear targeting sequence (Robbins *et al.*, 1991).

The most pronounced feature of Nop4p is the presence of four RNA recognition motifs, three tandemly arranged in the amino-terminal half of the protein, and a fourth truncated RRM located in the carboxyl-terminal half of Nop4p. Within the ~80–100 amino acid long canonical RRM are the highly conserved octapeptide RNP1 and the slightly less conserved hexapeptide RNP2 located 30–40 amino acids amino-terminal to RNP1. There are 10 other conserved individual residues located at specific positions within RRM (Bandziulis *et al.*, 1989; Mattaj, 1989; Keene and Query, 1991; Kenan *et al.*, 1991; Dreyfuss *et al.*, 1993). Structural studies of several RRM-containing proteins show that the RRM contains a $\beta\alpha\beta\beta\alpha\beta$ structure, in which the four β strands form an antiparallel β sheet, with RNP2 and RNP1 located in the inner β 1 and β 3 strands of the β sheet (Nagai *et al.*, 1990; Hoffman *et al.*, 1991; Wittekind *et al.*, 1992).

RRM1 and RRM3 of Nop4p both contain prototypical RNP1 and RNP2 sequences, and overall are quite similar in sequence to a number of other RRMs. Both the Chou–Fasman (Chou and Fasman, 1978) and the Garnier–Robson methods (Garnier *et al.*, 1978) predict that RRM1 and RRM3 of Nop4p have prototypical $\beta\alpha\beta\beta\alpha\beta$ structures, in which the RNP1 octamer and RNP2 hexamer are contained in the β 3 and β 1 strands.

RRM2 of Nop4p is somewhat imperfect. The highly conserved Lys/Arg1 and Phe/Tyr8 of RNP1 are Cys and Met, respectively, and the less conserved Phe/Tyr2, Gly/Lys4 and Leu6 of RNP2 are Ile, Arg and Met, respectively, in RRM2. Although the overall sequence similarity of the remaining 69 amino acids to the RRM family is reasonably high (~50%), the predicted secondary structure of RRM2 does not show any strong match to that of canonical RRMs. In particular, RNP1 is predicted to be within an α -helix. Thus, RRM2 of Nop4p may have diverged from a functional RRM.

RRM4 of Nop4p does not completely match the sequence of canonical RRMs. It contains an RNP1 sequence that matches the consensus perfectly, but there are only 20 residues amino-terminal and 14 residues carboxy-terminal to this RNP1 that are similar to those in other RRMs. There is no RNP2 sequence in RRM4.

The predicted RNA recognition motifs in Nop4p, as well as the requirement of Nop4p for pre-rRNA processing, suggest that Nop4p may be associated with snoRNAs, or may bind to rRNA precursors, processing intermediates, or products. Because Nop4p contains multiple RRMs, it might contact more than one RNA molecule simultaneously. Preliminary co-immunoprecipitation experiments with antibody 28.3a against Nop4p have failed to detect U3, U14 and snR10 snoRNAs in the precipitation complexes. When extracts were prepared from cells irradiated with UV light to crosslink RNA and protein, snR190 was co-immunoprecipitated by 28.3a antibody (C.Sun and J.Woolford, unpublished data). Thus, Nop4p might be a loosely associated component of a putative snoRNP containing snR190, or may be more indirectly

Fig. 9. Yeast small nucleolar RNAs and nucleolar proteins that function in different steps of pre-rRNA processing.

associated with snR190 in larger macromolecular structures in the nucleolus.

Some yeast nucleolar proteins are required for multiple steps in pre-rRNA processing and for biogenesis of both the 40S and 60S ribosomal subunits, whereas other nucleolar proteins are necessary for steps in pre-rRNA processing leading to the assembly of either 40S or 60S ribosomal subunits, but not both (Figure 9). Depletion of Nop1p or Nop3p results in delayed processing of the 35S primary rRNA transcript, diminished production of most rRNA processing intermediates and mature 18S rRNA, and eventually a deficit of mature 25S rRNA (Tollervey *et al.*, 1991; Russell and Tollervey, 1992). Depletion or inactivation of Gar1p, Sof1p or Nsr1p results in increased accumulation of the 35S pre-rRNA, and diminished amounts of the 32S, 27SA and 20S processing intermediates and mature 18S rRNA. However, production of both the 27SB precursor to 25S rRNA and the mature 25S rRNA is not significantly decreased (Girard *et al.*, 1992; Lee *et al.*, 1992; Jansen *et al.*, 1993). Depletion or inactivation of Spb4p, Drs1p or Nop4p impairs production of 25S rRNA, but not 18S rRNA (Sachs and Davis, 1990; Ripmaster *et al.*, 1992). Unlike the case for cells depleted of Nop1p, Gar1p, Sof1p, Nop3p or Nsr1p, there is no increase in the amount of 35S pre-rRNA or decrease in 27SA or 20S rRNA processing intermediates or mature 18S rRNA upon depletion of Nop4p. Rather, depletion of Nop4p results in a decrease in the amount of the 27SB rRNA processing intermediate and a significant decrease in the amount of mature 25S rRNA. This suggests that Nop4p functions in rRNA processing or ribosome assembly steps after formation of the 27SA processing intermediate, and is distinct from those requiring Nop1p, Nop3p, Gar1p, Nsr1p and Sof1p. The defect in pre-

Table I. Yeast strains

Strain	Genotype	Source
JM749	<i>MATa/MATα ura3-52/ura3-52 trp1-Δ101/trp1-Δ101 lys2-801/lys2-801 leu2-Δ1/leu2-Δ1 his3-Δ200/his3-Δ200</i>	J.Maddock
SC252	<i>MATa ura3-52 ade1 leu2-3,112 GAL2</i>	J.Hopper
JWY4905	<i>MATa ura3-52 ade1 leu2-3,112 GAL2 pYEp352NOP4</i>	This study
JWY4914	<i>MATa/MATα ura3-52/ura3-52 trp1-Δ101/trp1-Δ101 lys2-801/lys2-801 leu2-Δ1/leu2-Δ1 his3-Δ200/his3-Δ200 NOP4/nop4::TRP1</i>	This study
JWY4916	<i>MATa/MATα ura3-52/ura3-52 trp1-Δ101/trp1-Δ101 lys2-801/lys2-801 leu2-Δ1/leu2-Δ1 his3-Δ200/his3-Δ200 NOP4/nop4::TRP1 pRS316NOP4</i>	This study
JWY4917	<i>MATa ura3-52 trp1-Δ101 lys2-801 leu2-Δ1 his3-Δ200 nop4::TRP1 pRS316NOP4</i>	This study
JWY4919	<i>MATa ura3-52 trp1-Δ101 lys2-801 leu2-Δ1 his3-Δ200 nop4::TRP1 pUN90GAL1-NOP4</i>	This study

rRNA processing is consistent with the deficiency of 60S ribosomal subunits observed upon depletion of Nop4p. A more detailed examination will be required to identify the precise step or steps in pre-rRNA processing for which Nop4p is necessary.

Materials and methods

Strains, plasmids and media

Strains used in this study are described in Table I. Yeast cells were grown at 30°C in YEPglu (1% yeast extract, 2% peptone, 2% glucose), YEPgal (1% yeast extract, 2% peptone, 2% galactose) or synthetic medium (Sherman *et al.*, 1986) containing either glucose or galactose as carbon source. Methionine or uracil were omitted from the synthetic medium used in pulse-chase experiments to increase the efficiency of radioactive labelling. Yeast cells were transformed with DNA using the lithium acetate method (Ito *et al.*, 1983). Yeast strains SC252 and JWY4905 were used for indirect immunofluorescence microscopy. JWY4905 was derived from SC252 by transformation with the high copy plasmid YEp13NOP4.

A 6.6 kb *Pst*I DNA fragment containing *NOP4* was isolated from plasmid YEp13NOP4 and subcloned into plasmid vector YEp352 (Hill *et al.*, 1986) to generate YEp352NOP4. Plasmid pRS316NOP4 was constructed by subcloning a 6.4 kb *Bam*HI-*Sac*I DNA fragment isolated from YEp352NOP4 into plasmid pRS316 (Sikorski and Hieter, 1989).

A *nop4::TRP1* null allele lacking the 5' half of *NOP4* was constructed as shown in Figure 2B. YEp352NOP4 was digested with *Spe*I and *Cl*aI. The overhanging 5' ends were filled in with Klenow enzyme and a 1.3 kb *Pvu*II fragment containing the yeast *TRP1* gene (Jones and Prakash, 1990) was ligated between the filled in *Spe*I and *Cl*aI sites. The resulting construct was digested with *Bgl*II. The 10.5 kb *Bgl*II fragment containing this *nop4::TRP1* allele was gel purified and used to replace one copy of *NOP4* in diploid strain JM749 by transformation and homologous recombination (Rothstein, 1983), to create diploid strain JWY4914.

In order to construct JWY4917 containing the genomic *nop4::TRP1* null allele and wild type *NOP4* on a low copy plasmid pRS316, JWY4914 was transformed with plasmid pRS316NOP4 containing *URA3*. The resulting strain JWY4916 was sporulated and tetrads dissected. A *Trp*⁺ *Ura*⁺ haploid spore clone (JWY4917) was selected.

The *GAL1-NOP4* promoter fusion was constructed as follows. A 2.3 kb *Ssp*I-*Sca*I DNA fragment that contains the full length *NOP4* gene (Figure 2) was subcloned into the *Sma*I site of plasmid L1-118, constructed in our laboratory by subcloning the *Eco*RI-*Sa*I fragment of plasmid pBM258T (Johnston and Davis, 1984) containing the *GAL*I

promoter into plasmid pUN90 (Elledge and Davis, 1988). This plasmid containing the *GAL1-NOP4* promoter fusion and the yeast *HIS3* gene was transformed into haploid strain JWY4917. Transformants were plated on medium containing 5-FOA and galactose to select for loss of plasmid pRS316NOP4. The resulting strain JWY4919 contains only one functional *NOP4* gene, the *GAL1-NOP4* conditional allele.

Screening a λ gt11 yeast genomic expression library and a YEp13 yeast genomic library

A λ gt11 yeast genomic DNA expression library (a gift from Dr Robert Hamatake, NIEHS) was immunoscreened with the yeast nucleolar specific monoclonal antibody 28.3a (a gift from Drs Connie Copeland and Michael Snyder, Yale University) using the protocol of Young and Davis (1991). Phage were plated at $\sim 5 \times 10^4$ plaques per 150 mm plate. A 10-fold dilution of a 28.3a cell line culture supernatant was used as the primary antibody probe. This primary antibody was detected by alkaline phosphatase-conjugated secondary antibody (Promega Corp., Madison, WI), following the manufacturer's instructions.

Genomic DNA containing a portion of *NOP4* cloned in λ gt11 was amplified by PCR under standard conditions (Sherman *et al.*, 1989), using a pair of oligonucleotides (CS1: 5'-GGTGGCGACGACTCCTGG-AGCCCG-3' and CS2: 5'-TTGACACCAGACCACTGGTAATG-3'), complementary to the sequences flanking the *EcoRI* site in λ gt11. The PCR product was digested with *EcoRI* to remove phage DNA, labelled with 32 P by random primer reactions (Feinberg and Vogelstein, 1984) and used as a colony hybridization probe to screen a library of yeast genomic DNA cloned in plasmid YEp13, as described in Gergen *et al.* (1979).

DNA and RNA methods

Extraction of yeast DNA and RNA, Southern and Northern hybridization, and construction and analysis of recombinant DNA molecules were performed as described in Sambrook *et al.* (1989). DNA sequence was determined by the dideoxy nucleotide termination method (Sanger *et al.*, 1977), using single-stranded DNA templates cloned in the Bluescript plasmid (Stratagene, La Jolla, CA). Sequenase version 2.1 was used for sequencing reactions, following the manufacturer's instructions (USB Corp., Cleveland, OH). Sequence analysis, database searches and protein structure predictions were performed using the Sequence Analysis Software Package computer programs developed by the Genetics Computer Group at the University of Wisconsin Biotechnology Center (Devereux *et al.*, 1984). Computer software was accessed through the courtesy of the Pittsburgh Supercomputing Group.

Chromosome mapping of *NOP4*

A blot of electrophoretically separated yeast chromosomes and an ordered library of yeast genomic fragments cloned in bacteriophage λ and cosmids (a gift from Drs L.Riles and M.Olson, Washington University and University of Washington) were hybridized with the 32 P-labelled 2.2 kb *EcoRI* DNA fragment containing *NOP4*.

Growth curve and viability tests

Strain JWY4919 growing exponentially in 50 ml YEPGal medium was harvested by centrifugation at 4000 g for 5 min, washed once with an equal volume of YEPGlu medium to remove galactose, and inoculated into 50 ml of YEPGlu medium. Growth and viability of this strain in YEPGal medium and after shifting to YEPGlu medium were assayed as previously described (Moritz *et al.*, 1991). In order to maintain cultures in exponential growth, cells were diluted into fresh medium whenever the OD₆₁₀ reached 0.7 units.

Western immunoblot analysis

Western immunoblotting was performed as previously described (Moritz *et al.*, 1991), except that yeast cell lysates were prepared by boiling cells (10^9 cells per ml) directly in Laemmli sample buffer (65 mM Tris, pH 6.8, 10% glycerol, 1% SDS, 1% β -mercaptoethanol) for 5 min, and vortexing hard six times for 30 s with an equal volume of glass beads to minimize degradation of Nop4p. The cell lysate was subjected to electrophoresis on 10% SDS-polyacrylamide gels, and electrophoretically transferred to BA-S 85 nitrocellulose membranes (Schleicher & Schuell, Keene, NH). The blots were first incubated with 28.3a antibody for 2 h at room temperature, and then washed three times with TBST solution (10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween 20) to remove free primary antibody. The Nop4p antigen-antibody complexes were detected by alkaline phosphatase-conjugated secondary antibody as described above.

Fractionation and characterization of ribosomes

Ribosomal subunits, ribosomes and polyribosomes were assayed by sucrose gradient centrifugation of cell extracts, as described previously (Deshmukh *et al.*, 1993), except that yeast cells were grown in 100 ml medium and 40 A₂₆₀ units of cell lysate were layered on each 35 ml linear 7–47% sucrose gradient.

Pulse-chase analysis of pre-rRNA processing

The synthesis, processing, and turnover of pre-rRNA was assayed by pulse-chase analysis using L-([3 H]methyl)methionine, as described in Deshmukh *et al.* (1993). Similar procedures were followed for pulse-chase experiments using [3 H]uracil, except that JWY4919 containing the pRS316 vector bearing *URA3* was used, to enable growth in synthetic complete medium lacking uracil. Cells were pulse-labelled with 50 μ Ci [3 H]uracil (51 Ci/mmol) per ml for 2 min and chased for up to 30 min with 300 μ g/ml cold uracil. Yeast RNA was subjected to electrophoresis on 24 cm long gels for 6.5 h at 200 V.

Indirect immunofluorescence microscopy

Indirect immunofluorescence microscopy was performed on cells grown in YEPGlu, as described by Copeland and Snyder (1993). Purified 28.3a monoclonal antibody was used at a concentration of 20 μ g/ml. The secondary antibody used was rabbit anti-mouse immunoglobulin G conjugated with rhodamine (Cappel, Organon Teknika Corp., Durham, NC). Slides were viewed using a Zeiss Axiophot microscope equipped with a 100 $\times$ objective lens and Nomarski DIC optics. Photographs were taken with a CCD camera (Photometric Inc., Tucson, AZ) under constant illumination conditions. Photographs were made by transforming the digital image in the computer to Kodak Tmax-100 film using a Kodak slidewriter.

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