The Yeast Nucleolar Protein Nop4p Contains Four RNA Recognition Motifs Necessary for Ribosome Biogenesis*

Chao Sun‡ and John L. Woolford, Jr.§
From the Department of Biological Sciences, Carnegie Mellon University, Pittsburgh, Pennsylvania 15213

The Saccharomyces cerevisiae nucleolar protein Nop4p is necessary for processing of rRNA and assembly of 60 S ribosomal subunits. Nop4p is unusual in that it contains four RNA recognition motifs (RRMs) including one noncanonical RRM, as well as several auxiliary motifs, two acidic regions between the RRMs, and a carboxyl-terminal domain rich in lysines and arginines. To examine the functional importance of these motifs, we isolated random and site-directed mutations in NOP4 and assayed Nop4p function in vivo. Our results indicate that each RRM is essential for Nop4p function; mutations in conserved aromatic residues of Nop4p cause a temperature-sensitive lethal phenotype and diminished 60 S ribosomal subunit production. The carboxyl-terminal domain of Nop4p may serve as spacers or tethers to separate the RRMs.

Eukaryotic ribosomes are synthesized in the nucleolus, the nuclear subcompartment where rRNA is transcribed, modified, and processed during assembly with ribosomal proteins. The 18 S, 5.8 S, and 25–28 S rRNAs are produced in the nucleolus from a 35–40 S pre-rRNA by exo- and endonucleolytic processing at sites within the 5′ and 3′ external transcribed spacers and the two internal spacers of the pre-rRNA (1–3). 5 S rRNA is transcribed from separate genes.

A number of yeast and metazoan small nucleolar RNAs and associated proteins have been identified that are necessary for rRNA processing and ribosome assembly (3–6). Some of the snoRNAs, present in ribonucleoprotein particles (snoRNPs), are thought to function in concert in a multi-snoRNP complex called the processome (3, 7). This complex might function analogously to spliceosomes containing small nuclear RNPs assembled on pre-mRNAs, to direct proper folding of rRNA necessary for its processing or assembly with ribosomal proteins and nonribosomal nucleolar proteins.

In addition to snoRNP proteins, other nucleolar proteins necessary for ribosome biogenesis have been identified, especially using genetic schemes in yeast (1, 3, 6). These include the endonucleases MRP RNase and RNase III (Rnt1p), exonucleases Rat1p, Xrn1p, and Rrp4p, putative RNA-dependent AT-Pases/helicases Spb4p, Drs1p, Rrp3p, and Dbp3p, the rRNA methylase Dim1p, the putative shuttling protein Nsr1p, and the prolyl-peptidyl cis-trans-isomerase Npi46p/Fpr3p.

Nop4p/Nop77p is a yeast nucleolar protein that was identified both by screening an expression library with a monoclonal antibody specific for a yeast nucleolar antigen and independently in a screen for mutants synthetically lethal in combination with a nop1 mutation (8, 9). Depletion of Nop4p impairs production of 25 S rRNA and causes a deficit of 60 S ribosomal subunits. Nop4p contains five motifs that potentially bind RNA: four RNA recognition motifs (RRMs) (10–12) plus a carboxyl terminus rich in lysine and arginine residues. In addition, there are two regions in Nop4p rich in acidic residues, one between RRM1 and RRM2 and a second between RRM2 and RRM3 (Fig. 1A). Acidic domains are frequently found in nucleolar proteins and have been suggested to interact with other proteins, for example with basic regions of ribosomal proteins (13).

Understanding the role of Nop4p in ribosome biogenesis will be facilitated by analysis of the structural features of Nop4p that are necessary for its functions. To initiate a structural and functional analysis of Nop4p, we constructed site-directed mutations in each of the above-mentioned motifs of Nop4p and isolated random mutations in the NOP4 gene. We assayed the effect of these mutations on Nop4p function in vivo. We found that each RRM is essential and that the carboxyl-terminal charged domain is important for function of Nop4p. However, most of the glutamic or aspartic residues in the acidic motifs can be deleted or replaced with alanine residues without affecting function or nucleolar localization of Nop4p.

EXPERIMENTAL PROCEDURES

Strains and Media—Yeast strains used in this study are described in Table I. Yeast were grown in YEPD (1% yeast extract, 2% peptone, 2% glucose) or synthetic medium (14) containing glucose as a carbon source. Yeast were transformed with DNA using the lithium acetate method (15).

PCR Mutagenesis—Random mutations in the NOP4 gene were generated by PCR coupled with in vivo gap repair (16). PCR amplification was done under conditions predicted to cause misincorporation of nucleotides (17, 18). The wild type NOP4 gene used for generating gaps and as a substrate of PCR in this experiment is a 3.2-kilobase pair PstI/Smal fragment cloned in a modified pRS315 plasmid (JW3434) that has SpeI and XbaI sites in the polylinker destroyed by SpeI/XbaI double digestion and religation. The yeast strain used for transformation and the subsequent mutant screen is JYW4901, which has the chromosomal copy of NOP4 disrupted with the TRP1 sequence, and bears a wild type NOP4 on plasmid PUN65. Four different gaps on the NOP4 gene were generated individually by digestion with different pairs of enzymes, each of which recognizes a single site in the gene or

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The Journal of Biological Chemistry 272:25345–25352, 1997

Printed in U.S.A.
plasmid backbone. Gap 1 was created by Spel/Ncol double digestion; it spans nucleotides 250–754, corresponding to the amino-terminal region of Nop4p including the RRM1 and the first 7 amino acid residues of RRM2. Gap 2 was created by Ncol/Bln1 digestion, which removes sequences between nucleotides 754 and 1494, encoding RRM2 and RRM3. Gap 3 was created by Bln1/AarII digestion, which deletes sequences between nucleotides 1494 and 2103, corresponding to RRM4 and the linker region between RRM3 and RRM4. Gap 4 was generated by AarII/BamHI digestion, which removes sequences between nucleotides 2103 and 2469 that encode the 81 amino acid long carboxy-terminal region of Nop4p. To use the Ncol site at nucleotide 754, the Ncol site at nucleotide 114 was destroyed in plasmid JW3434 by partial digestion and end-filling with Klenow enzyme, resulting in plasmid JW3435. The sequences of the oligonucleotides used in the PCR mutagenesis experiment are presented in Table II.

The PCR products were purified by phenol extraction and transformed together with gel-purified gapped NOP4 plasmids into yeast cells. Conditional lethal nop4 mutants were identified by assaying the inability of cells to lose the NOP4 SUP11 URA3 plasmid, indicated by either a color sectoring phenotype or by sensitivity to 5-fluoroorotic acid at nonpermissive temperatures, and by direct assays of Ts phenotype on YEFD medium after loss of the URA3-bearing plasmid on 5-fluoroorotic acid plates. Yeast transformants were replica-plated to media that contained 0.01% 5-fluoroorotic acid and 0.1% proline as the only nitrogen source (19). After incubation for 3–4 days, plates were then replicated on YEFD medium and incubated at 15, 23, 30, and 37 °C. Colonies that could not grow at high or low temperatures were identified and patched on YEFD plates and confirmed by going through another round of plate replication and temperature sensitivity tests. The mutants were then subjected to a plasmid shuffling test to determine whether the mutant phenotype was due to mutations in the plasmid-borne NOP4 gene. By this means 30 temperature-sensitive mutants were obtained.

Site-directed Mutagenesis—Plasmids to be mutagenized were transformed into Escherichia coli strain CJ236, and single-stranded DNA was prepared from the transformants. Site-directed mutagenesis reactions were carried out as described by Kunkel et al. (20). The mutation was used to construct a mutant strain for use in the study. The mutation was confirmed by sequencing. The mutagenic oligonucleotides used for mutagenesis are listed in Table II.

Indirect Immunofluorescence Microscopy—Indirect immunofluorescence microscopy was performed on cells grown in YEFD, as described by Copeland and Snyder (21). A modified scheme was established to use two mouse monoclonal antibodies directed against different yeast nucleolar proteins in the same experiment. Yeast cells were fixed at room temperature for 1 h with an equal volume of 7.4% formaldehyde pre-
pared freshly from paraformaldehyde, and cell walls were removed by digestion with Zymolase 100-T (Seikagaku Corp.) at a concentration of 10 μg/ml and glucuronidase (Sigma) at a concentration of 40 μl/ml for 1 h at 30 °C. Approximately 1.5 × 10^6 cells were added to each well of a polylysine-coated glass slide; unattached cells were washed away by repeatedly adding buffer to the wells and aspirating with vacuum. Approximately 10 μl of the first primary antibody (anti-Nop1p) was applied to each well and incubated at 4 °C overnight. Cy3-conjugated goat anti-mouse IgG (Jackson ImmunoResearch) was used at 50 μl/ml and incubated for 1 h at room temperature. After washing away the unbound Cy3 antibodies, the Cy5-conjugated goat anti-mouse IgG (Jackson ImmunoResearch) was added at 20 μl/ml and incubated for 2 h at room temperature. The free Cy3 Fabs were then washed away by adding sorbitol buffer and 0.1% bovine serum albumin protein to the wells and aspirating with vacuum. Approximately 10 μl of the partially purified 28.3a (anti-Nop4p) at a concentration of 7 mg/ml was added to each well and incubated for 2 h at room temperature. After washing away the unbound 28.3a antibodies, the Cy5-conjugated goat anti-mouse IgG (Jackson ImmunoResearch) was applied to slides. Incubation proceeded at room temperature for 1 h before an extensive wash to remove the free Cy5 secondary antibody. A control experiment without application of the 28.3a antibody was used to ascertain whether the first primary antibody was saturated by the Cy5-conjugated Fab.

RESULTS

Each One of the Four RRMs in the Nop4 Protein Is Required for Function—To determine whether each of the four RRMs in Nop4 protein (Fig. 1A) is important for its function, we assayed the effects of mutations in NOP4 predicted to compromise RNA binding of each RRM. X-ray diffraction and NMR spectroscopic studies of several RRM-containing proteins indicated that the RRM consists of four anti-parallel β-strands forming a β-sheet and two α-helices. Solvent exposed aromatic side chains in the most conserved RNP-1 and RNP-2 domains of the β-sheet form ring stacking interactions with RNA bases (12, 22–30). Mutation of these aromatic residues disrupts RNA binding or otherwise inactivates RRM-containing proteins (22, 31–42). We constructed site-directed mutations in the most conserved Phe residues of RNP-1 in RRM1, RRM2, and RRM3 of Nop4 as shown in Fig. 1B. nop4-11 contains Phe-68 → Leu and Phe-70 → Leu mutations in RNP-1 of RRM1, nop4-18 contains a Phe-192 → Leu mutation in RNP-1 of RRM2, and nop4-19 contains a Phe-335 → Leu mutation in RNP-1 of RRM3. nop4-3, which was recovered in a screen for conditional lethal nop4 alleles (see below), contains a Phe-557 → Leu mutation in RNP-1 of RRM4.

The phenotype resulting from each of these mutations was assayed in strains containing a nop4::TRP1 genomic disruption and a low copy CEN plasmid bearing the mutant nop4 gene. Each of these nop4 mutants is temperature-sensitive for growth, i.e. grows well at 30 °C but is unable to grow at 37 °C. To ascertain whether these four Ts nop4 mutants were defective in ribosome biogenesis, lysates were prepared from each strain grown at 30 °C or after shifting to 37 °C for 4 h and subjected to sucrose gradient centrifugation to display 40 S and 60 S ribosomal subunits, monoribosomes, and polyribosomes. As shown in Fig. 2, each of the nop4 mutants is deficient in 60 S ribosomal subunits at 37 °C, as indicated by a decreased ratio of free 60 S ribosomal subunits to 40 S subunits compared with wild type NOP4 yeast, and accumulation of half-mer polyribosomes (9, 43, 44). Most of these nop4 mutants exhibit similar shortages of 60 S subunits 2 or 3 h after shifting to 37 °C, compared with 4 h post-shift, and some (nop4-3 and nop4-19) are moderately deficient in 60 S subunits even when grown at 30 °C (Fig. 2 and data not shown).

The ribosome deficiency phenotype of nop4-3 containing a Phe-557 → Leu mutation in RNP-1 of RRM4 indicates that RRM4 is important for Nop4p function. However, RRM4 may be an atypical RRM; the sequence of the amino-terminal 30 residues of RRM4 does not match the RRM consensus (10–12). In particular there is not a canonical RNP-2 sequence, and none of the conserved hydrophobic residues in predicted helix 1 are present in RRM4 (Fig. 1B). To further test the importance of RRM4, we mutated Tyr-514 to Leu (nop4-16). The position of this Tyr residue within RNP-2 of RRM4 is similar to that of the highly conserved Tyr implicated in RNA binding of canonical RRMs (12, 28, 29). The nop4-16 mutant containing the Tyr-514 → Leu mutation has a pronounced shortage of 60 S ribosomal subunits and accumulates half-mer polyribosomes at 37 °C (Fig. 4) but is not Ts− for growth (Fig. 3). In summary, in vivo phenotypes caused by these mutations in RRM1–RRM4 of Nop4p suggest that each of the four RRMs is important for Nop4p function.

We generated a set of temperature-sensitive nop4 mutants to further investigate functional domains of Nop4p. Thirty Ts− nop4 mutants were generated by PCR amplification and gap
repair of NOP4. Six of these mutants, nop4-1–nop4-6, were chosen for further study. Analysis of these nop4 mutants as well as other site-directed nop4 mutants further indicates the necessity of the RRMs for Nop4p function. nop4-1 contains two mutations, Arg-170 → Gly in RRM2 and Phe-310 → Leu in a conserved Phe residue in RRM3 (Fig. 1B). The nop4-1 mutant is Ts− for growth and for accumulation of 60 S ribosomal subunits (Figs. 3 and 4). To determine the significance of each individual amino acid change in nop4-1, mutants containing each of the single mutations were constructed by site-directed mutagenesis. nop4-8 contains Phe-310 → Leu and nop4-9 contains Arg-170 → Gly. Like the nop4-1 mutant, nop4-8 and nop4-9 mutants are deficient in 60 S ribosomal subunits. The nop4-8 mutant is Ts− for growth, and nop4-9 shows a very leaky Ts− phenotype. The nop4-8 mutant exhibits more extreme Ts− and 60 S ribosomal subunit deficiency phenotypes than nop4-9 but is still less pronounced than those of the nop4-1 double mutant (Figs. 3 and 4), indicating that mutations in RRM2 and RRM3 may cause an additive phenotype in the double mutant. The nop4-2 mutant contains an Arg-152 → Gly mutation in RNP-2 of RRM2 and a Glu-214 → Asp mutation in the carboxyl terminus of RRM2 (Fig. 1). nop4-10, which contains the single Arg-152 → Gly mutation, was constructed by site-directed mutagenesis. nop4-10 and nop4-2 have similar phenotypes, both are tight Ts− mutants, exhibit moderate deficiencies of 60 S ribosomal subunits at 30 °C, and have more pronounced shortages of 60 S ribosomal subunits at 37 °C (Figs. 3 and 4).

Three of the Ts− nop4 mutants examined, nop4-4, nop4-5, and nop4-6, contain single missense mutations in the interval between RRM3 and RRM4, indicating that the linker region between RRM3 and RRM4 is also important for Nop4p function. Each of these mutants is Ts− for growth and for 60 S ribosomal subunit biogenesis (data not shown).

To examine whether the observed nop4 mutant phenotypes described above simply result from decreased amounts of Nop4p, cell lysates were prepared from wild type and mutant strains at 30 °C and 3 h after shifting to 37 °C. Nop4p levels were examined by Western immunoblot analysis. The levels of mutant Nop4p were not significantly decreased compared with wild type protein, except nop4-10 and nop4-11 proteins (Fig. 5). This suggests that, in most cases, Nop4p degradation is not the primary cause of the ribosome biogenesis defects observed in these mutants.

The mutant phenotype of the alleles might result from mislocalization of the protein either as a result of inactivation of a nuclear localization sequence or as an indirect result of loss of binding to a nucleolar ligand. A nuclear localization sequence co-localizes with an RRM sequence in the U1 70K protein; mutations in an RRM prevent nuclear export of Npl3p and cause mislocalization of an Nsr1-β-gal fusion protein (42, 45, 46). To test these possibilities, the intracellular localization of the mutant Nop4 proteins encoded by nop4-1 and nop4-3 was examined by indirect immunofluorescence microscopy. Localization of the nucleolar protein Nop1p was included as a control for nucleolar structure and for accessibility of individual cells to antibodies. Mutants were examined at 30 °C and 3 h after shifting to 37 °C when ribosome deficiency phenotypes are more severe. Each mutant protein is localized to the nucleolus at both temperatures (Fig. 6B and data not shown), identical to wild type Nop4p (Fig. 6A). Nucleolar structure is not detectably perturbed in these nop4 mutants, as both anti-Nop1p and anti-Nop4p antibodies stained an identical crescent-shaped pattern, as seen in wild type cells. Thus, mislocalization of Nop4p is not apparent as a result of mutations in the RRMs.

Each of the Acidic Motifs in Nop4 Protein Is Not Essential—The importance of the two acidic domains of Nop4p between...
RRM1 and RRM2 and between RRM2 and RRM3 was tested by assaying the effects of deleting portions of either or both domains or replacing residues within each with alanine. Deletion of residues 131–140, eight of which are Asp or Glu (nop4-14 mutation), or deletion of amino acids 250–267, 14/18 of which are Asp or Glu (nop4-13), does not cause any discernible effect on growth or ribosome biogenesis (data not shown). However, deletion of both acidic motifs (nop4-15) is lethal. The acidic domains may play critical but redundant roles; alternatively, lethality of the double deletion may be caused by disruption of Nop4p structure. To test these possibilities and to discern the minimal length of acidic peptide sufficient for essential Nop4p function, additional deletion and alanine replacement mutations were constructed in which most of the acidic residues in one of the two acidic domains were deleted, and many of the acidic residues in the other motif were changed to alanine. The nop4-20 mutation contains the D250–267 deletion and residues 131–135 (EDEDD) are changed to alanine. nop4-21 contains the D131–140 deletion, and residues 250–256 (EDAEENH) are changed to alanine. nop4-22 contains the D250–267 deletion, and residues 131–140 (EDEDDADGED) are changed to alanine. All three mutants, nop4-20, nop4-21, and nop4-22, grow as well as wild type NOP4 yeast at 13, 23, 30, and 37 °C and have wild type ribosome and polyribosome gradient profiles at 30 and 37 °C (data not shown). Indirect immunofluorescence microscopy showed that Nop4p is properly localized to the nucleolus in the nop4-22 mutant (data not shown). Binding of synthetic nop4-14 mutant protein to homoribopolymers is nearly identical to wild type Nop4p, and binding of synthetic nop4-15 mutant protein, lacking both acidic intervals, is only slightly decreased (data not shown). Results of these experiments assaying acidic motif mutations are summarized in Fig. 7. We conclude that the entire stretch of acidic motifs in Nop4p is not critically important for function of Nop4p.

The Carboxyl-terminal Region Is Important for the Function and Proper Localization of Nop4p—The carboxyl-terminal region of Nop4p contains many charged amino acid residues, including four short sequences that resemble nuclear localization signal sequences (47). To test the functions of this domain, a nonsense codon was engineered at the codon for residue Lys-618 of NOP4 to generate a truncation of the carboxyl-terminal 68 amino acids. This nop4-12 mutant grows very slowly and has a severe 60 S ribosomal subunit deficit at all temperatures tested (data not shown). Indirect immunofluorescence microscopy showed that Nop4p is properly localized to the nucleolus in the nop4-22 mutant (data not shown). Binding of synthetic nop4-14 mutant protein to homoribopolymers is nearly identical to wild type Nop4p, and binding of synthetic nop4-15 mutant protein, lacking both acidic intervals, is only slightly decreased (data not shown). Results of these experiments assaying acidic motif mutations are summarized in Fig. 7. We conclude that the entire stretch of acidic motifs in Nop4p is not critically important for function of Nop4p.
mutants, indicating that the integrity of the nucleolus is not obviously compromised (Fig. 8A). Results of in vitro binding experiments with ribohomopolymers demonstrated that the carboxyl-terminal deleted Nop4p retains only 30% of wild type levels of RNA binding activity at both 30 and 37 °C (data not shown). This low RNA binding activity and the temperature insensitivity of binding are consistent with the poor growth and deficit of ribosome synthesis of nop4-12 at all temperatures. It is also very striking that the nop4-12 mutant protein accumulates at much higher levels than that of wild type Nop4p, as indicated by the high intensity of the protein band on Western

**DISCUSSION**

We have examined the importance of seven different domains of the modular multi-domain protein Nop4p. This yeast nucleolar protein is necessary for biogenesis of ribosomes, in
particular late steps in processing of rRNA or assembly of 60 S ribosomal subunits. Mutations in each of the four RNA recognition motifs of Nop4p, as well as deletion of the charged carboxyl-terminal 68 amino acids, cause a deficit of 60 S ribosomal subunits in vivo, indicating that each of these domains is important for Nop4p function in ribosome biogenesis. In contrast, deletion or replacement of acidic motifs between RRM1 and RRM2 or RRM2 and RRM3 has no discernible effect on Nop4p function.

To test whether each RRM in Nop4p is important for binding RNA, we assayed binding of wild type and mutant Nop4p proteins to ribohomopolymers in vitro. Proteins were synthesized in vitro by coupled transcription/translation. Mutant proteins containing Phe→Leu mutations in RNP-1 motifs, as well as nop4-1, nop4-8, and nop4-10 mutant proteins, bound poly(G) or poly(U) less well than wild type protein at 30 °C. More definitive evidence for the role of each RRM in Nop4p function requires identification of the natural RNA ligand(s) of Nop4p and assays of individual RRMs or combinations of RRMs.

The presence of four RRMs in one protein is unusual; only a few among the 200-plus known RRM proteins contain four RRMs: poly(A) binding protein, nucleolin, hnRNP L, and hnRNP I pyrimidine tract-binding protein (11, 48). It is likely that each of the four RRMs of Nop4p functions to bind RNA. Mutation of the conserved Phe in RNP-1 of each RRM inactivates Nop4p in vivo. These Phe residues have been implicated in binding RNA in several well-studied RRMs (12, 28, 29). Each of the Phe→Leu mutations in the conserved Phe of each RRM causes quantitatively different effects on growth and ribosome biogenesis, perhaps reflecting differences in structure and function of the four RRMs. Each RRM may bind independently to similar or distinct RNA sequences, either in one RNA or in more than one RNA molecule. For example, one of the RRMs of U1A protein binds to U1 small nuclear RNA and independently to similar sequences in the 3′-untranslated region of U1A mRNA, whereas the second RRM of U1A binds to a different RNA (31, 49). Binding of Nop4p to more than one ligand might explain why Nop4p containing a mutation in any one of the RRMs is retained in the nucleolus (Fig. 6 and data not shown). Even if binding to one RNA ligand were significantly decreased by these mutations, the mutant proteins may remain bound to other nucleolar ligands via the other RRMs. It is also possible that two or more of the RRMs of Nop4p may function cooperatively to bind a specific ligand, as observed for PABP, ASF/SF2, and hnRNP A1 (50–52).

As described previously (9), the sequences of RRM2 and RRM4 of Nop4p diverge significantly from the consensus RRM. In particular, RNP-2 in both RRMs is atypical, and RRM4 lacks several of the highly conserved hydrophobic residues between RNP-2 and RNP-1 thought to form a hydrophobic core of the RRM (22). Noncanonical RRMs in ASF/SF2 and U2AF65 bind RNA (37, 39, 53). Mutations in RNP-1 of atypical RRMs of Saccharomyces cerevisiae Npl3p and Schizosaccharomyces pombe Mei2 inactivate these proteins (42, 54). Our mutational analysis suggests that both of the atypical RRMs in Nop4p are functional and are likely to bind RNA. As mentioned above, Phe→Leu mutations in RNP-1 of RRM2 and RRM4 inactivate Nop4p in vivo and decrease binding of Nop4p to RNA in vitro. Mutating Tyr-514 in RNP-2 of RRM4 to Leu also inactivates Nop4p in vivo. This Tyr is positioned analogously to that in the prototypical RRMs and is thought to interact with RNA. Interestingly, the Arg-152 → Gly mutation in RNP-2 of RRM2, which creates a closer match to a consensus RNP-2, was isolated in a screen for Ts− nop4 mutants. More detailed structure/function analyses of the noncanonical RRMs of Nop4p may reveal other modes of RNA binding.

The first three RRMs of Nop4p are more closely spaced than are RRM3 and RRM4. RRM1 and RRM2 are 48 residues apart, RRM2 and RRM3 are 68 residues apart, and RRM3 and RRM4 are separated by 145 residues. This irregular spacing is reminiscent of that in a subset of the multi-RRM proteins. Shamo et al. (55) argued that linker regions between RRMs may be critical determinants for RNA binding, by modulating binding to single versus multiple RNA ligands (cis versus trans binding). They suggest a breakpoint between cis and trans binding may be approximately 70 residues. Three of the six Ts− nop4 mutants we characterize are in the long linker region between RRMs 3 and 4, suggesting an important role for this linker. Further experiments are necessary to test the importance of the long linker of Nop4p and to test whether RRM1–RRM3 and RRM4 constitute two separate functional domains.

The sequence of the carboxyl-terminal domain of Nop4p, downstream from RRM4, and the phenotypes resulting from truncation of the 68 carboxyl-terminal amino acids suggest that this domain may be required for binding RNA or for nuclear targeting or for both. Several peptide sequences in this region resemble arginine-rich peptides in human immunodeficiency virus Tat, human immunodeficiency virus Rev, or the phage P22, λ, or d21 N proteins that bind to specific RNA ligands (12). Among the 68 carboxyl-terminal amino acids, 9 are arginines and 16 are lysines. The RRM-containing proteins hnRNP A1, ASF/SF2, and eukaryotic initiation factor-4B contain similar positively charged peptides within domains that bind to RNA, independently of the RRMs (56, 57). As previously noted, the carboxyl-terminal domain of Nop4p contains five potential nuclear localization sequences (9). The mislocalization of the carboxyl-terminal truncated Nop4p to the cytoplasm could be due to the absence of nuclear localization sequences, such that it is not efficiently imported into the nucleus. Alternatively, mislocalization could result from diminished binding activity toward nucleolar RNA or protein ligands. The truncated Nop4 protein accumulates to levels severalfold higher than wild type Nop4p (Figs. 5 and 8). This may simply reflect either a mechanism for cells to compensate for partial loss of plasmid-borne mutant Nop4p function or a pathway of feedback regulation of NOP4 expression.

It was surprising that deletion or replacement of the acidic motifs of Nop4p did not appear to affect Nop4p function. It has been hypothesized that stretches of acidic and serine residues in nucleolar proteins are platforms for interactions with basic ribosomal proteins. Binding of such nucleolar proteins to both rRNA and ribosomal proteins might facilitate ribosome assembly (58, 59). Our experimental results suggest that such a model of interaction does not apply to Nop4p, although we cannot totally rule out the possibility that the few acidic residues remaining in the nop4-22 mutant protein are sufficient for binding to basic motifs. An interesting alternative explanation for the lethality of the double deletion mutant nop4-15 and for the wild type phenotype of the single deletion or deletion/alanine replacement mutants nop4-13, nop4-14, nop4-20, nop4-21, and nop4-22 is that proper spacing between either pair of RRMs is sufficient for Nop4p function.

How might Nop4p function in ribosome biogenesis if it contains multiple RNA binding motifs? Nop4p might bind to snoRNA or to rRNA to facilitate proper intra- or intermolecular base pairing of these RNAs during rRNA processing or packaging of rRNA with ribosomal proteins (60). Nop4p might serve

2 C. Sun and J. Woolford, unpublished observations.
as a “matchmaker” by binding to multiple different RNA sequences in one or more RNA molecules to facilitate formation of particular RNA-RNA base pairs. The original matchmaker model posits that this occurs via protein-protein as well as RNA-protein interactions (61). Multiple RNA recognition motifs within Nop4p provide a means for it to function thus as a matchmaker without protein-protein interactions. Such has been proposed for eIF-4B binding simultaneously to rRNA and mRNA to facilitate translation initiation (62) and for U1A signal of SV40 mRNA to couple splicing and polyadenylation (63) and for U1A binding to both U1 small nuclear RNA and a polyadenylation signal of SV40 mRNA to couple splicing and polyadenylation (49). Nop4p might function as a “ribochaperone,” like yeast mitochondrial splicing factor blocks to formation of proper structure (64). Nop4p might function as a “riboeffector,” like yeast mitochondrial splicing factor, to prevent RNA misfolding by maintaining unstructured RNA conformations, thus overcoming kinetic blocks to formation of proper structure (63, 64). Nop4p might also be a “riboeffector,” like yeast mitochondrial splicing factor CBP2, that binds to and stabilizes a particular active RNA tertiary structure, thus overcoming the thermodynamic block to RNA folding by minimizing formation of alternative inactive conformers (65). Identification of the RNA ligands of Nop4p will facilitate tests of these models.

Acknowledgments—We are grateful to members of our laboratory and Drs. Maurice Swanson and Michael Cusick for valuable discussions and critical reading of the manuscript.

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