Assembly of 5S Ribosomal RNA Is Required at a Specific Step of the Pre-rRNA Processing Pathway

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Abstract. A collection of yeast strains surviving with mutant 5S RNA has been constructed. The mutant strains presented alterations of the nucleolar structure, with less granular component, and a delocalization of the 25S rRNA throughout the nucleoplasm. The 5S RNA mutations affected helix I and resulted in decreased amounts of stable 55 rRNA and of the ribosomal 60S subunits. The shortage of 60S subunits was due to a specific defect in the processing of the 27SB precur-

Key words: 5S ribosomal RNA • pre-rRNA • ribosomes • yeasts • cell nucleolus

Eukaryotic ribosome biogenesis requires the coordinate assembly of 70–80 ribosomal proteins with four ribosomal RNA s to yield the two mature ribosomal subunits. It takes place in the nucleolus where the large 35S rRNA precursor encoding the 18S, 5.8S, and 25S rRNAs (the sizes are from Saccharomyces cerevisiae) is synthesized by RNA polymerase I (for review see Woolford and Warner, 1991). The fourth rRNA, 5S RNA, is encoded by independent genes transcribed by RNA polymerase III. In most eukaryotic organisms the 5S RNA genes are located outside the nucleolus (Hadjiolov, 1985), with the exception of S. cerevisiae in which the 5S RNA genes are located in the nucleolus between the large ribosomal transcription units and transcribed in the opposite orientation (Warner, 1989). The synthesis of the 55 ribosomal RNA by a distinct form of RNA polymerase raises the question of its coordinated preparation with the other rRNA components. As yet, there is no indication that coregulation exists at the level of transcription, even in S. cerevisiae with its special form of ribosomal gene organization.

5S ribosomal RNA, before its incorporation into the ribosome, can bind to different proteins and be translocated in different cellular compartments. In eukaryotes, newly synthesized 55 rRNA interacts transiently with the protein La before its binding to the ribosomal protein rPL5 (Steitz et al., 1988; Guddat et al., 1990; Yoo and Wolin, 1994). In X enopus oocytes, because of the extraordinary demand for ribosome production, 5S RNA is produced in large quantities and is complexed, in addition to rPL5, with other proteins including the transcription factor TFIIIA (Picard and Wegnez, 1979; Picard et al., 1980). The 55/ RNP particles are exported to the cytoplasm where they are stored. During vitellogenesis, rPL5 replaces TFIIIA in association with 55 rRNA, which migrates back to the nucleus where it is directed to the nucleolus for incorporation into ribosomes (Rudt and Pieler, 1996). The transit of the 5S RNA through the cytoplasm is likely due to the necessity to store large amounts of 5S RNA during the early stages of the X enopus development. In somatic cells, the nuclear rPL5–55 particle is probably directed immediately to the nucleolus and does not transit through the cytoplasm (Allison et al., 1995; Michael and Dreyfuss, 1996). Similarly, in S. cerevisiae, where 55 RNA is synthesized in the nucleolus, the RNA is probably directly incorporated into ribosomes and does not transit through the cytoplasm, as suggested by the fact that the rPL5–55 complex is found mostly in the nucleus upon the disruption of the large ribosomal subunit assembly in rPL16 mutants (Deshmukh et al., 1993).

Ribosome assembly occurs simultaneously with the maturation of the 35S precursor rRNA that includes methyla-
tion, pseudouridylation, and cleavage reactions. The cleavage steps of the rRNA have been particularly well studied in S. cerevisiae and serve as landmarks to describe the whole pathway, but the step at which the 55–rpl5 particle is incorporated to the ribosome is not well known (Venema and Tollervey, 1995). In H. E. L cells, 55 RNA can be found in ribosomal precursor particles that have not yet undergone the processing step that makes 5.8S rRNA (Warner and Soeiro, 1967). Similarly, indirect methods using yeast-labeled cells have suggested that 55 RNA is present in pre-60S particles, precursors of the large subunits, and may already be present in the less matured pre-90S particles that contain 35S pre-rRNA (Trapman et al., 1976). These two studies suggest that 5S RNA incorporation is an early event of the ribosome assembly/maturational pathway. On the other hand, the fact that a 55–rpl5 complex could be easily dissociated from the large 60S ribosomal subunit without disrupting it, suggested that the 55–rpl5 complex was added at a late step on the surface of the subunit (Blobel, 1971; Nazar et al., 1979). In vitro ribosome reconstitution experiments using proteins and mature rRNA purified from E. Scherichia coli did not clarify the situation as the 55 RNA could be incorporated at any step, but was, nevertheless, necessary at a late step to form an active ribosomal particle (Dohme and Nierhaus, 1976). The location of 55 RNA in mature ribosomal particles has been the subject of extensive studies in E. coli (Bogdanov et al., 1995). The 55 RNA is located in the central protuberance of the large subunit, in close proximity to the small ribosomal subunit. Cross-linking experiments suggest a direct interaction between residues in the D-loop of the 55 RNA and at least two regions of 235 rRNA that are of major functional importance: the GTPase-associated region, involved in EF-G binding, and the peptidyltransferase center (Ontosova et al., 1994; Sergiev et al., 1998).

In the present study, by using mutant 55 RNA, we show that 55 RNA is incorporated at a late step of preribosomal RNA maturation and that the recruitment of the 55 RNA is necessary for the efficient processing of the 275S prerNA. The participation of 55 RNA in the prerRNA processing pathway would ensure that stoichiometric amounts of the three prerRNAs of the large subunit are produced. In addition, we found that alterations of the 60S subunit formation are accompanied by modifications of the nuclear localization of these subunits and of the nucleolar granular compartment.

**Materials and Methods**

**Strains, Plasmids, and Media**

Yeast genetic techniques and media were as described by A. Usual et al. (1987). The wild-type strain YRW1 was M A Tα can1-100 his3-11 leu2-3,112 trp1-1 ura3-1 ade2-1 TFC2 LEU2 pJ A 230 (URA3, TFC2, and CEN3) (A. R. Chambault et al., 1992). The YSC14 strain was derived from YRW1; it Survived without the TRFL1 factor because of the presence of a multicopy plasmid (pRS3) containing a 5S RNA gene under the control of the RNA polymerase III RPR1 promoter as described in Camier et al. (1995). The RPR1-5S gene was mutagenized as described below to create a collection of pOK plasmids. Mutant cells carrying these new plasmids were obtained by transforming YRW1 and selecting the cells that survived without pJ A 230. The mutant strains contained the following plasmids: YOK 69 contained pOK 1 plasmid; YOK 71 (pOK 3); YOK 72 (pOK 4); YOK 73 (pOK 5); YOK 74 (pOK 6); YOK 76 (pOK 7); and YOK 77 (pOK 8).

**Mutagenesis of the RPR1-5S Gene**

The four nucleotides (GGCA) at the 5′ end of the mature 55 RNA in YSC14 strain were mutagenized according to the two-step PCR method described by Higuchi et al. (1988). The sequence at the junction between the RPR1 promoter and the 55 DNA was GATTCGGCAGGT; the italic sequence corresponds to the 5′ end of the wild-type 55 RNA and the underlined sequence corresponds to the sequence that was mutagenized. Two overlapping PCR fragments, one corresponding to the 5′ part of the RPR1-5S (from the 5′ end of the gene to downstream of the mutation site), and the other to the 3′ part of the RPR1-5S (from upstream of the mutation site to the 3′ end of the gene) were synthesized. These two PCR products were obtained using mutagenic primers and 5′ and 3′ primers containing a BamHI restriction site. Mutagenic PCR products were used as templates for a third PCR reaction to synthesize a complete RPR1-5S gene with mutations on both strands. The mutagenic primers for the synthesis of the 5′ fragments were: d(TGGCA TT(TJC)TTCCT GTGCGCCATAT) for the preparation of plasmids pOK 1, pOK 3, pOK 5, and pOK 6, or d(TGGCA TTGTTGCGCCATAT) for the deletion of the GGC sequence (plasmid pOK 7). For the synthesis of the 3′ PCR fragments, the primers were d(GCAACGAC(A)G(A)G(A)GCTC GCTCCC) for pOK 1, 4, 5, and 6, or d(GCAACCAATCGCA GCTCCC) for the preparation of pOK 7. The third PCR combined 1 pmol of each 5′ and 3′ PCR products and the 5′ and 3′ primers containing the BamHI restriction site. The resulting PCR fragments were digested with BamHI. The pOK plasmids derived from the pRS3 plasmid by exchange of the BamHI fragment containing the wild-type 55 RNA gene with the RPR1-5S fragment of a mutant RPR1-5S gene. The nature of the modification was determined by sequencing. The pOK 3 and pOK 8 plasmids contained two repeats of the BamHI fragment from pOK 4 and pOK 1, respectively.

**RNA Analysis**

RNA was extracted as described by Schmitt et al. (1990) with some modifications. Cells corresponding to 10 ml of culture at an OD 600nm 0.4–0.6 were resuspended in 250 μl of 50 mM sodium acetate, pH 5.3, 10 mM EDTA, plus 25 μl of 50% SDS, mixed with 150 μl of acid-washed glass beads and 250 μl of phenol, vortexed vigorously, and incubated for 5 min at 65°C with intermittent vortexing. RNAs were precipitated with 0.3 M sodium acetate, pH 5.3, and 3 vol of ethanol at −20°C overnight. Small RNAs were separated on a denaturing 8% polyacrylamide gel and visualized by ethidium bromide staining.

**Quantiﬁcation of Small RNA**

Small RNA species ranging from 1 to 5 μg were analyzed on 8% denaturing polyacrylamide gels and visualized by ethidium bromide staining. The gel picture was registered with an enhanced analysis system (E.A.S.Y.; Herolab) and the amount of each species was measured by densitometry using the NIH Image program. The curves for each RNA species were used to measure the average ratio between the amount of two RNA species (55 RNA and tRNA and 5.8S rRNA and 5S RNA). These experiments were reproduced at least three times for each strain.

**Analysis of High Molecular Weight rRNAs**

High molecular weight rRNAs were analyzed on 12% agarose gels after denaturation with glyoxal and dimethylsulfoxide as described in A. Usual et al. (1987). A 14 h run at 55 V in 10 mM NaHPO4, pH 7.0, RNA was transferred to a positively charged nylon membrane (Boehringer Mannheim) and cross-linked with UV. rRNAs were visualized by spraying the membrane with ENHANCE® (DuPont) and autoradiographed at −70°C.

**Analysis of the 5′ and 3′ Ends of Small RNA**

The 5′ end of 55 RNA was identified by primer extension using the MMLV reverse transcriptase and the internal 55 RNA oligonucleotide GTCAGGCCTCTACGACTTAAC as described in Jacquet et al. (1989). The identification of the 3′ end of the mutant 55 RNA was carried out by sequencing using either chemical modifications or ribonuclease digestions as described below. The mutant 55 RNA's were isolated from denaturing gels and 3′ end was labeled with [32P]J P C using T4 RNA ligase. The two labeled bands were separated on an 8% sequencing polyacrylamide gel, isolated, and analyzed separately. The chemical sequence was carried out using standard reactions as described in England et al. (1980) using diethylpyrocarbonate for the modification of A, dimethylsulfate for G, aqueous hydrazine for U, and 3 M NaCl in anhydrous hydrazine for C. After the modifications of the bases, the RNAs were digested with aniline and separated on a sequencing gel. The ribonuclease sequence of the 3′ end–labeled 55 RNA was performed as described in Donis-Keller et al. (1977) with mod-
from a chimeric gene that does not require TFIIIA. The non-LHP1 part of the 25S rRNA probe, or the 1,954-bp EcoRI-EcoRI fragment for the 18S rRNA probe. The probes were diluted (final, 2 ng/ml) in a hybridization medium containing 50% formamide and 10% dextran sulfate in 1× SSC. 20 μl of probe was added on the grids and denatured for 5 min at 75°C. Hybridization was carried out overnight in a wet chamber at 37°C. The grids were washed three times for 5 min in 50% formamide/2× SSC at 45°C, three times for 5 min in 0.1× SSC at 60°C, and a few seconds in 4× SSC at room temperature. Nonspecific immunological sites were blocked for 30 min with 5% BSA in 4× SSC. The probe was detected with an avidin-biotin-peroxidase complex and diaminobenzidine as chromogen. The grids were washed three times for 5 min in 50% formamide/2× SSC at 37°C. The grids were washed three times for 5 min in 2× SSC at room temperature and dehydrated through an ethanol series of 70, 90, and 100%.

Results

5S Mutations Result in Less Stable 5S RNA and Decreased Growth Rate

A collection of yeast strains surviving with mutated 5S rRNA has been constructed using a genetic system described previously (Camier et al., 1995). In these strains, transcription of the endogenous 55S R N A genes was abolished by inactivating the gene coding for the transcription factor TFI11A. The cells survive with 5S RNA expressed from a chimeric gene that does not require TFI11A. The RPR1 promoter fused to the 55S RNA gene is transcribed by RNA polymerase III to give an RPR1-5S RNA precursor that is processed into 55S RNA molecules with extended 5′ and 3′ ends. The 5′ extremity contains four additional 5′ nucleotides GGG (Table I, strain YSC14) and the 3′ extremity is heterogeneous, with 1–3 additional residues (data not shown). In wild-type 55S RNA, the 5′ and 3′ ends of the molecules are paired in a stem called helix I; the additional nucleotides could be part of an extended helix I and protected from degradation during RNA processing (Moore, 1996). To remove the extra nucleotides, mutations, which could potentially decrease interactions between the 5′ and 3′ ends of the 55S RNA, were intro-

Strains were grown under selective conditions in a synthetic medium without uracil (−Ura) at an OD600nm of 0.2, diluted 10–100-fold and spotted on −Ura plates. The plates were incubated at 30°C or 37°C for various periods of time as indicated in the figure legend. Photographs were scanned and arranged with Adobe Photoshop to have a homogenous black background.

Electron Microscopy

The preparation of cells for electron microscopy and freeze electron substitution was as described by Leger-Silvestre et al. (1997). For all observations, the grids were contrasted with saturated aqueous uranyl acetate alone or combined with lead citrate (freeze substitution grids) and imaged in a JEOL 1200EX electron microscope operating at 80 kV.

Scores were additive and quantified by electron microscopy. The grids for immunoelectron microscopy were pretreated for 15 min with PBS buffer, pH 7.6, containing 2% BSA and incubated for 1 h at room temperature with anti-Nop1 mAb (1:100) provided by Dr. J. Aris (University of Florida, Gainesville, FL). The grids were washed for 30 min with PBS buffer containing 1% BSA and transferred for 1 h to colloidal gold-conjugated goat anti-mouse diluted 1:80 in the same buffer. A ferroconjugate, the grids were washed for 20 min in PBS buffer and 10 min in water, and air-dried. Controls were performed using gold-labeled antiserum alone. No labeling was detected on these grids.

For in situ detection of ribosomal RNA, a 35S pre-rRNA probe was synthesized by nick-translation in the presence of digoxigenin-11dUTP (Boehringer Mannheim) from a plasmid containing an rDNA unit. The probe for the 25S and 18S rRNA were synthesized by random priming using a DIG-High Prime kit (Boehringer Mannheim) from isolated restriction DNA fragments: the 3,533-bp KpnI-HindIII fragment for the 25S rRNA probe, or the 1,954-bp EcoRI-EcoRI fragment for the 18S rRNA probe. The probes were diluted (final, 2 ng/ml) in a hybridization medium containing 50% formamide and 10% dextran sulfate in 1× SSC. 20 μl of probe was added on the girds and denatured for 5 min at 75°C. Hybridization was carried out overnight in a wet chamber at 37°C. The grids were washed three times for 5 min in 50% formamide/2× SSC at 45°C, three times for 5 min in 0.1× SSC at 60°C, and a few seconds in 4× SSC at room temperature. Nonspecific immunological sites were blocked for 30 min with 5% BSA in 4× SSC. The probe was detected with an avidin-biotin-peroxidase complex and diaminobenzidine as chromogen. The grids were washed three times for 5 min in 50% formamide/2× SSC at 37°C. The grids were washed three times for 5 min in 2× SSC at room temperature and dehydrated through an ethanol series of 70, 90, and 100%.

Ribosome Analysis

Yeast ribosomes were fractionated by sucrose gradients (Baim et al., 1985). Cells were grown in 100 ml of yeast extract/peptone/glucose (YPD) medium at 30°C. At 0.8–0.9 of the culture, which was immediately transferred to ice. A ferroconjugate, the cells were collected by centrifugation and washed by lysing buffer containing 10 mM Tris-Cl, pH 7.4, 100 mM NaCl, 30 mM MgCl2, 50 μg/ml of cycloheximide, and 200 μg/ml of heparin. They were resuspended in two volumes of lysate buffer and one volume of acid-washed glass beads. The cells were disrupted by vortexing the suspension eight times for 15 s with a 30 s freezing of the cells (1 min) was included in the chase times. The pellets were centrifuged at room temperature. Cell pellets were immediately frozen in a dry ice/ethanol bath and stored at –70°C until use.

Analysis of RNA Extremities Resulting from Processing in ITS2. The 5′ end of the 25S rRNA was analyzed by primer extension and the 3′ end of the TS-PrRNA precursor was analyzed by an RNase protection assay. Primer extension analysis was done according to the method described by Hong et al. (1997). The 5′ end 32P-labeled oligonucleotide for the analysis of the 5′ end of the 25S rRNA (cleavage at site C1) was 5′ TACCTGAGC AATCCCGGT TG G (Hong et al., 1997). The RNase protection assay was carried out using a 35S-labeled transcript complementary to the ITS2 sequence, prepared with T7 RNA polymerase. The T7 template corresponds to a DNA fragment encoding the major part of the ITS2 sequence (nucleotides 1–215), amplified by PCR, using oligonucleotides 5′-CCTTCT TACA AACTTCTGTTT GTT GAG and 5′-GGCTT AGCGGCTT CTCCCC as primers and cloned under a T7 RNA polymerase promoter. Total yeast RNA was hybridized with T7RNA transcripts and the resulting RNA duplexes were digested with a mixture of RNase A and RNase T1 and analyzed on a denaturing gel (A ubel et al., 1987).

Pulse-Chase Experiments

For pulse-chase labeling of pre-rRNA, 10 ml of cells growing in a synthetic medium without methionine at an OD600 of 0.3–0.4 were labeled with 70 μCi/ml of [35S]-methionine for 1 min at 30°C. U4 labeled methods to a DNA fragment that was sequenced by the ITS2 sequence. The 5U end of the 25S rRNA was analyzed by primer extension and the 3′ end of the TS-PrRNA precursor was analyzed by an RNase protection assay. Primer extension analysis was done according to the method described by Hong et al. (1997). The RNase protection assay was carried out using a 35S-labeled transcript complementary to the ITS2 sequence, prepared with T7 RNA polymerase. The T7 template corresponds to a DNA fragment encoding the major part of the ITS2 sequence (nucleotides 1–215), amplified by PCR, using oligonucleotides 5′-CCTTCTT CCTTCAA AACTTCTGTTT GTT GAG and 5′-GGCTT AGCGGCTT CTCCCC as primers and cloned under a T7 RNA polymerase promoter. Total yeast RNA was hybridized with T7RNA transcripts and the resulting RNA duplexes were digested with a mixture of RNase A and RNase T1 and analyzed on a denaturing gel (A ubel et al., 1987).

Ribosomal Subunit Quantiﬁcation was done according to the method described by Moritz et al. (1991). Cycloheximide, heparin, and MgCl2 were omitted from all buffers, and ribosomal subunits were separated on high salt (0.5 M KCl) sucrose velocity gradients.

55S Mutations Result in Less Stable 5S RNA and Decreased Growth Rate

A collection of yeast strains surviving with mutated 5S RNA has been constructed using a genetic system described previously (Camier et al., 1995). In these strains, transcription of the endogenous 55S RNA genes was abolished by inactivating the gene coding for the transcription factor TFI11A. The cells survive with 5S RNA expressed from a chimeric gene that does not require TFI11A. The RPR1 promoter fused to the 55S RNA gene is transcribed by RNA polymerase III to give an RPR1-55S RNA precursor that is processed into 55S RNA molecules with extended 5′ and 3′ ends. The 5′ extremity contains four additional 5′ nucleotides GGG (Table I, strain YSC14) and the 3′ extremity is heterogeneous, with 1–3 additional residues (data not shown). In wild-type 55S RNA, the 5′ and 3′ ends of the molecules are paired in a stem called helix I; the additional nucleotides could be part of an extended helix I and protected from degradation during RNA processing (Moore, 1996). To remove the extra nucleotides, mutations, which could potentially decrease interactions between the 5′ and 3′ ends of the 55S RNA, were intro-

1. A abbreviations used in this paper: ITS2, internal transcribed spacer 2; YPD, yeast extract/peptone/glucose.
Table I. Characteristics of Yeast Strains Surviving with 5S rRNA Mutants

| Strain | 5′ sequence | Number of RPR1-5S repeats | Doubling time at 30°C | Growth at 37°C | tRNA/5S ratio | 5.8S/5S ratio |
|--------|-------------|---------------------------|----------------------|----------------|---------------|--------------|
| WT     | —           | —                         | 1.5                  | +++++          | 2.1 ± 0.4     | 1.2 ± 0.1    |
| YSC14  | GCGA        | 1                         | 3.8                  | 5.8 ± 0.5      | 1.4 ± 0.1     |
| YOK69  | CAGT        | 1                         | 2.3                  | ++             | 3.4 ± 0.4     | 1.1 ± 0.1    |
| YOK71  | TCCT        | 1                         | 3.4                  | —              | 4.7 ± 0.6     | 1.1 ± 0.1    |
| YOK72  | CTCT        | 1                         | 3.4                  | —              | 4.8 ± 0.5     | 1.1 ± 0.2    |
| YOK76  | GATT        | 1                         | 3.7                  | —              | 6.1 ± 0.2     | 1.3 ± 0.1    |
| YOK74  | TTCT        | 1                         | 5.3                  | —              | 7.2 ± 0.5     | 1.4 ± 0.1    |
| YOK77  | CCCT        | 2                         | 2                    | +++)           | 3.1 ± 0.2     | 1.1 ± 0.1    |
| YOK71  | CTCT        | 2                         | 3.3                  | —              | 4.1 ± 0.4     | 1.1 ± 0.1    |

*Sequence immediately upstream of the 5S rRNA gene in the RPR1-5S construct.
†5.8S rRNA, 5S rRNA, and tRNA were separated by electrophoresis in polyacrylamide gel and their amounts were determined by scanning the gels after ethidium bromide staining (see Materials and Methods). The results are from at least three independent experiments.
‡The original 5S rRNA mutant derived from the RPR1-5S construct with nonmutagenized 5′ sequence.

Duplicated in the sequence upstream of the 5S RNA gene (Table I, strains YOK69, YOK71, YOK72, and YOK74). A deletion mutant was also constructed that removed the last four nucleotides of the RPR1 sequence at the junction with the SS RNA gene (Table I, strain YOK76). All the mutations gave rise to functional 5S RNA, since the cells could grow in the absence of TFI1A, at 30°C (Table I). Small RNAs were prepared from all mutants and analyzed on a denaturing polyacrylamide gel (Fig. 1). No mutation restored the normal size of the 5S RNA. The 5S RNA of all mutants migrated as two RNA bands of 125- and 127-nt long. The analysis of their 5′ and 3′ extremities by primer extension and RNA sequencing, respectively, showed that all the mutated 5S RNA still contained the 5′ and 3′ nucleotide extensions (data not shown).

The cells surviving with mutant 5S RNA displayed very different growth rates both at 30°C and 37°C and presented defects in cell and colony morphology (Table I and Fig. 2). The cells had a hyperpolarized growth and did not separate well after division, which resulted in filaments. These defects were particularly pronounced in slow growing mutants (Fig. 2, strains YSC14 and YOK74). The fact that the growth rate at 30°C and 37°C, and that the cell morphology could be improved by varying the mutations in the RPR1-5S gene indicated that these defects were due to the mutated 5S RNA rather than to the absence of TFI1A.

Duplication of the RPR1-5S insert in two different mutants resulted in a growth rate improvement both at 30°C and 37°C (Table I and Fig. 3), compare YOK69 and YOK7, or YOK72 and YOK71, indicated by asterisks in Fig. 3). This result suggested that the different growth rates reflected in part the amount of stable 5S RNA in the cell. The ratio between the amounts of tRNA and 5S RNA was determined by densitometry and found to be ~2 in the wild-type cells and between 3 and >7 in the mutants (Table I). The decrease in the amount of 5S RNA correlated well with the decrease in the cell growth rate. A partially, mutated 5S RNA accumulated at different levels depending on the sequence located at the 5′ end of the molecule, and the different levels of 5S RNA determined the growth rate of the mutant cells.

Overproduction of Two 5S RNA Binding Proteins, rpL5 and Lhp1p, Partially Rescues 5S RNA Defects

Woolford and co-workers have shown that free 5S RNA was not stable in vivo and that its stabilization required the binding of ribosomal protein rpL5 (Deshmukh et al., 1993). One of the major binding sites of rpL5 to 5S RNA is helix I; the presence of extra nucleotides adjacent to that helix could perturb the binding of rpL5 and, therefore, result in decreased amount of stable 5S RNA. We found that in the slow growing mutants (YOK74, YOK76, YOK72, YOK73, and YOK71), the overexpression of the gene encoding rpL5 improved the growth rate at 30°C and 37°C (Fig. 3). The amount of 5S RNA, analyzed in the most altered mutant (YOK74), was increased upon rpL5 overexpression, which is likely the cause of the improved growth rate (Table II). Similar experiments were conducted with the LHP1 gene encoding the yeast homologue of the Lp protein, known to interact with the 3′ end of RNA polymerase III transcripts (Stefano, 1984). Lp overexpression also led to an improved growth rate and resulted in an increased amount of stable 5S RNA (Fig. 3, Table II). In this case, the nature of the mutant 5S RNA was modified, and the 5S RNA now migrates as a single band, corresponding to the slow migrating band of the doublet on denaturing polyacrylamide gels (data not shown). The 5′ end of 5S RNA, as determined by primer extension, was unchanged, suggesting that the increase in size was due to the protection of the extra 3′ nucleotides by bound Lhp1p. The increase in the amount of 5S RNA by overexpressing the LHP1 gene could reflect the existence of an in vivo pool of...
5S RNA-Lhp1p. Alternatively, the slow migrating RNA band generated by LHP1 overexpression could have a higher affinity for rpL5 than the fastest migrating band and, therefore, be better stabilized.

**Mutations in 5S RNA Result in Decreased Synthesis of the Ribosomal 60S Subunit**

When analyzing the small RNA species present in 5S mutants, we noticed that whereas the ratio between tRNA and 5S RNA increased severalfold in slow growing cells, the ratio between 5.8S rRNA and 5S RNA remained practically constant, similar to the wild-type value (Table I). This result indicated that the amount of 5.8S rRNA decreased concomitantly with the amount of 5S RNA. Since 5S RNA and 5.8S rRNA are part of the same large 60S ribosomal subunit, we analyzed the content in ribosomal subunits in the mutant strains. Polyribosomes and free ribosomal particles were separated on sucrose velocity gradients from wild-type cells and two different 5S mutants (Fig. 4).

Mutant ribosomal profiles showed marked alterations that were more accentuated in the slow growth mutant, YSC14, than in a better growing mutant, YOK77. First, the total amount of polyribosomes was significantly reduced in both mutants, suggesting a less efficient protein synthesis rate compared with wild-type cells. Second, the ratio between the amount of free 60S and free 40S ribosomal subunits was reversed in the mutants because of the accumulation of 40S subunits and a reduction in 60S particles. The imbalance between the 60S and 40S subunits was also evidenced by the presence of additional peaks sedimenting more quickly than the 80S monoribosomes or the polyribosomes. These peaks (Fig. 4, arrows) are likely to represent halfmers polyribosomes that contain mRNA associated with an integral number of ribosomes plus a stalled 48S preinitiation complex. The presence of halfmers can be an indication of a decreased amount of 60S subunits. To confirm the deficiency in 60S subunits, ribosomal profiles were run under conditions that dissociate the ribosomes. The ratio between the 60S and 40S subunits, which is \( z^2 \) for wild-type cells, decreased to 1 in YSC14 cells (data not shown). Therefore, the decreased amount of 5S RNA observed in the mutant cells is accompanied by a decrease in the total amount of 60S subunits. This decrease very likely alters the protein synthesis rate that results in decreased growth rate.

**The Level of 5S RNA Affects Pre-rRNA Processing**

The shortage in mature 60S subunits could reflect the misincorporation of the mutant 5S RNA in the pre-60S particles, which in turn would lead to the accumulation of partially assembled 60S subunits. This possibility was particularly interesting since in all the mutant profiles analyzed, we reproducibly observed an additional peak sedimenting more quickly than the 80S monoribosomes or the polyribosomes. These peaks (Fig. 4, arrows) are likely to represent halfmers polyribosomes that contain mRNA associated with an integral number of ribosomes plus a stalled 48S preinitiation complex. The presence of halfmers can be an indication of a decreased amount of 60S subunits. To confirm the deficiency in 60S subunits, ribosomal profiles were run under conditions that dissociate the ribosomes. The ratio between the 60S and 40S subunits, which is \( z^2 \) for wild-type cells, decreased to 1 in YSC14 cells (data not shown). Therefore, the decreased amount of 5S RNA observed in the mutant cells is accompanied by a decrease in the total amount of 60S subunits. This decrease very likely alters the protein synthesis rate that results in decreased growth rate.

**Table II. Overexpression of RPL5 or LHP1 Increases Both the Amount of 5S rRNA and the Growth Rate**

| Strain       | Doubling time at 30°C | Growth at 37°C | tRNA/5S ratio | 5.8S/5S ratio |
|--------------|-----------------------|----------------|---------------|---------------|
| YOK74 + vector | 5.5                   | -              | 7.2 ± 0.5     | 1.4 ± 0.1     |
| YOK74 + RPL5-2μ | 4                     | +              | 5.8 ± 0.5     | 1.4 ± 0.1     |
| YOK74 + LHP1-2μ | 2.9                   | ++             | 4.6 ± 0.4     | 1.3 ± 0.1     |

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menting as a 35S particle, absent in the wild-type cells and whose intensity was particularly high in the YSC14 strain. However, the analysis of the RNA content of the peak showed that it did not contain any ribosomal RNA, but instead an RNA species of 2600 nt that corresponded to the 20S single-strand viral RNA (data not shown) (Wejksnora and Haber, 1978; Matsumoto et al., 1990; Rodriguez-Cousino et al., 1991). We did not identify any other peak that could represent the partially assembled 60S subunit. The analysis of the 5S RNA content in the 60S subunits showed that the different mutant 5S RNA species were all incorporated in the subunits, which shows that the decrease in 60S was not due to the selective incorporation of only some of these species.

We explored the possibility that 5S RNA could play a role in the biogenesis of the 60S subunit and analyzed the preribosomal RNA processing steps in the mutants (Fig. 5). The fate of the ribosomal precursors was followed in the WT strain and the YOK77 and YSC14 mutants by pulse-chase experiments after labeling with methyl-[^3]H]methionine. The methylation patterns were quantified and the efficiencies of maturation of the different species were measured (Table III). The efficiency of maturation of the 20S pre-RNA into 18S mature rRNA was similar in the mutants and the WT, ~70% after a 2.5-min chase and 90% after a 5.5-min chase (Table III, ε₁₈). In sharp contrast, the accumulation of the mature 25S rRNA took much longer in the mutant cells compared with the WT, with a more pronounced defect in the YSC14 strain: after a 2.5-min chase no mature 25S rRNA was detected in YSC14 cells, only 15% in YOK77 cells, whereas the maturation was 70% complete in the WT cells (Table III, ε₂₅).

Table III. Maturation Efficiencies (ε) of 27SA RNA into 27SB RNA (ε₂₇), 27S (A + B) RNA into 25S RNA (ε₂₅), and 20S RNA into 18S RNA (ε₁₈)

| Time   | ε₂₇ | ε₂₅ | ε₁₈ |
|--------|-----|-----|-----|
| 2.5 min| ε₂₇ | ε₂₅ | ε₁₈ |
| 5.5 min| ε₂₇ | ε₂₅ | ε₁₈ |
| 10 min | ε₂₇ | ε₂₅ | ε₁₈ |
| 30 min | ε₂₇ | ε₂₅ | ε₁₈ |

This table is a quantification of pulse-chase experiments realized as in Fig. 5, from two sets of experiments. The time points refer to the chase times. The maturation efficiency was calculated as the percentage of matured species relative to the amount of unmatured and matured product. ε₂₇, amount of 27SB RNA/amount of (27SA + 27SB) RNA × 100; ε₂₅, amount of 25S RNA/amount of (27S + 25S) RNA × 100; ε₁₈, amount of 18S RNA/amount of (20S + 18S) RNA × 100.
nated at least in part from the lower amount of stable 5S RNA, increasing the amount of 5S RNA by overexpression of the RPL5 or LHP1 genes should speed up the processing reaction. We analyzed the ribosomal processing in the strain YOK74 (the most affected mutant) transformed with either gene (Fig. 6). When either rpl5 or lhp1p was overproduced, the processing rate of the 27SB precursor was improved, since it was 67-90% complete after a 5.5-min chase compared with only half complete in the absence of overproduced protein. We conclude that the amount of stable 5S RNA in the cell influenced the rate of processing of the 27SB precursor, which suggests that the 5S RNA is recruited at this step of the processing pathway and that its recruitment is necessary for the processing.

### Nucleolar Alterations and Abnormal Nuclear Localization of the 60S Subunit RNA

In S. cerevisiae, 5S RNA genes are present in the nucleolus. The absence of endogenous chromosomal 5S transcription and the defects in 60S subunit formation prompted us to analyze the structure of the nucleolus in these mutants. The small size of yeast and its tough cell wall considerably hindered precise structural analysis of its nucleolus by fluorescence and electron microscopy. Recently, by combining cryofixation and cryo-substitution, it has been possible to identify distinct substructures similar to the components of nucleoli of higher eukaryotes: the fibrillar centers, the dense fibrillar component, and the granular component (Leger-Silvestre et al., 1997). These techniques coupled with conventional in situ techniques at the electron microscopy level were applied to the analysis of some of the 5S mutants described here.

In all mutants, a nucleolus could be morphologically identified. Its structure was similar for all the mutants but differed from a wild-type nucleolus since it appeared less dense, mostly fibrillar with less granular component (Fig. 7 A). These characteristics were more pronounced in slow growing mutants. To appreciate the degree of disorganization of the nucleolus in the 5S mutants, we analyzed the localization of a nucleolar marker, the Nop1 protein, and the ribosomal RNA. The immunolocalization of Nop1p was normal and corresponded to the dense region of the nucleolus referred to as the nucleolus (Fig. 7 B). Therefore, the nucleolus of the mutants was not strongly disorganized. The ribosomal RNA was detected by in situ hybridization with digoxigenin-labeled probes complementary either to the 35S rRNA precursor (Fig. 8 A) or to the mature 25S rRNA (Fig. 8 B). The results were identical for both probes but differed depending on the strain analyzed. For the wild-type strain, the nuclear labeling was, as expected, concentrated in the nucleolus. No significant labeling appeared in the nucleoplasm except for a few gold particles in the nuclear pores, which likely corresponded to preribosomal particles exported to the cytoplasm. For one of the most altered strains, YSC14, the precursor or the 25S rRNA was totally dispersed throughout the nucleus. For YOK69, one of the fast growing mutants, the situation was intermediate, with the nucleolus being mostly labeled plus regions at the periphery of the nucleoplasm. A accumulation of gold particles near the nuclear envelope was observed with both probes for all the 5S mutants analyzed.

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2.5-min chase, most of the 27SA pre-RNA had been converted into 27SB pre-RNA in the three cell types (Table III, e8). No 32S pre-RNA or 35S pre-RNA was detected in the experiments, which suggests that the maturation of these species was not significantly altered in the mutants. Similar pulse-chase experiments performed in the presence of [3H]thymidine instead of [3H]methionine also showed a specific delay in the processing of the 27SB precursor, which was again more pronounced in the YSC14 strain than in the YOK74 strain (data not shown). Therefore, the delay observed was not due to a delay in the methylation reaction. The processing of the 27SB precursor consists in the removal of a part of the internal transcribed spacer 2 (ITS2) between sites C1 and C2, through a still unknown mechanism (Venema and Tollervey, 1995). We analyzed the C1 and C2 cleavage sites by primer extension and RNAse protection experiments, respectively. No difference was found between the 5S mutants and the wild-type cells (data not shown). Therefore, the 5S RNA mutations did not alter the cleavage sites in the precursor but had a strong effect on the processing reaction rate. The decreased processing rate of the RNA of the large subunit is likely at the origin of the decreased growth rate. There is indeed a good correlation between the processing rate and the growth rate, as shown for example in the YSC14 mutant, which grows four times more slowly than the WT, and processes the 27SB pre-RNA four times more slowly (half complete maturation of the 25S rRNA is observed after a 6-min chase compared with 1.5-min in the WT).

As described above, one main consequence of the 5S mutations was to decrease the amount of stable 5S RNA in the cells. If the low processing rate in the mutants origi-
When similar experiments were conducted with a probe hybridizing to the 18S rRNA of the small ribosomal subunit, its localization was exclusively nucleolar for both wild-type and 5S mutant cells (data not shown). The ribosomal RNA mislocalized in the nucleus of 5S mutants corresponded, therefore, to the RNA of the large ribosomal subunit. The nuclear localization of the small subunit RNA remained unaffected.

Discussion

The novelty of this paper consists in the observation that 5S RNA plays a role in the processing of the large ribosomal subunit RNA. We show that there is a direct correlation between the rate of processing of the 27SB pre-rRNA and the amount of 5S RNA present in the cells. We propose that 5S RNA binds to the preparticle containing the 27SB pre-rRNA and that this binding is necessary for the processing to proceed at a normal rate and give rise to the mature rRNA of the large subunit. This mechanism could participate in a quality control process ensuring that all newly formed mature 60S ribosomal subunits contain stoichiometric amounts of the three rRNA components.

We have studied a collection of yeast strains surviving with mutant 5S RNA. The 5S mutations that correspond to nucleotide extensions at the 5' and 3' ends of 5S RNA result in decreased accumulation of 5S RNA in the cells, which is possibly due to the lower affinity of the ribosomal
protein rpL5 for the mutant 5S RNA. In all the 5S mutants studied here, the decrease in the amount of 5S RNA was paralleled by a decrease in the amount of 60S subunits. No partially assembled 60S subunits devoid of 5S RNA were detected, suggesting that either they were degraded or their formation was impaired. The analysis of the preribosomal processing in the mutants favors the second hypothesis. We found that the rate of processing of 27SB pre-rRNA leading to mature 25S rRNA and 7S pre-rRNA (a precursor to the 5.8S rRNA) was considerably decreased in the 5S mutants. The slower rate was, at least in part, a consequence of the decrease in the amount of 5S RNA since it could be improved by the overproduction of rpL5 or Lhp1p that increased the amount of stable 5S RNA in the cells. We propose that 5S RNA is recruited by the preribosomal particles containing the 27SB precursor and that its binding allows processing to proceed at a normal rate. In the presence of limiting amounts of 5S RNA, 27SB pre-rRNA does not appear to accumulate, as judged by Northern analysis (data not shown) showing that it is eventually degraded.

Our observation that 5S RNA plays a role in the formation of the mature 60S subunits is in agreement with previous observations made by other groups. Nazar and collaborators found that expressing mutant 5S RNA in otherwise wild-type cells (i.e., also containing wild-type 5S RNA) led to a decrease in the production of 60S subunits suggesting that the 5S RNA was necessary for the formation or the stability of the subunit (van Ruyk et al., 1992). Aditional evidence came from the work of Woullford and collaborators who found that in the absence of rpL5 synthesis, no mature 60S subunits were produced, and of Lee and collaborators who observed a decreased production of 60S subunits in the presence of mutant rpL5 (Dechampsme et al., 1993; Ye et al., 1995a,b; Ye et al., 1996). Although these experiments altered rpL5 levels, they probably reflected the role of 5S RNA as well since rpL5 and 5S RNA are associated in an RNP that binds to the preribosomes. In yeast cells depleted of rpL5 no specific defect of the large ribosomal RNA processing was detected, but the whole processing pathway was slowed down. The delay of all the processing steps probably does not mean that rpL5 plays a role in all these steps. It may rather be due to a feedback mechanism, as proposed for two other yeast mutants (Nip7 and Spb4) altered in the processing of 27SB pre-rRNA and that displayed a delay in the processing of 35S and 32S pre-rRNA s (Zanchin et al., 1997; de la Cruz et al., 1998b).

The maturation step affected by 5S RNA corresponds to two cleavage steps in the ITS2, at site C1 (the 5’ end of mature 25S rRNA) and site C2 (located within ITS2) that release the mature 25S rRNA and the 7S pre-rRNA, which is further processed to yield the mature 5.8S rRNA. No mutant has been isolated that presented a defect in only one of the cleavage steps, which suggests that the two cleavages are connected. The maturation of 27SB pre-rRNA is one of the slowest steps of the pathway, reflecting probably important structural rearrangements and the need to recruit several components (Gaullier et al., 1997), among which is probably the 5S–rpL5 complex. 5S RNA probably does not have a direct role in the cleavage reactions but could well be involved in the correct assembly of a maturation-competent preparticle. Other yeast mutants slowed down in the processing of 27SB pre-rRNA are assembly mutants altered in the ribosomal proteins rpL16 or rpL32 (Moritz et al., 1991; Vilardeil and Warner, 1997), mutants in putative RNA helicases (Ripmaster et al., 1992; de la Cruz et al., 1998a) and mutants in a methylase (Hong et al., 1997).

In parallel with the ribosomal processing defects, the 5S
RNA mutants presented alterations of the nucleolar structure. Their nucleolus appeared less dense and with a less granular component than a wild-type nucleolus. The modification of the granular compartment is expected since this compartment corresponds to a concentration of preribosomal particles in the late stages of maturation (Shaw and Jordan, 1995), and the 5S mutants are altered in one of these stages. More unexpected was the delocalization of the large ribosomal subunit RNA throughout the nucleoplasm, which shows that the transport of the preparticles from the nucleolus to the cytoplasm is altered in these mutants. There is not a general defect in the nuclear transport since the localization of the small ribosomal subunit RNA was normal. Interestingly, in yeast mutants altered in a nucleoporin and, therefore, in the transport across the nuclear pores, a similar delocalization of 35S ribosomal RNA throughout the nucleus has been observed (Gas, N., unpublished observations). We propose that mutant pre-particles not properly processed are not exported to the cytoplasm, which perturbs the whole trafficking of the pre-subunits in the nucleus. Very few data are available on the mechanism of transport of the preribosomal particles across the nucleoplasm and the nuclear envelope. There are some indications that transport and nucleolar organi-

Figure 7. The nucleolus of 5S RNA mutants is partially disorganized. (A) Electron micrograph after freeze electron substitution of the wild-type and YSC14 strains grown at 30°C. Substructures similar to the components of the nucleolus of higher eukaryotes are visualized: the fibrillar center (FC), the dense fibrillar component (DFC), and the granular component (GC). The fibrillar centers are not visible on the WT section. N, nucleoplasm. (B) Immunogold localization of the nucleolar protein Nop1 in the wild-type and YSC14 strains. Nop1 was detected using anti-Nop1 mAbs revealed with a secondary gold-conjugated antibody. Gold particles colocalized with the nucleolus in the two strains. N, nucleoplasm. Bars, 500 nm.
zation could be linked, since mutants affected in proteins of the mRNA export pathway exhibit alterations of the nucleolus and defects in the preribosomal RNA processing (Kadowaki et al., 1994).

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Figure 8. Nuclear delocalization of the 25S rRNA in 5S RNA mutants. The localization of the 35S pre-rRNA and the 25S mature rRNA was analyzed by in situ hybridization using digoxigenin-11dUTP-labeled probes specific for the 35S pre-rRNA (A) or the 25S rRNA (B) in the wild-type (WT), YOK69, and YSC14 strains. The probes were detected with antidigoxigenin antibodies gold conjugate. In A, as a control, the grids were pretreated with DNase-free RNase (YSC14 + RNase) before hybridization. N, nucleoplasm; Nu, nucleolus; NE, nuclear envelope; and P, cell wall. Bars, 500 nm.
