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A System for the Analysis of Yeast Ribosomal DNA Mutations

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To develop a system for the analysis of eucaryotic ribosomal DNA (rDNA) mutations, we cloned a complete, transcriptionally active rDNA unit from the yeast *Saccharomyces cerevisiae* on a centromere-containing yeast plasmid. To distinguish the plasmid-derived ribosomal transcripts from those encoded by the rDNA locus, we inserted a tag of 18 base pairs within the first expansion segment of domain I of the 26S rRNA gene. We demonstrate that this insertion behaves as a neutral mutation since tagged 26S rRNA is normally processed and assembled into functional ribosomal subunits. This system allows us to study the effect of subsequent mutations within the tagged rDNA unit on the biosynthesis and function of the rRNA. As a first application, we wanted to ascertain whether the assembly of a 60S subunit is dependent on the presence in *cis* of an intact 17S rRNA gene. We found that a deletion of two-thirds of the 17S rRNA gene has no effect on the accumulation of active 60S subunits derived from the same operon. On the other hand, deletions within the second domain of the 26S rRNA gene completely abolished the accumulation of mature 26S rRNA.

Eucaryotic ribosomes are highly structured supramolecular complexes consisting of about 80 different constituents. These complexes are manufactured largely within the nucleolus, where the rRNA genes are transcribed by RNA polymerase I and the nascent pre-rRNA molecules assemble with a large set of both ribosomal and nonribosomal proteins (10). The preribosomal particles undergo a number of maturation steps, including processing and modification of the rRNAs, and are subsequently transported into the cytoplasm. Very little is known as yet about the structural elements in the pre-rRNA involved in the processing and assembly of preribosomes.

The pre-rRNA molecule is encoded by a transcription unit which, together with its flanking sequences, is tandemly repeated 50 to 1,000 times in the chromosome of most eucaryotic organisms (14). Each transcription unit has the following general structure: 5′ ETS-17/18S rRNA-ITS1-5.8S rRNA-ITS2-25/28S rRNA-3′ ETS (Fig. 1), in which ETS and ITS designate external and internal transcribed spacers, respectively. Evolutionary studies and phylogenetic comparisons of the eucaryotic ribosomal transcription units (8) have revealed that the regions coding for the mature rRNAs are rather well conserved whereas the transcribed spacers show only a low degree of evolutionary constraint (23). The pattern of conservation within the rRNA sequences appears to be mosaic, alternating highly conserved regions with more variable regions that have no counterpart in the corresponding secondary structure of *Escherichia coli* rRNA and are called "expansion segments" (24). While there is general agreement that the conserved regions within the rRNAs are likely to play an important role in assembly or functioning of the ribosome (or both), the role of the expansion segments and transcribed spacers is still being debated. One view is that these regions harbor elements important for species-specific functions; the other is that they are dispensable, but do not interfere with the proper formation and functioning of the ribosome. However, so far no detailed studies on the role of either the conserved or the variable regions of eucaryotic rRNA have been carried out. To perform such studies, systems for the analysis of ribosomal DNA (rDNA) mutations are required. Very elegant mutational approaches have been developed by Dahlberg (5) for *E. coli* rRNA, but they cannot easily be adapted to eucaryotic organisms. Because of its impressive molecular and genetic facilities, the yeast *Saccharomyces cerevisiae* is an obvious candidate for the application of a mutational approach to the study of the eucaryotic ribosome. The yeast rDNA unit (Fig. 1) is present 100 to 200 times on chromosome XII. From this unit RNA polymerase I transcribes a 37S pre-rRNA molecule that is processed into 17S, 5.8S, and 26S rRNA (19). One major problem for mutational analysis of rDNA is that it is impossible to replace all units by mutated copies, the more so since ribosomes are essential for the viability of the cell. We therefore chose to introduce into yeast cells an extra set of rDNA units that can be subjected to in vitro mutagenesis. To be able to distinguish between (pre-)ribosomes derived from these extra rDNA units and the majority of endogenous (pre-)ribosomes, the former have to be tagged somewhere within the mature rRNA sequence. In this paper, we describe the construction of a fully active, plasmidborne operon which has been tagged within the 26S rRNA. We show that the tag does not perturb the biosynthesis and function of the ribosome. Using this system, we demonstrate that a deletion of the major part of the 17S rRNA gene does not block the formation of mature 60S subunits. On the other hand, we show that deletions of 90 and 678 base pairs (bp), respectively, within the second domain of 26S rRNA completely abolish the accumulation of mature 26S rRNA.

MATERIALS AND METHODS

Enzymes, strains, and plasmids. Restriction enzymes were purchased from Bethesda Research Laboratories, Gaithersburg, Md., and Pharmacia, Uppsala, Sweden. Polynucleotide kinase, *E. coli* DNA polymerase I (Klenow fragment), T4 DNA ligase, T4 DNA polymerase, and *Bal*31 exonuclease were obtained from Bethesda Research Laboratories. DNase RQ1 was from Promega; zymolyase-100T was from Seikagaku Kogyo Co., Tokyo, Japan; and helicase was from Biologique Française, Clichy, France. Oligonucleotides were synthesized with an Applied Biosystems 381A DNA

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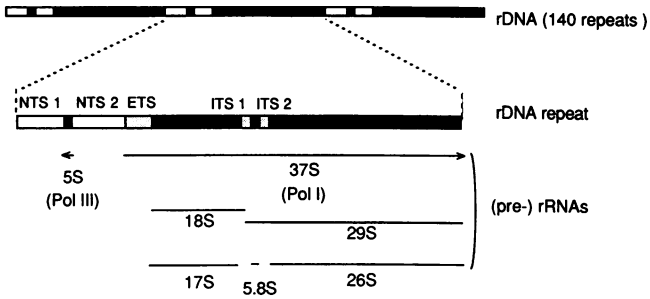

FIG. 1. Genetic organization of yeast ribosomal DNA and simplified scheme of the processing of rRNA. Nontranscribed and external and internal transcribed spacers are indicated by NTS, ETS, and ITS, respectively.

synthesizer, *Pst*I linkers were obtained from Pharmacia, and *Hin*dIII linkers were from Bethesda Research Laboratories.

E. coli DH1 (F⁻ *recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* λ⁻) and JM101 {*supE thi* Δ(*lac-proAB*) [F′ *traD36 proAB lacI*^{q} ZΔM15]} were used for transformation and propagation of plasmid and M13 phage DNAs, respectively. *S. cerevisiae* MG34 (*leu2 trp1 rad2*) and HR2 (α *his4 leu2 trp1*::*URA3*) were used for transformation of pORCS and related plasmids.

Plasmid pMY60 contains a 9.1-kilobase (kb) partial *Hin*dIII fragment of rDNA from *S. carlsbergensis* NCYC74 in pBR322 (J. Meyerink, Ph.D. thesis, Vrije Universiteit, Amsterdam, The Netherlands, 1979) (see Fig. 2).

Construction of a plasmid-contained rDNA unit. pRCS, the plasmid containing a ribosomal centromeric DNA unit of *S. carlsbergensis*, was constructed by inserting one rDNA unit (9.1 kb) plus 600 bp of adjoining sequences in the yeast-*E. coli* shuttle vector YCp7. The inserted rDNA unit was reconstructed from pMY60 in four intermediate cloning steps depicted in Fig. 2A. This was necessary because the rDNA unit in pMY60 is interrupted within the 37S pre-rRNA operon. Step 1 involved the insertion of the *Sma*I-*Hin*dIII fragment (0.7 kb) in the multiple cloning site of pSP65 leading to pML65. In step 2, this insert was extended to the *Xba*I site within the 17S rRNA gene. To that end, a *Hin*dIII linker was ligated into the *Xba*I site of pMY60 and the 0.3-kb *Hin*dIII-*Hin*dIII (ex-*Xba*I) fragment was ligated in the proper orientation into the *Hin*dIII site of pML65, resulting in plasmid pML1. Subsequently, the 7.0-kb *Sac*I-*Sma*I fragment was ligated into pML1, leading to pML2. Finally, pRCS combined the *Bcl*I-*Nsi*I 3.5-kb fragment of pML2 and the 6.2-kb *Nsi*I-*Hin*dIII fragment taken from pMY60. The resulting rDNA fragment was inserted between the *Bam*HI and *Hin*dIII sites in the Tc^r gene of YCp7 and extended from a *Bcl*I site at −328 to a *Hin*dIII site at +269, relative to the 3′ end of the 26S rRNA gene (Fig. 2). The *Sma*I site in YCp7 flanking the *CEN3* fragment was destroyed by the insertion of a *Pst*I linker.

Tagging of the rRNA operon. Two complementary synthetic 18-mers with *Kpn*I-compatible ends were annealed and ligated into the unique *Kpn*I site within the 26S rRNA gene of pRCS. Insertion of the double-stranded oligonucleotide destroyed the *Kpn*I site and introduced a (unique) *Xho*I site (see Fig. 5). Oligonucleotide-containing pRCS was linearized by extensive *Xho*I digestion to remove multiply inserted oligonucleotides and subsequently purified by gel electrophoresis and electroelution. After recircularization, the plasmid was again transformed into *E. coli* DH1. The resulting plasmid with the oligonucleotide-containing rRNA operon was named pORCS (oligonucleotide-tagged ribosomal centromeric rDNA unit of *S. carlsbergensis*). The orientation of the oligonucleotide was assessed by Northern (RNA) blot hybridization of the rRNA with the separate single-stranded 18-mers.

Construction of deletion mutants. (i) pORCSΔp: deletion of the promoter for RNA polymerase I. A promoter deletion was constructed in the rDNA subclone pML1 (Fig. 2A). A 50-μg amount of pML1 DNA was linearized by 1.6×10^{-3} U of DNase RQ1 in the presence of 0.5 mM Mn^{2+} essentially as described previously (11). This method produces double-stranded breaks in a random distribution. After gel electrophoresis, the linearized plasmid molecules were excised, electroeluted, and incubated for 30 min at 25°C with 1.5 U of *E. coli* DNA polymerase I (Klenow fragment) to create blunt ends. Then the plasmid was recircularized in the presence of a *Bam*HI linker (kindly provided by J. H. van Boom, Leiden, The Netherlands) and transformed to *E. coli* DH1. From the resulting collection of randomly inserted *Bam*HI linkers in the *Sma*I-*Xba*I fragment of the rDNA (Fig. 2A), a recombinant was chosen with the *Bam*HI linker at position −190 relative to the transcriptional start. This plasmid, pML[Bam −190] was digested with *Bam*HI and *Bgl*II (position +128 relative to the site of transcription initiation). The large fragment was purified by gel electrophoresis and electroelution and recircularized by ligation. From the resulting plasmid, pMLΔp, the *Sma*I-*Nsi*I 640-bp fragment (Fig. 2) containing the deleted promoter was used to replace the analogous fragment from pORCS, resulting in plasmid pORCSΔp. The size of the deletion was confirmed by restriction enzyme mapping.

(ii) pORES: deletion of *CEN3*. pORCS was partially digested with the restriction enzyme *Pst*I. The 15.3-kb partial *Pst*I fragment was purified by gel electrophoresis and electroelution and recircularized by ligation, resulting in plasmid pORES.

(iii) pORCSΔ17: deletion in the 17S rRNA gene. pORCS was digested with *Sac*I and *Nsi*I, and the 16-kb fragment was purified by gel electrophoresis and electroelution. The 3′ protruding ends of this fragment were removed by T4 DNA polymerase treatment (15), and the fragment was recircularized by ligation. The position and size of the deletion (1,195 bp) were confirmed by mapping a set of flanking restriction sites.

(iv) pORCSΔ26-1 and pORCSΔ26-2: deletions in the 26S rRNA gene. A 25-μg portion of pORCS DNA was linearized by digestion with the restriction enzyme *Mlu*I and subsequently treated with 0.5 U of *Bal*31 exonuclease at 37°C in 20 mM Tris hydrochloride, pH 8.1, containing 12 mM $CaCl_2$, 12 mM $MgCl_2$, 600 mM NaCl, and 1 mM EDTA for an appropriate time (sampling between 5 and 30 min). The shortened fragments were recircularized with T4 DNA ligase and transformed to *E. coli* DH1. The size of the deletion was determined for two mutants, pORCSΔ26-1 (90 bp) and pORCSΔ26-2 (678 bp), by sequencing by the method of Maxam and Gilbert (16).

Isolation of polysomes from *S. cerevisiae*. Transformed yeast cells were grown in 600 ml of selective medium (1 liter contains 20 g of glucose · H_2O and 6.7 g of yeast nitrogen base without amino acids, supplemented with the additional requirements of the strain). At a cell density of 2×10^7 to 3×10^7 cells per ml (A_{550} of 0.4), the culture was given a nutritional shiftup by addition of 0.25 volume of fivefold-concentrated rich medium (rich medium contains, per liter, 11 g of glucose · H_2O, 3 g of yeast extract [Difco Laboratories, Detroit, Mich.], 3 g of malt extract [Difco], and 5 g of

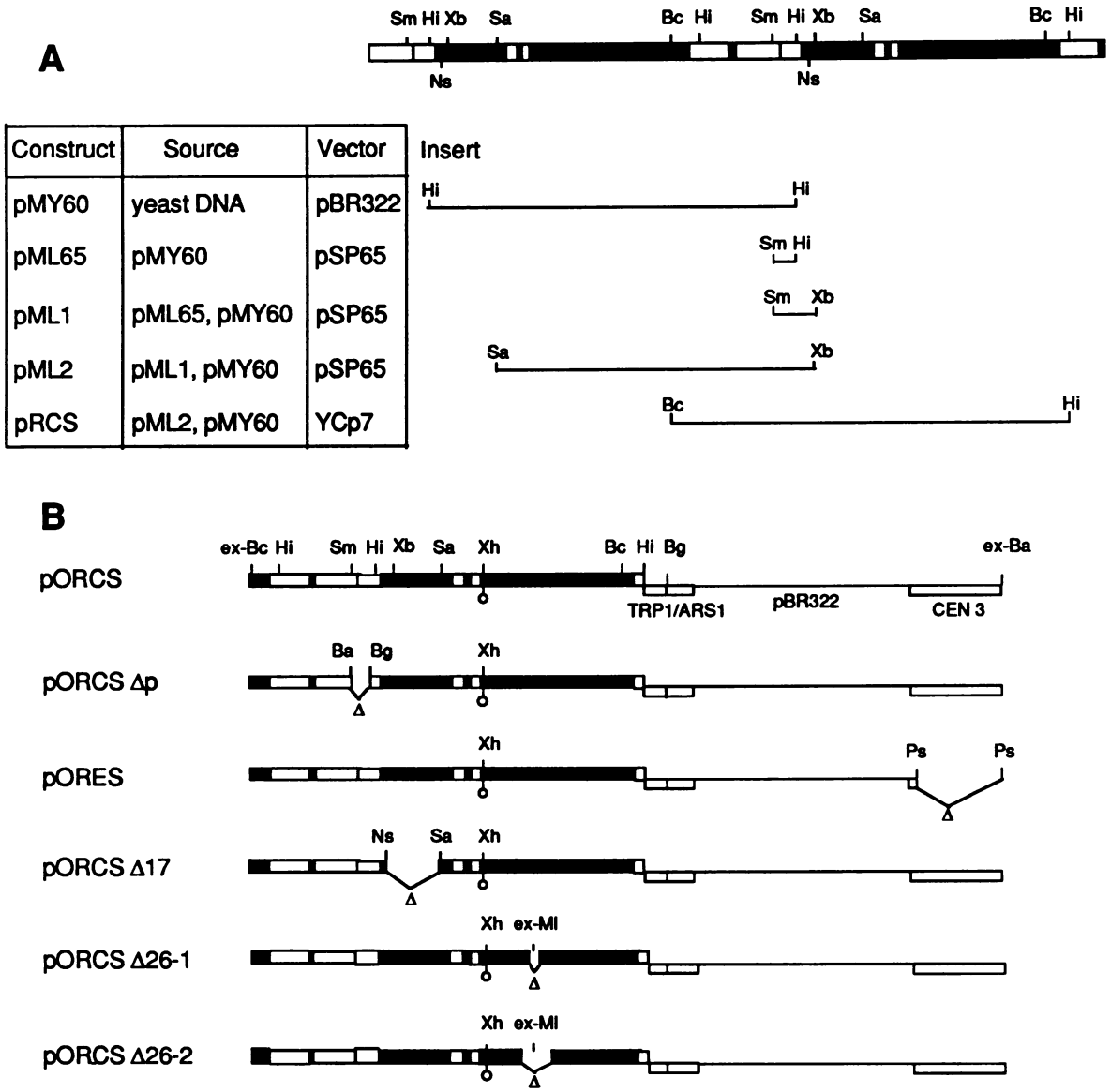

FIG. 2. Construction of pRCS (A) and maps of the plasmids derived from its oligonucleotide-containing variant pORCS containing specific deletions (B). Panel A shows two tandemly repeated rDNA units aligned with the rDNA inserts of the various recombinants used for the reconstruction of the uninterrupted rDNA insert of pRCS from a single, interrupted one in pMY60. (B) The tag in pORCS is represented by (○), and the various deletions are shown by (△). Black bars represent the genes for the mature rRNAs, and white bars represent transcribed and nontranscribed spacers (cf. Fig. 1). Abbreviations used for restriction enzyme sites are as follows: Sm, *Sma*I; Hi, *Hin*dIII; Ns, *Nsi*I; Xb, *Xba*I; Sa, *Sac*I; Bc, *Bcl*I; Xh, *Xho*I; Bg, *Bgl*II; Ba, *Bam*HI; Ps, *Pst*I; Ml, *Mlu*I.

neutralized peptone [Oxoid Ltd., London, U.K.]). The culture was grown for one more generation time. Cycloheximide was then added to a final concentration of 0.1 g/liter, and incubation was continued for 30 min. The cells were harvested, washed once with STB (10 mM Tris hydrochloride, pH 6.5, 60 mM KCl, 10 mM magnesium acetate, 0.05 g of polyvinyl sulfate per liter, 1 mM dithiothreitol, 0.36 mM cycloheximide, 5.7 mM spermine), and finally suspended in 3 ml of STB to which 70 U of RNAsin (Promega) had been added. The cells were shaken with 10 g of glass beads (diameter, 0.45 to 0.52 mm) in a Braun shaker for two 45-s periods. The homogenate was centrifuged twice for 15 min each time in an Eppendorf centrifuge, and the supernatant was layered onto a 10 to 40% (wt/vol) sucrose gradient and centrifuged in an SW28 rotor (Beckman Instruments, Inc., Fullerton, Calif.) for 16 h at 12,000 rpm. Fractions were pooled and precipitated with ethanol, and the RNA was isolated by phenol and phenol-chloroform extractions.

Labeling of probes. (i) Oligonucleotide. A 50-ng portion of the single-stranded oligonucleotide (18-mer) was labeled, using 5 U of polynucleotide kinase and 70 μCi of [γ-^{32}P]ATP (Radiochemical Centre, Amersham, U.K.). The reaction was stopped by the addition of 30 mM EDTA–1 M NH_4 acetate, and the mixture was extracted with phenol-chloroform. Free label was removed by using the spin column procedure (15). Specific activities of the oligonucleotide ranged from 1×10^6 to 5×10^6 cpm/pmol.

(ii) pBR322. A 200-ng amount of pBR322 DNA was labeled with 10 μCi of [α-^{32}P]dATP (Radiochemical Centre) and a nick translation kit (Radiochemical Centre). The reaction

mixture was extracted with phenol-chloroform, and free label was removed by NH_4 acetate precipitation. The specific activity of the probe was in the order of 40×10^6 cpm/pmol.

(iii) M13-TRP1. The *TRP1*-specific probe was obtained by subcloning the *Cla*I-*Bgl*II fragment of *TRP1* from YCp7 in M13mp10. A 50-ng portion of single-stranded DNA was annealed with a pentadecamer (a gift from J. H. van Boom) as a primer for Klenow DNA polymerase. The reaction was carried out for 15 min at 25°C in the presence of 10 μCi of [α-^{32}P]dATP followed by a 15-min chase. The mixture was extracted with phenol-chloroform, and free label was removed by NH_4 acetate precipitation. Specific activities ranged from 50×10^6 to 100×10^6 cpm/pmol.

Miscellaneous techniques. Yeast transformation was performed by the method of Beggs (2) with minor modifications. Total DNA was isolated from transformed yeast cells, essentially according to reference 18. RNA from transformed yeast cells was isolated by the method of Kraig et al. (13) with minor modifications. All blotting techniques were performed as described in reference 15, except that Hybond-N (Radiochemical Centre) was used instead of nitrocellulose. Southern blots were hybridized at 65°C in 6× SSC (1× SSC is 0.15 M NaCl plus 0.15 M sodium citrate)–0.5% sodium dodecyl sulfate–5× Denhardt solution–1 mM EDTA–0.1% sodium pyrophosphate–100 μg of denatured herring sperm DNA per ml. Blots were washed once in 2× SSC–0.1% sodium dodecyl sulfate–1× Denhardt solution–1 mM EDTA–0.1% sodium pyrophosphate for 1 h at 65°C, followed by two washes in similar solutions, but now containing 1× SSC and 0.1× SSC, respectively. Northern blots were hybridized at 48°C in the same solution used for Southern blots. They were washed twice for 1 h at 48°C in 2× SSC in the same solution used for washing the Southern blots.

RESULTS

Our design for a system to analyze yeast rDNA mutations consists of one or more stably maintained extrachromosomal copies of an active ribosomal transcription unit producing rRNA that is distinguishable from the bulk of endogeneous rRNA by virtue of a specific oligonucleotide sequence within the 26S rRNA. Figure 2A shows how such a transcriptionally active rDNA unit was reconstructed from a plasmid (pMY60) containing one complete 9.1-kb yeast rDNA repeating unit, which, however, is interrupted within the transcription unit. To ensure maximum transcription, we have preserved both the upstream and the downstream copies of the rDNA enhancer in our construct since it is not yet known whether the enhancer exerts its influence upon the downstream or the upstream transcription unit (6, 7). The reconstructed rDNA unit, having a 600-bp direct repeat at both ends, was cloned in the *CEN3*-containing yeast vector YCp7 and called pRCS. After insertion of the oligonucleotide tag (see below), the resulting plasmid was designated pORCS.

The presence of a centromere sequence is essential to select against integration of the plasmid, which may lead to physical separation of the tag from the mutation to be studied. Southern analysis of DNA from yeast cells transformed with either pORCS (Fig. 3, lanes 2 to 5) or a plasmid similar to pORCS, but having its centromere deleted (pORES; Fig. 3, lanes 6 and 7), clearly showed that only the centromere-containing pORCS is maintained extrachromosomally. The reason for this is that the integration of pORCS leads to a dicentric chromosome, which is highly detrimental to the viability of the cells (9).

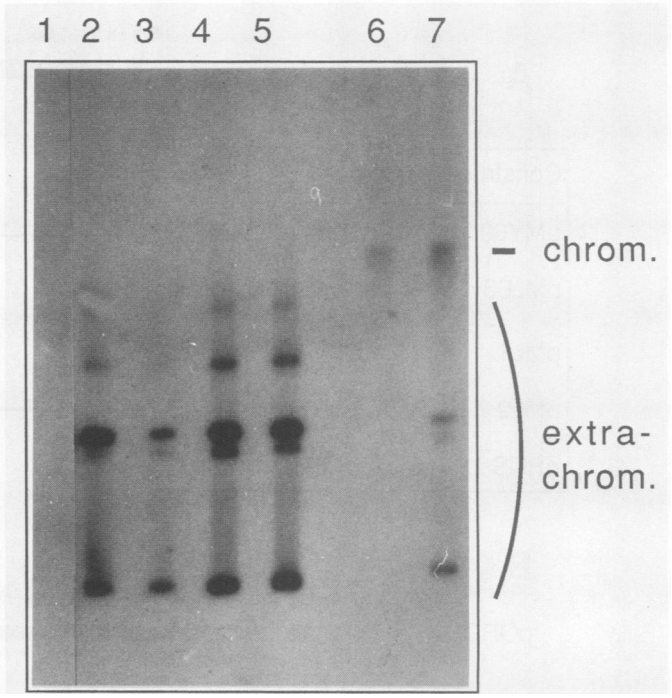

FIG. 3. Effect of *CEN* sequence on extrachromosomal maintenance of pORCS plasmids. Total undigested DNA isolated from the host *S. cerevisiae* MG34 (lane 1), from four independent pORCS transformants (lanes 2 to 5), and from two independent pORES transformants carrying a deletion of the *CEN3* sequence (lanes 6 and 7) were fractionated by electrophoresis in a 0.3% agarose gel, blotted onto Hybond N, and hybridized with nick-translated pBR322.

In addition to having a high mitotic stability, *CEN*-containing plasmids are generally present in 1 or 2 copies per cell. Surprisingly, the copy number of pORCS and its derivatives varied over a much broader range. Figure 4A shows a Southern blot of DNA isolated from a number of different pORCS transformants obtained in a single transformation experiment. The hybridization signal of the single-copy chromosomal *TRP1* gene is used as a reference. Comparison of this signal with the plasmid-derived *TRP1* hybridization signals led to the conclusion that the pORCS copy number ranges from 1 or 2 ("low" copy number: lanes 2, 5, and 6) to about 10 ("high" copy number: lanes 1, 3, and 4). Analysis of the isolated plasmids showed that low- and high-copy transformants contain identical plasmids (data not shown). Apparently in the MG34 host, pORCS and related plasmids are able to escape the mechanism that normally keeps the number of *CEN*-containing plasmids at 1 or 2 (see Discussion). Although the high-copy-number transformants would facilitate the quantification of the effect of specific mutations on the accumulation of tagged 26S rRNA, the observed variation in copy number among the different pORCS transformants made it necessary to ascertain whether the copy number of a given transformant remains constant. Figure 4B shows that there is no detectable change in the copy number exhibited by either a high- or a low-copy pORCS transformant when it is grown for 30 generations under nonselective conditions.

Construction of a tagged 26S rRNA gene. The insertion of a tag in the mature rRNA should not interfere with the processes we want to study. Therefore, an expansion segment was chosen as the site of insertion for the tag because,

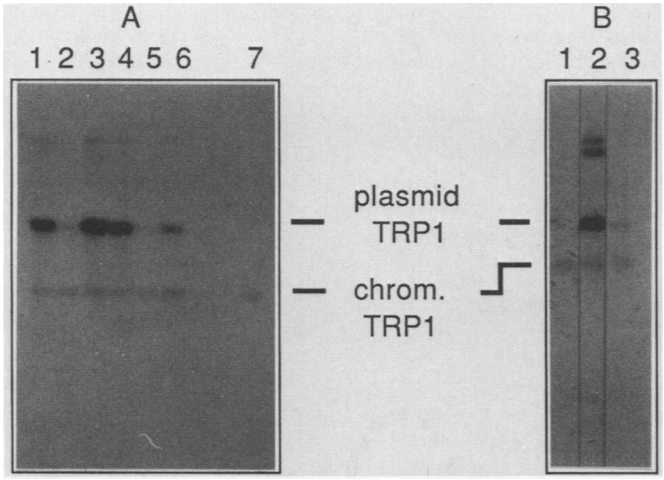

FIG. 4. Copy number analysis and mitotic stability of pORCS in yeast cells. (A) *Bgl*II-digested total DNA isolated from the host *S. cerevisiae* MG34 (lane 7) and from six independent pORCS transformants (lanes 1 to 6) was fractionated on a 1% agarose gel, blotted onto Hybond N, and hybridized with a probe specific for the *TRP1* gene (M13-TRP). (B) *Bgl*II-digested total DNA isolated from a high-copy-number pORCS transformant (lane 2, about 10 copies; cf. panel A, lane 3) and a low-copy-number transformant (lane 3, about 1 copy; cf. panel A, lane 2) after growth in batch culture for 30 generations in minimal medium supplemented with tryptophan (60 mg/liter) and leucine (400 mg/liter). MG34 DNA is shown in lane 1. Bands visible in the upper part of the gel are partial digestion products.

as compared with procaryotic rDNA, expansion segments can be viewed as natural insertions which in a number of cases vary in length and sequence among different eucaryotes (8). As the target site for our tag (an 18-bp synthetic oligonucleotide; Fig. 5B), we chose the first expansion segment in domain I of the 26S rRNA gene (Fig. 5A). With the aid of a computer, the 18-bp oligonucleotide tag was designed in such a way that it differs as much as possible from the yeast rDNA sequence, given the prerequisites that it contains an *Xho*I site (to remove multiple copies of the tag) and disturbs the proposed secondary structure of the 26S rRNA at the site of insertion (22) as little as possible (Fig. 5B).

Tagged 26S rRNA is easily detectable and can be quantitated relative to the amount of total 26S rRNA. Total RNA from pORCS transformants was analyzed by Northern hybridization, using the ^{32}P-labeled oligonucleotide as a probe (Fig. 6). In high-copy pORCS transformants, a strong hybridization signal is found at a position corresponding to that of normal 26S rRNA. In addition, in high-copy pORCS transformants, hybridizing RNAs are seen at roughly the position of 17S rRNA, which are apparently degradation products of 26S rRNA. Upon longer exposure, additional hybridizing transcripts were found (see Fig. 10, lane 1, for a clear example), one of which runs at the position of 37S pre-rRNA, suggesting that the tagged 26S rRNA is derived from a regular RNA polymerase I transcript and not from a fortuitous transcript from the mutated transcription unit by another RNA polymerase, e.g., polymerase II. Additional evidence that the tagged RNA has been transcribed by polymerase I, initiating at the proper initiation site, stems from the analysis of yeast cells containing a pORCS plasmid from which a fragment known to harbor the polymerase I promoter (12) has been deleted (pORCSΔp; Fig. 2B). RNA isolated from these transformants shows only negligible amounts of tagged 26S rRNA (Fig. 6, lanes 6 and 7), indicating that Polymerase I is indeed responsible for the production of virtually all of the tagged transcripts. These observations imply that the insertion of the oligonucleotide per se does not interfere with either the transcription of 37S pre-RNA or its processing into 26S RNA.

In an attempt to measure the proportion of tagged 26S rRNA in the cell, we visualized both tagged and untagged RNA species in one single experiment. Primer extension starting from an oligonucleotide complementary to 26S rRNA sequences located just downstream of the site of insertion of the tag resulted in a major 154-nucleotide-long extension product derived from the wild-type cellular 26S rRNA, as well as an extension product that was 18 nucleotides longer, derived from the pORCS plasmid. The latter product was observed only in transformed cells (Fig. 7, lanes 1 and 2). Assuming that the primer hybridizes equally well with the tagged and the wild-type 26S rRNA and that the extension reaction proceeds equally efficiently in both cases, the relative intensities of the final extension products in Fig. 7 indicated that, in a high-copy pORCS transformant, the amount of tagged 26S rRNA is roughly 0.2% of the total amount of 26S rRNA.

Tagging the 26S rRNA gene does not interfere with its participation in the formation of translationally active ribosomes. None of the steps along the pathway leading to a complete translationally active ribosome was hampered by the presence of the tag in 26S rRNA (Fig. 8). Figure 8 shows a Northern blotting experiment of RNA isolated from polysomal fractions of yeast cells transformed with pORCS. A 26S rRNA signal due to hybridization with the oligonucleotide probe was found in monosomes (lane 2) as well as in disomes, trisomes, and polysomes (lanes 3, 4, and 6, respectively). The same blot, but now probed for total 26S rRNA (Fig. 8, bottom panel), demonstrates that the ratio of tagged to total 26S rRNA was roughly the same in all fractions. Furthermore, the polysome profile was not significantly altered in the pORCS transformant. Therefore, we can conclude that the tagged 26S rRNA is correctly assembled into (pre-)ribosomal particles which are transported out of the nucleus into the cytoplasm, where they can bind to mRNA molecules. Ribosomes deficient in elongation and translocation will block movement of other ribosomes. The presence of such functionally impaired ribosomes is thus expected to lead to a lower ratio of tagged/total 26S rRNA in the fractions containing the higher polysomes. Since tagged 26S rRNA is present in higher polysomes in the same proportion as in 80S monosomes, we conclude that the tagged ribosomes are not detectably deficient in their initiation and elongation properties.

An intact 17S rRNA gene is not required for correct processing of 26S rRNA. Having established that the oligonucleotide insertion into the 26S rRNA gene does not interfere with the production of functional ribosomes, we wanted to show that the pORCS system is indeed capable of revealing *cis*-acting elements within the rDNA unit that might be involved in the different steps of ribosome biogenesis. First, we investigated whether there is an interdependence between the assembly of the 17S and 26S rRNA sequences into functional ribosomes. In other words, does the formation of a 60S subunit require in *cis* any of the steps leading to the assembly of a 40S subunit? To answer that question, we deleted a large part (66%) of the 17S rRNA coding region (Fig. 2B); the resulting plasmid was designated pORCSΔ17. RNA isolated from low-copy-number pORCSΔ17 transformants gave rise to a hybridization signal

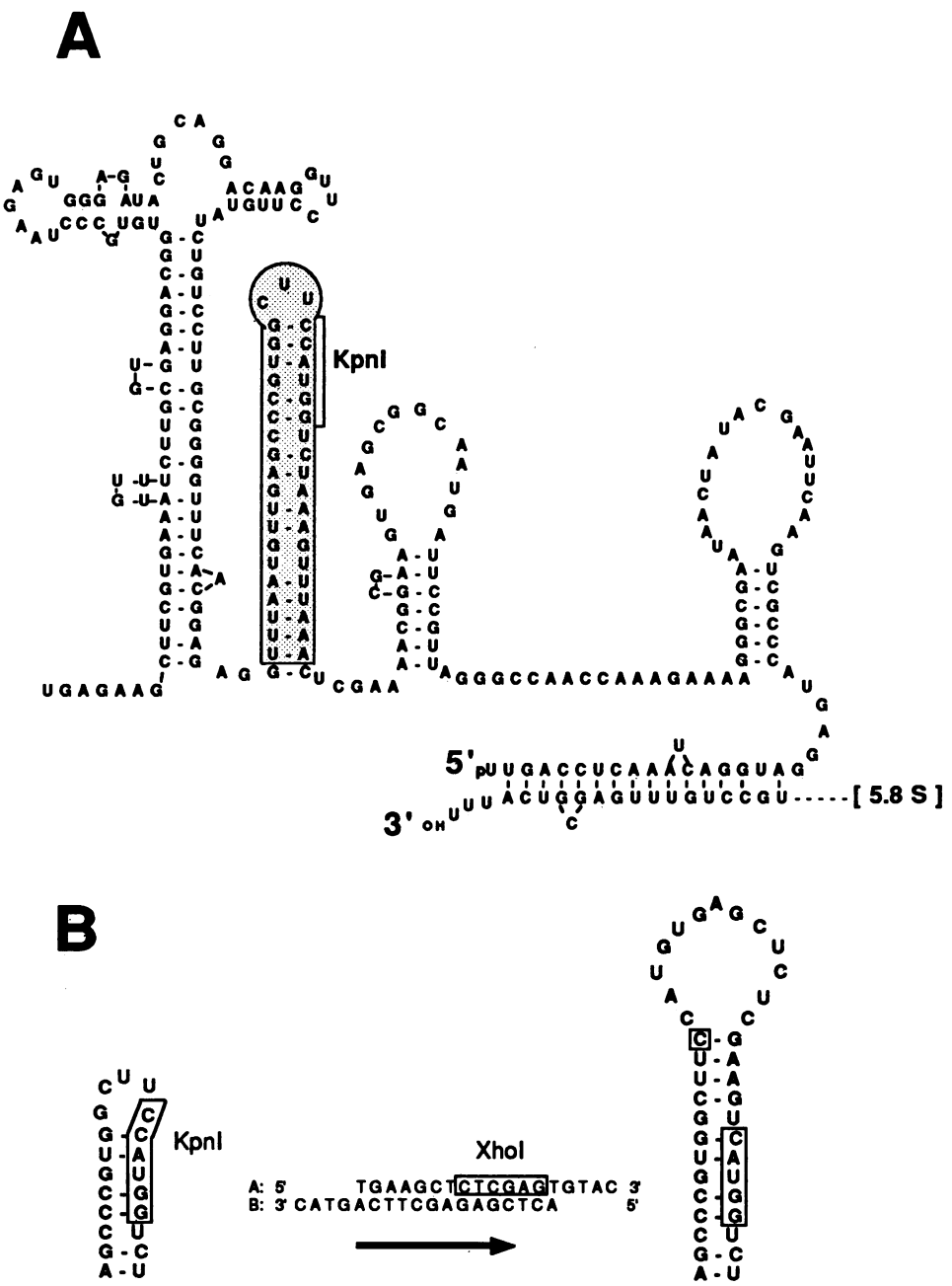

FIG. 5. Site of insertion of the 26S rRNA tag. (A) Proposed secondary structure of domain I of yeast 26S rRNA (22). The expansion segment is shaded and the *Kpn*I site is indicated. (B) Structural change of the expansion segment caused by insertion of the oligonucleotide tag into the *Kpn*I site.

at the position of 26S rRNA (Fig. 6, lanes 8 and 9), indicating that correctly processed 26S rRNA was produced from the tagged precursor rRNA carrying the 17S rRNA deletion. Comparison of the yield of tagged 26S rRNA with that in a low-copy-number pORCS transformant (lane 3) showed that the two operons produce the same amount of 26S rRNA. This demonstrates that the presence of an intact 17S rRNA gene within the same precursor is not required for the correct processing of 37S pre-rRNA into 26S rRNA. To estimate whether the 17S rRNA deletion has any effect on the assembly of the large subunit and its transport into the cytoplasm, we analyzed the RNA isolated from the polysomal fractions of a pORCSΔ17 transformant (Fig. 9). Again, tagged RNA was found in all polysomal fractions. Upon comparison with panel B of Fig. 9, which shows the ethidium bromide-stained gel prior to blotting, we can conclude that tagged 26S rRNA produced from a precursor carrying a deletion in the 17S rRNA gene still ends up in translationally active 60S ribosomal subunits.

Deletions within the 26S rRNA coding region block the production of 26S rRNA but not of 37S pre-rRNA and 29S pre-rRNA. Next, we analyzed two arbitrarily chosen deletions within conserved regions of domain 2 of 26S rRNA to see whether such deletions would cause the accumulation of

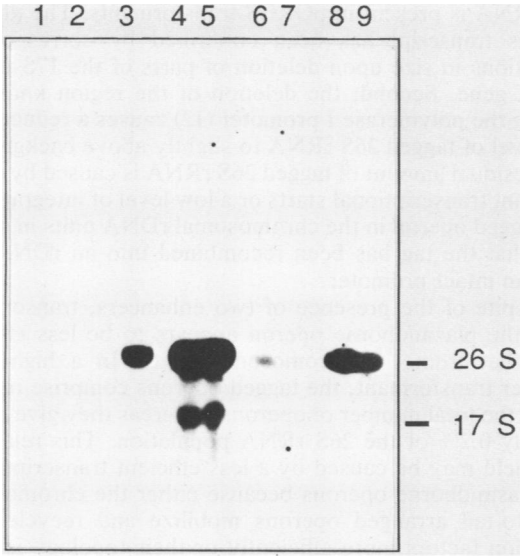

FIG. 6. Identification of tagged 26S rRNA. Total RNA (20 μg) from wild-type MG34 and various transformants was fractionated on a 1% agarose gel, blotted onto Hybond N, and hybridized with the ^{32}P-labeled oligonucleotide complementary to the tag in 26S rRNA. The signal at 17S rRNA was due to trapping of a low amount of 26S rRNA degradation products at this position. Lane 1, MG34; lane 2, pRCS; lane 3, pORCS (copy number, 1 to 2); lanes 4 and 5, pORCS (copy number, 8 to 10), lanes 6 and 7: pORCSΔp (copy number, 8 to 10); lanes 8 and 9, pORCSΔ17 (copy number, 1 to 2). Independent transformants were used in all duplicate experiments.

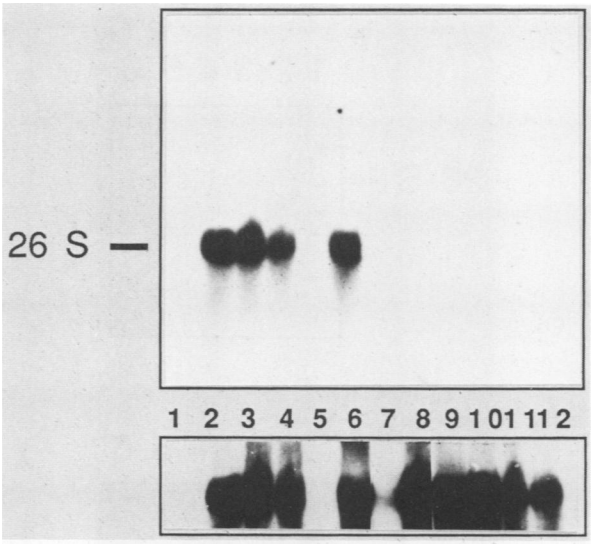

FIG. 8. Distribution of tagged 26S rRNA in polysomal fractions of a pORCS transformant. Polysomes were isolated from a pORCS (lanes 1 to 6) and a pRCS (lanes 7 to 12) transformant and fractionated by sucrose gradient centrifugation. RNA was isolated from the polysomal fractions, fractionated by electrophoresis on a 1% agarose gel, blotted, and hybridized with the oligonucleotide tag. (Upper panel) Lanes 1 and 7, top fraction of the gradient; lanes 2 and 8, 80S monosomes; lanes 3 and 9, disomes; lanes 4 and 10, trisomes; lanes 5 and 11, tetrasomes; lanes 6 and 12, penta- and higher polysomes. (Lower panel) The same blot after washing and reprobing with nick-translated pMY60, showing the total 26S rRNA in all lanes; the sample in lane 5 was lost before loading.

deficient ribosomal particles in the pORCS system. Northern analysis of RNA produced by these mutants shows that both a small 90-bp (pORCSΔ26-1) and a large 678-bp (pORCSΔ26-2) deletion had a similar effect: neither transformant contained a detectable amount of fully processed, tagged 26S rRNA (Fig. 10, lanes 2 and 3). However, the mutated operon was still transcribed, since we can clearly see tagged RNA migrating at the position of 37S pre-rRNA (lane 2). The size of the 37S pre-rRNA was proportionally reduced in the pORCSΔ26-2 transformant (lane 3). The presence of either deletion apparently does not interfere with the processing of the mutant 37S pre-rRNA into the corresponding 29S pre-rRNA, since this latter precursor could be clearly distinguished (Fig. 10, lane 2). In the case of the unmutated pORCS (lane 1), the 29S pre-rRNA band was obscured by the strong hybridization of the 26S rRNA. In the pORCSΔ26-2 mutant (lane 3), the shortened 29S pre-rRNA comigrated with the untagged 26S rRNA. These deletions seemed to inhibit some step of the rRNA maturation following the appearance of 29S pre-rRNA. However, no significant accumulation of deficient ribosomal particles could be observed, probably due to their rapid degradation.

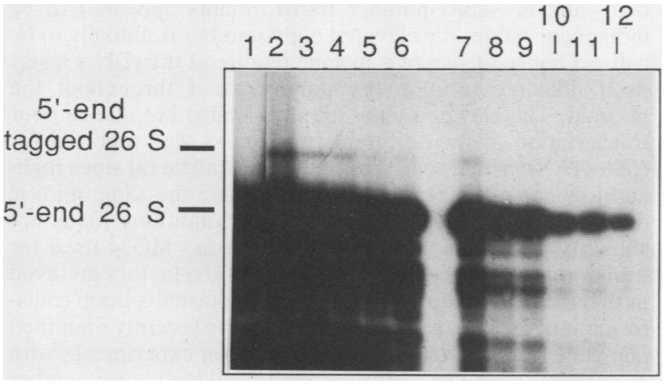

FIG. 7. Quantification of tagged 26S rRNA. A 19-mer, complementary to positions +136 to +154 of 26S rRNA (22), was annealed to total RNA isolated from MG34 and various transformants and extended by reverse transcriptase. Lane 1, MG34; 2 to 12, pORCS (8 to 10 copies per cell). Lanes 3 to 6 are extensions in the presence of ddGTP, ddATP, ddTTP, and ddCTP, respectively, mainly to show the band representing the 5′ end of the tagged 26S rRNA. In lanes 7 to 12, 1.6-, 6-, 10-, 20-, 50-, and 100-fold dilutions of the sample in lane 2 were applied.

DISCUSSION

System for analyzing rDNA mutations. We have described the first experimental system that allows mutational analysis of a eucaryotic rDNA unit. The system uses tagging of the pre-rRNA operon to monitor the fate of its transcripts after in vitro mutagenesis and subsequent transformation into yeast cells. For obvious reasons, we wanted to insert the tag within the regions coding for 26S or 17S rRNA in such a way that it behaves like a silent mutation with respect to processing, assembly, transport, and function of the ribosomal particle. We have demonstrated that the insertion of an 18-mer in the first expansion segment of 26S rRNA meets these demands since the resulting tagged 60S subunits are homogeneously distributed among all polysomal fractions (Fig. 8). Moreover, this observation supports the notion that the precise structure of this expansion segment is not crucial for ribosome biogenesis or function, although an effect on the kinetics of the maturation process cannot be ruled out.

To direct the transcription of plasmid-encoded 37S pre-rRNA, we have cloned a full yeast rDNA repeating unit of

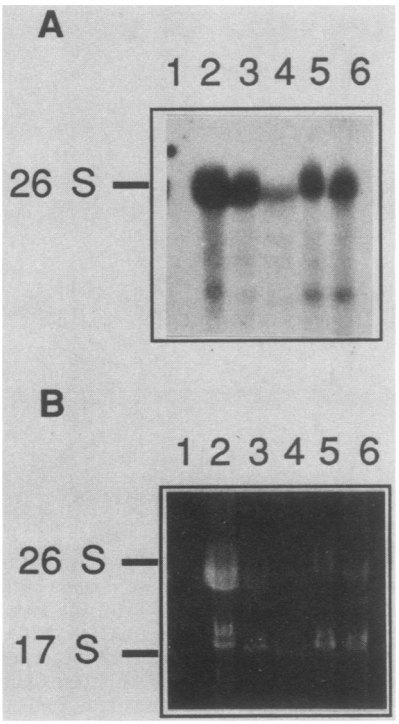

FIG. 9. Distribution of tagged 26S rRNA in polysomal fractions of pORCSΔ17. (A) Polysomes of a pORCSΔ17 transformant were isolated and fractionated on a sucrose gradient. RNA from the top fraction (lane 1), 80S monosomes (lane 2), disomes (lane 3), trisomes (lane 4), tetrasomes (lane 5), and higher polysomes (lane 6) was analyzed as described in the legend to Fig. 8. (B) Ethidium bromide-stained 26S and 17S rRNA pattern of the same gel used in panel A, prior to blotting.

9.1 kb plus 0.6 kb of adjoining sequences. In this way we ensured that both the upstream and the downstream enhancer regions were included, in order to have optimal transcription by polymerase I. Evidence that the plasmid-borne 37S pre-rRNA operon is indeed transcribed by polymerase I comes first from the observation that tagged 37S

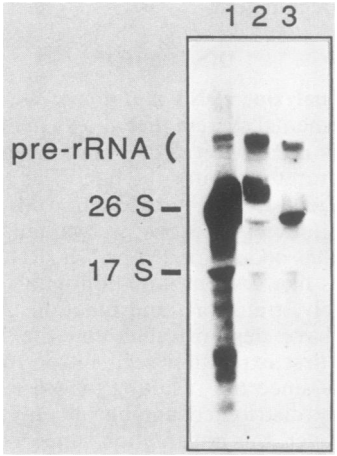

FIG. 10. Tagged transcripts from pORCS containing deletions in the 26S rRNA gene. Northern blot hybridization of RNA from pORCS (lane 1), pORCSΔ26-1 (lane 2), and pORCSΔ26-2 (lane 3) was performed as described in the legend to Fig. 6. The autoradiograph was overexposed to visualize the tagged rRNA precursors.

pre-rRNA is present in pORCS transformants. The identity of this transcript has been confirmed by corresponding reductions in size upon deletion of parts of the 17S or 26S rRNA gene. Second, the deletion of the region known to harbor the polymerase I promoter (12) causes a reduction in the level of tagged 26S rRNA to slightly above background. The residual amount of tagged 26S rRNA is caused by either aberrant transcriptional starts or a low level of integration of the tagged operon in the chromosomal rDNA units in such a way that the tag has been recombined into an rDNA unit with an intact promoter.

In spite of the presence of two enhancers, transcription from the plasmidborne operon appears to be less efficient than that from the chromosomal units. In a high-copy-number transformant, the tagged operons comprise roughly 5% of the total number of operons, whereas they give rise to roughly 0.2% of the 26S rRNA population. This relatively low yield may be caused by a less efficient transcription of the plasmidborne operons because either the chromosomal head-to-tail arranged operons mobilize and recycle transcription factors more efficiently or their topology is more favorable (20). The impaired transcription might also be due to localization of (a number of) the pORCS plasmids outside the nucleolus. An alternative possibility that cannot be ruled out is an increased turnover of the tagged 26S rRNA.

The inclusion of a centromere-bearing fragment appeared to be essential for obtaining genetically well-characterized and stable transformants. Without the *CEN* fragment we observe integration in the rDNA locus at a high frequency, resulting in loss of the extrachromosomal plasmids. Such integration is highly undesirable since it might uncouple the tag from the mutation under study. The *CEN* fragment provides a strong selection against integration since dicentric chromosomes are unstable in yeast cells (9).

Surprisingly, we found pORCS transformants of *S. cerevisiae* MG34 with copy numbers ranging from 1 or 2 to about 10 (Fig. 4). The reason for this variation remains unclear. Since both high- and low-copy transformants appeared to be mitotically stable, this situation is different from the inactivation of the *CEN* function upon deliberate transcription through the centromere by RNA polymerase II. Such transcription is known to result in a high copy number but also in mitotic instability of the plasmid (4). Since plasmids from high- and low-copy-number transformants appeared to be indistinguishable, the elevated copy number is unlikely to be a direct consequence of a specific feature of the rDNA insert itself, like transcription by polymerase I throughout the plasmid. The elevated copy number is also not due to poor transcription of the selectable marker as was reported for *CEN-ARS* plasmids carrying the *his3-Δ4* allele (3) since high- and low-copy-number transformants have the same mitotic division rate under selective growth conditions (data not shown). We rather think that *S. cerevisiae* MG34 used for transformation is less stringent in one of the factors involved in the accommodation of *CEN*-bearing plasmids upon transformation, for instance, the product of the recently identified *cop* gene (21). Preliminary transformation experiments with *S. cerevisiae* HR2 as a host did not reveal high-copy-number transformants with pORCS plasmids.

Applications of the system. The pORCS system has a high potential for unraveling the role of *cis*-acting elements within the rDNA unit in the maturation of eucaryotic ribosomes and for examining relationships among rRNA processing, subunit assembly, and the interaction of rRNA and ribosomal proteins. First, we have deleted two-thirds of the 17S rRNA gene to resolve whether there is any interdependence in the

assembly of eucaryotic 40S and 60S subunits. Such an interdependence has not been observed in procaryotes, in which processing of the transcript into individual pre-rRNA species takes place even before transcription of the rRNA is completed. It might, however, exist in yeast cells, in which the 37S pre-rRNA is first assembled into a 90S preribosomal particle, which is the common precursor of both subunits. Abovitch et al. (1) and Nam and Fried (17) have shown that the stoichiometric production of the two subunits can be disturbed upon a controlled decrease in the synthesis of specific yeast ribosomal proteins. Our experiments demonstrate that the formation of an intact 60S subunit does not depend on the simultaneous assembly of a 40S subunit from the same pre-rRNA. However, since domain 4 and most of domain 3 of the 17S rRNA gene are still present in pORCSΔ17, we cannot fully exclude the possibility that these sequences are involved in some critical primary step in the assembly of the mutilated 17S rRNA, indispensable for correct assembly of the 60S subunit. Therefore, final proof will have to be obtained from a deletion of the complete 17S rRNA gene, including its processing sites. The construction of such a mutant, however, has to await the identification of the *cis*-acting elements required for the processing of 17S, 5.8S, and 26S rRNAs.

Two arbitrarily chosen deletions were created in conserved parts of domain 2 of 26S rRNA to explore the fate of putative deficient ribosomal particles. Both mutants produced easily detectable, and probably somewhat enhanced levels of, 37S and 29S pre-rRNAs as compared with pORCS (Fig. 2B and 10), but they did not accumulate mature tagged 26S rRNA. At some stage after the formation of 29S pre-rRNA, the tagged RNA is apparently subjected to degradation. We infer that this degradation is evoked by a severe defect in the assembly of the 60S subunit. This suggests that the deleted sequences are directly or indirectly involved in the formation of stable and specific rRNA–r-protein complexes. More subtle mutations will now be generated to try to accumulate assembly intermediates or ribosomal subunits deficient in some step of the translation process.

In conclusion, we have developed a system for introducing mutations into a plasmid-encoded rDNA unit and for studying the expression and fate of the mutant rRNAs in vivo. Further mutations are now under study that will give new insights into the function of transcribed spacers and expansion segments and into the functional conservation of specific rRNA domains.

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