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The conserved GTPase center and variable region V9 from *Saccharomyces cerevisiae* 26S rRNA can be replaced by their equivalents from other prokaryotes or eukaryotes without detectable loss of ribosomal function

(evolution/protein–RNA interaction/expansion segment)

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ABSTRACT **Using the "tagged" rRNA gene system, which allows *in vivo* mutational analysis of *Saccharomyces cerevisiae* rRNA, we studied the role of two distinct structural elements of 26S rRNA in ribosome biogenesis and function—namely, the evolutionarily highly conserved "GTPase center" located in domain II and the eukaryote-specific variable region V9 in domain III. Replacement of the *S. cerevisiae* GTPase center with its counterpart from *Escherichia coli* did not affect the assembly of the mutant 26S rRNA into functional (as judged by their polysomal distribution) 60S subunits, indicating that the *E. coli* GTPase center functions efficiently in the context of the heterologous rRNA. Removal of most of the *S. cerevisiae* V9 region or replacement of this segment by the equivalent segment from mouse 28S rRNA also did not affect the formation of functional 60S subunits carrying the mutant 26S rRNA. Therefore, the V9 region does not seem to play a role in the biological functioning of the yeast 60S subunits, and these subunits appear to be able to accommodate V9 regions of various size and secondary structure without apparent loss of function.**

Over the past few years considerable evidence has accumulated substantiating the notion that rRNAs do not serve merely as an inert scaffold for the correct spatial assembly of the ribosomal proteins (r-proteins) but that these rRNAs themselves play a, if not the, central role in the biological functioning of the ribosome (for reviews, see refs. 1 and 2). This evidence derives from several different experimental approaches, the foremost being *in vivo* and *in vitro* mutational analysis of rRNA (3–5). Until very recently, however, this type of analysis could only be applied to prokaryotic rRNAs, *Escherichia coli* usually being the organism of choice. Similar *in vivo* studies on eukaryotic cells were severely hampered by the inherently high copy number of the rRNA genes, which blankets any effect of the relatively small number of mutant copies that can be introduced into these cells. *In vitro* mutational analysis of eukaryotic rRNA is as yet impossible due to the lack of an *in vitro* reconstitution system for eukaryotic ribosomes. Thus, virtually the only information presently available on functional aspects of eukaryotic rRNA derives from the mapping of antibiotic-resistance mutations (reviewed in ref. 6) and from our observation that two evolutionarily conserved structural elements in LSU-rRNA†, identified as the binding sites for r-proteins EL11¶ (7) and EL23 (8) in *E. coli* 23S rRNA, also act as recognition sites for the binding of the homologous r-proteins L15 and L25 to *Saccharomyces cerevisiae* 26S rRNA (9, 10).

Recently, however, systems for *in vivo* mutational analysis in two eukaryotic organisms—namely, *Tetrahymena* (11) and yeast (12, 13)—have been developed. The latter, developed in our laboratory, is based upon "tagging" of a *S. cerevisiae* rDNA unit by insertion of a unique oligonucleotide into either the 17S or 26S rRNA gene or both. The production and fate of the tagged transcripts derived from this extrachromosomal unit can easily be ascertained, even in the presence of a large excess of wild-type rRNA, by Northern hybridization using oligonucleotides complementary to the tags as probes. We have shown before that neither tag impairs the assembly of the pertinent rRNA into ribosomal subunits or the biological function of these subunits, as judged by their normal polysomal distribution (12, 13). Consequently, an additional mutation can be introduced into a tagged gene to study its effect on ribosome biogenesis and function. We have used this system to analyze the structure–function relationship of two elements within the *S. cerevisiae* 26S rRNA by replacing these elements with their counterparts from other, distantly related species. One element is the evolutionarily highly conserved "GTPase center" within domain II to which yeast r-protein L15 binds (9); the other is the eukaryote-specific variable region V9, which is part of the otherwise conserved binding site for r-protein L25 within domain III (10). A preliminary report of a portion of this work has been published (2).

EXPERIMENTAL PROCEDURES

Enzymes, Strains, and Plasmids. Polynucleotide kinase, T4 DNA ligase, and restriction enzymes were purchased from Bethesda Research Laboratories, except for *Eag* I and *Bst*BI, which were from New England Biolabs, and *Apa* I, which was from Boehringer Mannheim. Helicase was obtained from Biologie Française (Clichy, France). Sequencing reactions were performed by using a Sequenase 2.0 kit (United States Biochemical). A Bio-Rad Muta-Gene kit was used for performing site-directed mutagenesis according to the method of Kunkel *et al.* (14). *E. coli* DH1 (F^-, *recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1*, λ^-) was used for plasmid constructions. *E. coli* CJ236 [*dut, ung, thi, relA, pCJ105* (Cm^r)] and MV1190 {Δ(*lac-proAB*), *supE, thi,*

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Abbreviations: LSU, large subunit of the ribosome; r-protein, ribosomal protein.

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†The 16S–18S and 23S–28S rRNA species of the small and large ribosomal subunit, respectively, are generically referred to as SSU- and LSU-rRNA.

¶*E. coli* r-proteins are referred to with the prefix E. For yeast r-proteins, the nomenclature of Kruiswijk and Planta (27) is used throughout.

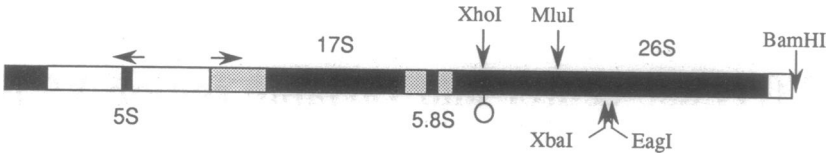

FIG. 1. Schematic representation of the *S. cerevisiae* rDNA insert of pORCS-Bam indicating the restriction sites employed in constructing the various mutants of the 26S rRNA gene used in this study. pORCS-Bam was derived from pORCS described previously (12) by creating a *Bam*HI site in the polylinker downstream of the DNA encoding rRNA (rDNA) insert. The regions encoding the mature and precursor sequences are represented by the black and stippled bars, respectively. Open bars correspond to nontranscribed spacer sequences. The site at which the tag has been inserted into the 26S gene is indicated by the open circle. Arrows show the start and direction of transcription by RNA polymerases I (large rRNA operon) and III (5S rRNA).

Δ(*srl-recA*)*306*::Tn*10* (tet^r) [F′:*traD36*, *proAB*, *lacI^q*, *ZΔM15*]} were used for site-directed mutagenesis, as described by the manufacturer of the kit. *S. cerevisiae* MG34 (*leu2*, *trp1*, *rad2*, *cir$^+$*) was used for expression of pORCS and related plasmids.

The pORCS plasmid, which carries a complete rDNA unit of *S. cerevisiae* that has been tagged by the insertion of an oligonucleotide into the 26S rRNA gene, has been described elsewhere (12). Oligonucleotides were synthesized by using an Applied Biosystems 381A DNA synthesizer.

GTPase Center Replacement. A unique *Bam*HI site was created downstream of the *S. cerevisiae* 26S rRNA gene by inserting a *Bam*HI linker into the pBR322-derived *Cla* I site of pORCS, yielding pORCS-Bam (Fig. 1). An *Mlu* I linker was inserted into the *Hin*dII site of pUC9, and the 3.0-kilobase *Mlu* I–*Bam*HI fragment from pORCS-Bam, comprising a large part of the yeast 26S rRNA gene, was cloned into the resulting plasmid, giving rise to pUC-GTPase. A 62-base-pair fragment, encoding the GTPase center-associated rRNA element of yeast 26S rRNA, was removed from pUC-GTPase by cutting with *Xba* I and *Eag* I (compare Figs. 1 and 3). In its place we inserted a 62-base-pair-long synthetic DNA fragment encoding helix 39–40 of the GTPase center of *E. coli* 23S rRNA and helix 38 of the *S. cerevisiae* GTPase center (numbering according to ref. 15; compare Figs. 2 and 3). The 3.0-kilobase *Mlu* I–*Bam*HI fragment of the resulting pUC-GTPase-Ec plasmid was used to replace the analogous fragment in pORCS-Bam, yielding pORCS-GTPase-Ec. The sequence of the DNA encoding the chimeric GTPase center was experimentally verified.

Replacement of Variable Region V9 of 26S rRNA. An *Mlu* I linker was inserted into the *Sma* I site of pTZ18U (16), and the 3.0-kilobase *Mlu* I–*Bam*HI fragment from pORCS-Bam was inserted into the resulting plasmid, yielding pTZ-V9. This plasmid was transformed into *E. coli* CJ236, and single-stranded DNA was prepared by using M13K07 (17) as a helper phage. By using suitable oligonucleotides with a length of 30 nucleotides, a deletion of 29 base pairs was created in the region encoding variable region V9 by loop-out mutagenesis (see Fig. 5) to give plasmid pTZ-ΔV9. The extent of the deletion was checked by sequence analysis. The site of the deletion is marked by a unique *Bst*BI site. pTZ-ΔV9 was linearized with *Bst*BI and recircularized in the presence of a synthetic DNA fragment encoding the mouse V9 sequence (39 base pairs; see Fig. 5). The resulting plasmid, pTZ-V9-M, was checked by sequencing. The 3.0-kilobase *Mlu* I–*Bam*HI fragments of pTZ-ΔV9 and pTZ-V9-M were used to replace the analogous fragment of pORCS-Bam, yielding pORCS-ΔV9 and pORCS-V9-M (see Fig. 5).

Miscellaneous. Yeast transformation was performed by the method of Beggs (18) with minor modifications. RNA was isolated from yeast cells essentially as described by Kraig *et al.* (19). Methods for polysome isolation, labeling of oligonucleotides, and Northern blotting have been described in detail elsewhere (12).

RESULTS

Replacement of the GTPase Center. Fig. 2 shows the secondary structure model for domains I–III of *S. cerevisiae* 26S rRNA in which the GTPase center as well as the binding site for r-protein L25, which includes the V9 region, have been highlighted. Helix 39–40 (Fig. 2) has been shown to constitute an important part of the GTPase center, which is involved in all phases of translation (see ref. 6 for a review). Furthermore, the helix 39–40 region of *E. coli* 23S rRNA is part of the binding site for r-protein EL11 (7) as well as the EL8 complex (20). At least one of these r-protein–rRNA interactions has been conserved across the prokaryote–eukaryote evolutionary boundary, since yeast r-protein L15 binds to the equivalent region in *S. cerevisiae* 26S rRNA (9). Moreover, *E. coli* EL11 faithfully recognizes the equivalent of its binding site in both mouse and yeast LSU rRNA *in vitro* (9) and can even functionally substitute for its L15 yeast equivalent *in vitro* (21). To assess whether the strong conservation of the helix 39–40 region as an r-protein binding site

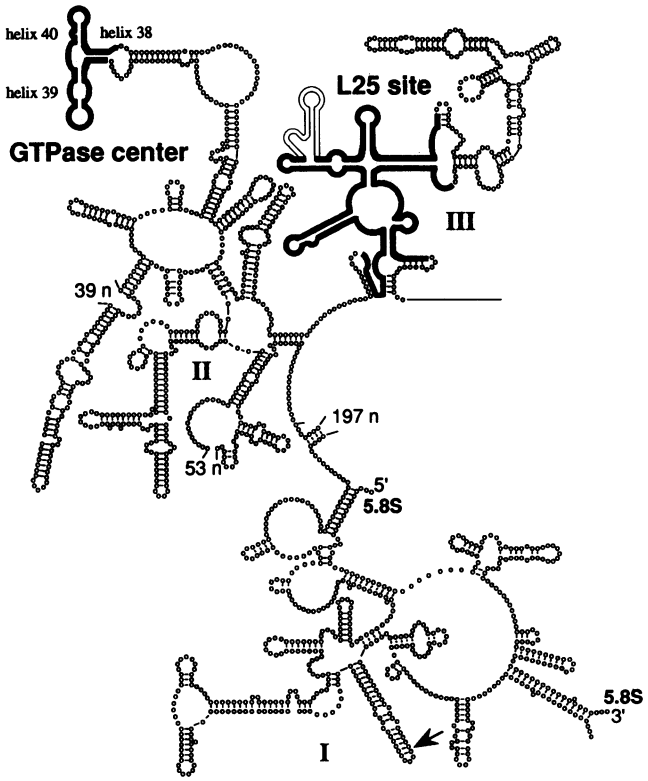

FIG. 2. Schematic representation of the secondary structure of *S. cerevisiae* 26S rRNA (6). Only domains I–III are shown (indicated by Roman numerals). The GTPase center and the L25 binding site are highlighted. The V9 variable region in the latter site is shown in white. Arabic numerals indicate the size of variable regions (in nucleotides) that have not been modeled. The arrow indicates the point at which the tag was inserted into domain I.

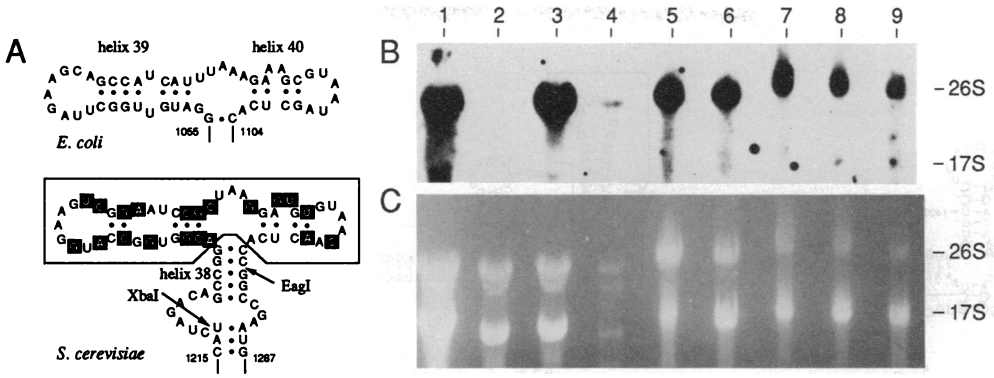

FIG. 3. Functional replacement of the GTPase center of *S. cerevisiae* 26S rRNA by its counterpart from *E. coli*. (*A*) Structure of the GTPase center of the two organisms. The region in the *S. cerevisiae* structure bounded by the *Xba* I and *Eag* I sites was replaced by a synthetic oligonucleotide encoding helix 38 of *S. cerevisiae* 26S and helices 39–40 of *E. coli* 23S rRNA. The nucleotides in the *S. cerevisiae* structure that are changed by this replacement are shown in reversed contrast. (*B*) Intracellular levels of tagged 26S rRNA in yeast cells transformed with the pORCS-Bam control plasmid (lane 1), untransformed host cells (lane 2), and cells transformed with pORCS-GTPase-Ec (lane 3) as determined by Northern hybridization using the oligonucleotide complementary to the tag as probe. Lanes 4–9 show the polysomal distribution of tagged 26S rRNA in pORCS-GTPase-Ec-transformed cells. Lane 4, top fraction of the sucrose gradient. Lanes 5–9, mono-, di-, tri-, tetra-, and larger polysomes. (*C*) Ethidium bromide staining pattern of the same gel as shown in *B* prior to blotting. Note that the relative intensities of the 17S and 26S rRNA bands do not reflect their relative molecular weights because ethidium bromide staining of RNA depends on conformational features.

extends to conservation of its function *in vivo* even in a heterologous context, we replaced these helices in tagged *S. cerevisiae* 26S rRNA by their counterparts from *E. coli*, using a synthetic deoxyoligonucleotide (Fig. 3). The level of plasmid-derived 26S rRNA in cells transformed with pORCS-GTPase-Ec carrying the chimeric, tagged, 26S rRNA gene is the same as in cells transformed with the pORCS-Bam control plasmid (Fig. 3 *B* and *C*, lanes 1 and 3)—i.e., 0.2–0.5% of the amount of its wild-type counterpart (12). Although the replacement introduces 20 point mutations into the yeast 26S rRNA (Fig. 3*A*), all of the mutant rRNA is assembled into ribosomes as indicated by the almost complete absence of a hybridization signal in lane 4 of Fig. 3 *B*, representing the top fraction of the gradient, the OD profile of which is shown in Fig. 4. Furthermore, about 70% of the total amount of mutant 26S rRNA is present in disomes and larger polysomes and thus is supposedly part of actively translating ribosomes (compare Fig. 3*B*, lanes 5–9 to lane 1). Finally, a visual comparison of the strength of the hybridization signal (Fig. 3*B*) with the staining intensities of the rRNA bands in the same fraction (Fig. 3*C*) indicates that the ratio of mutant to wild-type rRNA remains virtually constant across the whole polysome profile. This conclusion was confirmed by determining the radioactivity of the bands in Northern blots like those shown in Fig. 3*B* and normalizing to the concentration of total rRNA in the gradient fraction in question, measured as OD_{260} (Fig. 4). Therefore, we conclude that 60S subunits containing the chimeric 26S rRNA are capable of both initiation and elongation of translation.

Mutations in Variable Region V9. Variable region V9 (Fig. 2) is part of yet another r-protein binding site that has been strongly conserved during evolution. In *S. cerevisiae* 26S rRNA this site is recognized by r-protein L25 (10), whereas in *E. coli* 23S rRNA the corresponding site, in which the equivalent of the V9 region is only 3 nucleotides long (Fig. 5), acts as the binding site for EL23 (8). Moreover, either of these proteins faithfully recognizes the equivalent of its cognate binding site in the heterologous rRNA *in vitro* (10). On the other hand, both proteins fail to bind *in vitro* to the structural equivalent of their binding site in mouse 28S rRNA (22), which contains a larger V9 region (Fig. 5).

To assess the possible role of the V9 region *in vivo*, we removed most of this segment from a tagged *S. cerevisiae* 26S rRNA gene by *in vitro* loop-out mutagenesis, resulting in a structure more closely resembling the corresponding region of *E. coli* 23S rRNA (Fig. 5). Furthermore, we also replaced most of the *S. cerevisiae* V9 region by its counterpart from mouse 28S rRNA (Fig. 5). In both cases the intracellular level of the plasmid-derived (tagged) 26S rRNA is the same as that obtained with the pORCS-Bam control plasmid (compare lanes 1 and 3 in Fig. 6 *A* and *B*). Again, no mutant 26S rRNA can be detected at the top of the gradient (lane 4), and the large majority is present in disomes and larger polysomes (lane 5–9) with a distribution very similar or identical to that of its wild-type counterpart quantified by either Northern hybridization (Fig. 6*A*), ethidium bromide staining (Fig. 6*B*), or measurement of the OD_{260} of the various fractions (data not shown). We obtained similar results upon replacement of

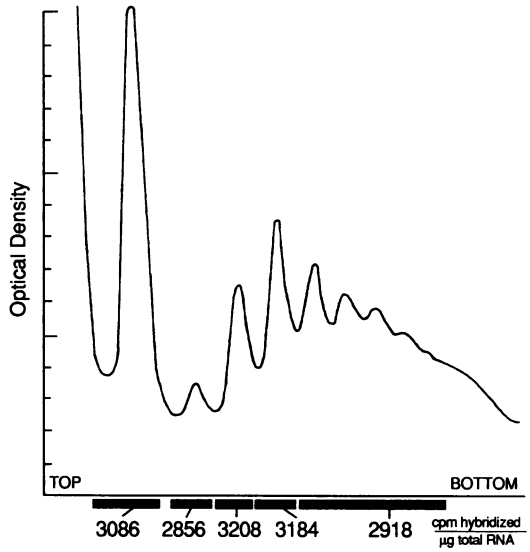

FIG. 4. Polysomes from *S. cerevisiae* cells transformed with pORCS-GTPase-Ec were separated on a sucrose gradient and pooled as indicated, and samples of the various fractions were subjected to Northern hybridization using excess probe specific for the mutant 26S rRNA (see Fig. 3*B*). After autoradiography the bands were cut out and assayed in a liquid scintillation counter. The amount of radioactivity in each band was normalized to the concentration of total RNA in the gradient fraction in question as determined from its OD_{260}. The values are shown below the polysome profile and are the means of two independent determinations.

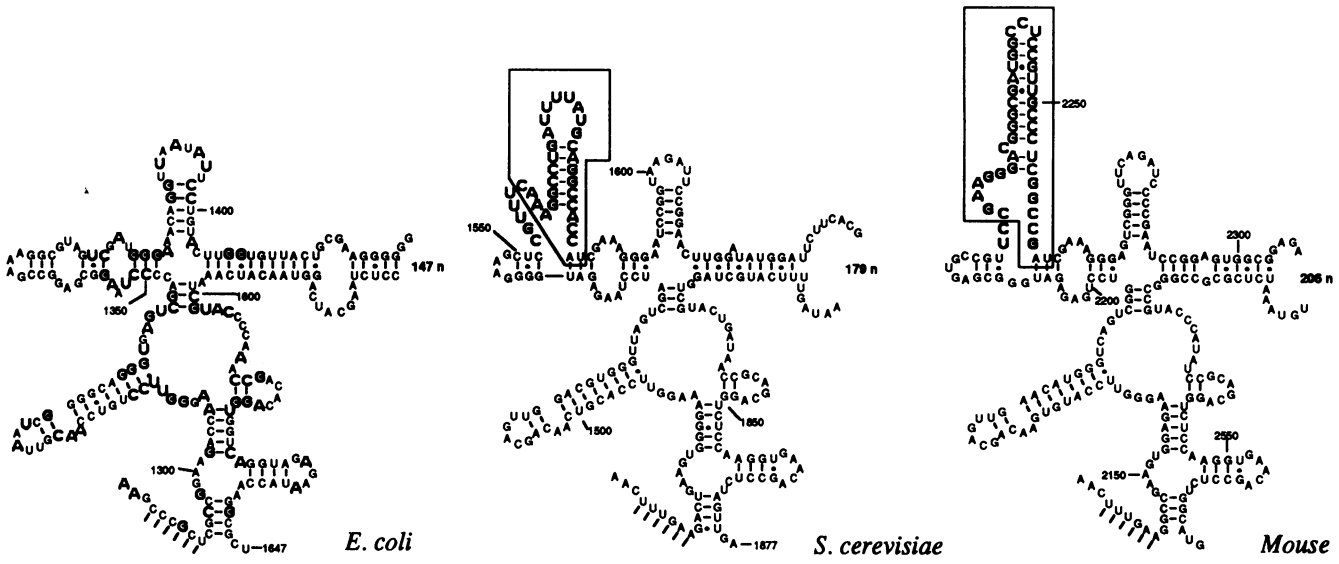

FIG. 5. Structural comparison of the L25 binding site in *S. cerevisiae* 26S rRNA to its equivalents in *E. coli* 23S and mouse 28S rRNA. Nucleotides conserved in all three sequences are shown in boldface type in the *E. coli* structure. Numbers indicate the position relative to the 5′ end of the LSU-rRNA in question. The eukaryote-specific variable region V9 is shown in boldface type in the *S. cerevisiae* and mouse sequences. The boxed region in the *S. cerevisiae* structure indicates the extent of the deletion in the V9 region in plasmid pORCS-ΔV9. In plasmid pORCS-V9-M, this boxed region has been replaced by the similarly boxed sequence of the mouse V9 region.

the yeast V9 region by the corresponding (unprocessed) region from *Tetrahymena thermophila*, the structure of which differs from its counterparts in both yeast and mouse (data not shown). Thus, neither the absence of the V9 region nor its replacement with structurally different equivalents from other eukaryotic organisms has a discernable deleterious effect on the function of the *S. cerevisiae* 60S subunits. Apparently, within the detection limits of our experiments, the V9 region does not play a significant role in the biological function of the yeast ribosome *in vivo*.

DISCUSSION

GTPase Center. The helix 39–40 region in domain II of the LSU-rRNA, which has been implicated in virtually all translational processes from initiation through termination, is one of the most strongly conserved elements within this type of rRNA (reviewed in ref. 6). It can be clearly distinguished even in mitochondrial LSU-rRNA from *Trypanosomes*, which lacks about two-thirds of the basic LSU-rRNA structure as represented by *E. coli* 23S rRNA (6). We have previously shown this region to be one of two elements that are functionally interchangeable as far as *in vitro* binding of r-protein from either *E. coli* or yeast is concerned (9, 10). The experiments reported in this paper demonstrate that the helix 39–40 region of *S. cerevisiae* 26S rRNA can be replaced by its *E. coli* counterpart without detectable loss of overall ribosomal function *in vivo*. To our knowledge, this constitutes the first example of such a functional interchange *in vivo* of a portion of rRNA between a prokaryote and a eukaryote. Since, in the pORCS-GTPase-Ec transformants, the polysomal distribution of the tagged chimeric 26S rRNA is indistinguishable from that of wild-type 26S rRNA (Figs. 3 and 4), ribosomes containing this chimeric rRNA must be competent in translational elongation. Moreover, because the proportion of mutant 60S subunits does not decrease with polysome length, they appear to elongate at normal or near normal rate. Our data, however, do not exclude a possible effect of GTPase center exchange on fidelity of translation or termination by the mutant ribosomes. A certain loss of efficiency in the initiation step of translation also cannot be ruled out. Such a loss should manifest itself as a reduction in the ratio of polysomal vs. nonpolysomal tagged rRNA, compared to the ratio shown by its wild-type counterpart. In our experiments this ratio could not be determined with sufficient accuracy to detect small differences.

Interestingly, one of the 20 sequence alterations caused by replacing the *S. cerevisiae* helix 39–40 region with its *E. coli* counterpart involves a nucleotide (*E. coli* adenosine 1067/*S. cerevisiae* guanosine 1241; Fig. 3*A*) considered to be of pivotal importance to the GTPase center (23). The sensitivity of *E. coli* ribosomes to thiostrepton, a drug that blocks elongation, has been closely linked to this adenosine residue, which is part of the binding site for the drug (23). Methylation of adenosine 1067 confers resistance to thiostrepton (24). Thiostrepton does not bind to eukaryotic ribosomes, which almost invariably contain a guanosine at this position. Thompson *et al.* (25) have shown, however, that the insensitivity of eukaryotic ribosomes to thiostrepton cannot be attributed solely to this sequence difference, since *E. coli* ribosomes containing an adenosine → guanosine mutation at position 1067 remain sensitive to the action of the drug. It would be interesting to establish whether yeast ribosomes containing the chimeric 26S rRNA are sensitive to thiostrepton.

Variable Region V9. Eukaryotic SSU- as well as LSU-rRNA contain several so-called variable regions, or expansion segments, located at distinct positions within the basic structure. Both the origin and functional significance of these regions are still being debated (see ref. 6 for a recent review). Our experiments indicate that variable region V9, located in domain III of the LSU-rRNA, is dispensable for correct assembly and functioning of the yeast 60S ribosomal subunit. Furthermore, the yeast V9 region can be replaced by its counterpart from other eukaryotes, having different primary and secondary structures, without discernable effect (Fig. 6). Thus, in *S. cerevisiae* at least this particular variable region seems to be a neutral appendage whose presence is tolerated because it does not disrupt ribosomal function. Nevertheless, the occurrence of processing within the V9 segment in a number of insects and lower eukaryotes (see ref. 6 for references) may indicate recruitment of this region for some specific purpose in these organisms.

As mentioned above, the V9 region is situated in the middle of the highly conserved binding site for r-protein L25 of yeast 26S rRNA. L25 binding, however, is not significantly af-

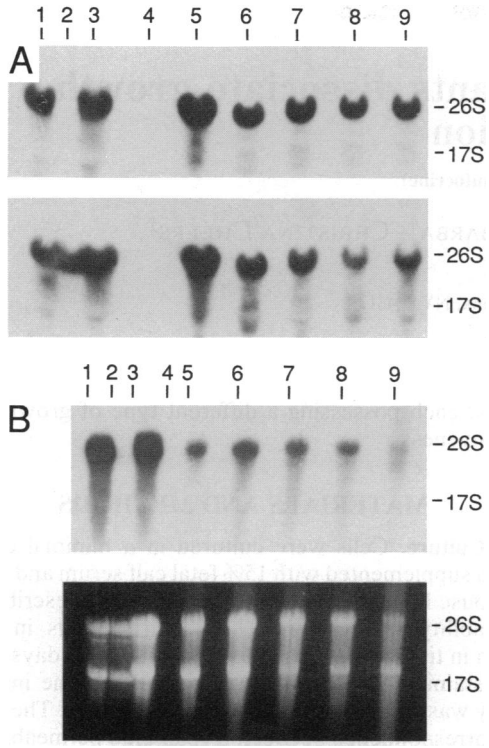

FIG. 6. Intracellular levels and polysomal distribution of tagged *S. cerevisiae* 26S rRNAs carrying the various structural alterations of the V9 region. (*A*) pORCS-ΔV9. (*B*) pORCS-V9-M. (*A* and *B Upper*) Northern hybridization using the oligonucleotide complementary to the 26S rRNA tag as probe. (*A Lower*) Northern hybridization of the same filter with a probe that does not discriminate between plasmid-derived and chromosomally derived 26S rRNA. (*B Lower*) Ethidium bromide staining pattern of the same gel prior to blotting. Note that the relative intensities of the 17S and 26S rRNA bands do not reflect their relative molecular weights because ethidium bromide staining of RNA depends on conformational features. Lanes: 1, total RNA from cells transformed with the pORCS-Bam control plasmid; 2, total RNA from untransformed host cells; 3, total RNA from cells transformed with the plasmid carrying the mutant 26S rRNA gene; lanes 4–9, polysomal distribution of the mutant 26S rRNA. Lane 4 contains RNA from the top fraction of the sucrose gradient. Lanes 5–9 contain mono-, di-, tri-, tetra-, and larger polysomes, respectively.

fected by changes in the structure of the V9 region. The yeast 60S subunits assembled with the mutant 26S rRNA molecules must contain L25, because we have shown this protein to be essential to cell survival (26), indicating that lack of L25 blocks either 60S subunit assembly or function. Moreover, *in vitro* experiments have shown that L25 efficiently recognizes a binding site that either lacks the V9 region or contains a foreign (mouse or *Tetrahymena*) counterpart (2).

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