Depletion of *Saccharomyces cerevisiae* ribosomal protein L16 causes a decrease in 60S ribosomal subunits and formation of half-mer polyribosomes

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We constructed yeast strains containing deletion–insertion null alleles of the *RPL16A* or *RPL16B* genes encoding the 60S ribosomal subunit protein L16 to determine the role of L16 in the synthesis and function of ribosomes. Strains lacking a functional *RPL16A* gene grow as rapidly as wild type, whereas those containing a null allele of *RPL16B* grow more slowly than wild type. RNA analysis using *RPL16* probes revealed that both *RPL16* genes are transcribed and that *RPL16B* transcripts accumulate to twice the level of *RPL16A* transcripts. No evidence was obtained for the occurrence of dosage compensation at the level of *RPL16* mRNA accumulation in either mutant. Strains lacking both *RPL16* genes are apparently inviable, demonstrating that L16 is an essential yeast ribosomal protein. Introduction of an extra copy of either *RPL16* gene into *rpl16b* mutants restored wild-type growth rates, indicating that the two forms of the L16 protein are interchangeable. *rpl16* mutants are deficient in 60S ribosomal subunits relative to 40S subunits. 43S preinitiation complexes accumulate in half-mer polyribosomes in the absence of sufficient 60S subunits. We postulate that the slow-growth phenotype of *rpl16* mutants results from the perturbation of initiation of protein synthesis.

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Ribosomal subunits are assembled in the nucleolus of eukaryotic cells from some 75 cytoplasmically synthesized protein components and from 4 RNA molecules that are processed in the nucleus from two distinct transcripts. Equimolar quantities of these ribosomal components accumulate at a variable rate that is characteristic of the physiological state of the cell (Gorenstein and Warner 1976; reviewed in Fried and Warner 1984; Warner et al. 1986). Little is known about the role of individual ribosomal proteins in the biosynthesis, assembly, and function of eukaryotic ribosomes. We have chosen to study the regulation of ribosome biogenesis and the function of ribosomal components in translation by a molecular genetic analysis of several ribosomal proteins of the budding yeast *Saccharomyces cerevisiae*. As a first step in this analysis, several investigators have cloned yeast ribosomal protein genes (Woolford et al. 1979; Bollen et al. 1981; Fried et al. 1981; Larkin and Woolford 1983). Surprisingly, most yeast ribosomal protein genes are duplicated. Most (25/29) of the cloned yeast ribosomal protein genes hybridize to restriction fragments from two separate loci in the yeast genome (reviewed in Planta et al. 1986). In several cases, both copies of these dupli-

cated genes are known to be expressed (Abovich et al. 1985; Johnson and Warner 1987; this study; A.G. Paulovich, J.C. Larkin, J.G. Anthony, E.R. Reynolds, and J.L. Woolford, in prep.). Ribosomal protein genes from other eukaryotes are either single-copy genes or exist as one functional intron-containing gene and multiple processed pseudogenes, with the exception of the two functional L1 genes of *Xenopus laevis* (Lorenz et al. 1985).

The existence of duplicated yeast ribosomal protein genes raises several questions. Are both copies of these genes expressed equally under various physiological conditions? Are the products of the duplicate genes the same and do they perform interchangeable functions? Alternatively, are there 'specialized ribosomes' containing one or the other form? How is the expression of the single-copy ribosomal protein genes balanced with that of the duplicated genes, given that the yeast ribosomal protein mRNAs accumulate in equimolar amounts (Kim and Warner 1983)?

Using cloned copies of the yeast *RPL16A* and *RPL16B* genes, we have initiated a genetic analysis of the synthesis and function of L16, a constituent of the 60S ribosomal subunit (Woolford et al. 1979; Rotenberg and Woolford 1986). We previously established that both *RPL16* genes are transcribed by isolating cDNA clones specific for each and by distinguishing the two *RPL16*

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transcripts using S1 mapping. We also characterized the structure of the *RPL16* genes and transcripts by S1 mapping, primer extension, and sequencing of the cDNA and genomic *RPL16* clones (J.L. Woolford, R. Last, M. Rotenberg, and A. Levy, in prep.). The polypeptides predicted from the nucleotide sequences of *RPL16A* and *RPL16B* are nearly identical, differing only at their third amino acid residues, threonine and alanine, respectively (Leer et al. 1984; Teem et al. 1984; J.L. Woolford, R. Last, M. Rotenberg, and A. Levy, in prep.). In this report we describe the use of mutant *S. cerevisiae* strains lacking

functional copies of one or the other or both of the *RPL16* genes to study the expression and function of ribosomal protein L16.

Results

Disruption of the *RPL16A* and *RPL16B* genes

Deletion-insertion mutations of *RPL16A* or *RPL16B* were constructed in diploids by the one-step gene replacement method of Rothstein (1983). The *RPL16A* gene was disrupted by inserting the yeast *URA3* gene

Figure 1. Replacement of the *RPL16A* gene with an *rpl16a::URA3* disruption allele. (A) Construction of the *rpl16a::URA3* deletion-insertion null allele. A synthetic *XhoI* site was inserted into a plasmid containing the 5' end of *RPL16A* to create a 2-bp deletion plus *XhoI* linker insertion within codon 30 of *RPL16A*. The yeast *URA3* gene was inserted into that *XhoI* site to produce plasmid *p116aΔURA*. A *BamHI* fragment containing the *rpl16a::URA3* allele was isolated from *p116aΔURA* and used to transform yeast diploid JW100 such that one wild-type allele of *RPL16A* was replaced by the deletion-insertion allele. The sizes of genomic *EcoRI* restriction fragments from the *RPL16A* wild-type allele and predicted fragments from the null allele are shown. Restriction enzyme sites: (Bg) *BglII*; (X) *XhoI*; (R1) *EcoRI*; (B) *BamHI*; (Ha) *HaeIII*. (B) Gel transfer hybridization analysis of yeast DNA from strains transformed with the *rpl16a::URA3* construct. Total DNA was purified from a diploid transformant (lane 1), from four progeny spore colonies from each of two tetrads derived from the transformed diploid (lanes 2–9), and from the untransformed parental diploid JW100 (lane 10). The DNA was digested with *EcoRI*, subjected to electrophoresis on a 1% agarose gel, transferred to nitrocellulose, and probed with a ^{32}P -labeled 1.2-kb *BamHI*–*BglII* fragment containing *RPL16A*. This probe hybridizes to both a 3.0-kb and a 4.4-kb *EcoRI* fragment, each containing part of *RPL16A* as well as to an 11.0-kb *RPL16B* *EcoRI* fragment, as shown. The 4.4-kb fragment is converted to a 5.6-kb *EcoRI* fragment in the disruption allele. The Ura phenotype of each diploid strain and haploid spore clone is shown above the lanes.

between the endpoints of a deletion from nucleotide +87 to nucleotide +89, i.e., within codon 30 of the *RPL16A* protein-coding region (Fig. 1A). An *rpl16b::LEU2* disruption was constructed by deleting the sequences from nucleotide +259 to +443 of *RPL16B* (codons 87–148) and replacing them with the yeast *LEU2* gene (Fig. 2A). Linear DNA fragments containing each of these *RPL16* deletion–insertion mutations were used to separately transform the diploid strain JW100, selecting for *URA3* or *LEU2*.

The *Ura*⁺ or *Leu*⁺ diploid transformants were sporulated, and tetrads were analyzed. Most tetrads yielded four viable spores in which all markers displayed the ex-

pected segregation patterns, including 2 : 2 segregation for either the *URA3* or *LEU2* disruption markers. Replacement of the wild-type *RPL16* alleles with the *rpl16a::URA3* or *rpl16b::LEU2* alleles was confirmed by gel transfer hybridization experiments (Figs. 1B and 2B). In each case, DNA from the untransformed diploid, transformed diploid, and four haploid spore clones from each of two tetrads was analyzed. *Ura*⁺ cosegregated with replacement of the 4.4-kb *RPL16A* *EcoRI* fragment by a 5.6-kb *EcoRI* fragment predicted to contain *rpl16a::URA3* (Fig. 1B). Similarly, *Leu*⁺ cosegregated with the 8.0-kb *HindIII* fragment containing *rpl16b::LEU2* (Fig. 2B). The transformed diploids were

Figure 2. Replacement of the *RPL16B* gene with an *rpl16b::LEU2* disruption allele. (A) Construction of the *rpl16b::LEU2* deletion–insertion null allele. A 185-bp deletion within the coding region of *RPL16B* was constructed using BAL31 exonuclease. Synthetic *XhoI* linkers were added to the deletion endpoints, and the yeast *LEU2* gene was inserted into the *XhoI* site to create plasmid *pl16bΔLEU2*. A linear *HindIII*–*BamHI* fragment containing the *rpl16b::LEU2* allele was transformed into yeast diploid JW100 such that one wild-type allele of *RPL16B* was replaced with *rpl16b::LEU2*. The sizes of genomic *HindIII* fragments from the *RPL16B* wild-type and mutant alleles are shown. Restriction enzyme sites: (H) *HindIII*; (X) *XhoI*; (B) *BamHI*; (K) *KpnI*. (B) Gel transfer hybridization analysis of yeast DNA from strains transformed with the *rpl16b::LEU2* construct. Total DNA was purified from four progeny spore colonies from each of two tetrads derived from the transformed diploid (lanes 1–8), from the transformed diploid (lane 9), and from the untransformed parental diploid JW100 (lane 10). The DNA was digested with *HindIII*, subjected to electrophoresis on a 1% agarose gel, transferred to nitrocellulose, and probed with a ³²P-labeled 3.0-kb *BamHI*–*HindIII* fragment containing *RPL16B*. This fragment hybridizes to a 6.0-kb *HindIII* fragment containing *RPL16B* and to an 11.0-kb *RPL16A* *HindIII* fragment (visible only in the original autoadiogram). The 6.0-kb *RPL16B* fragment is converted to an 8.0-kb *HindIII* fragment as a result of the deletion–insertion mutation. The *Leu* phenotype of each strain is shown above the lanes.

heterozygous for the expected *RPL16* restriction fragments, whereas the untransformed diploids and *Leu⁻Ura⁻* haploids contained only wild-type *RPL16* fragments. From these results, we conclude that neither *RPL16* gene is individually required for spore germination or mitotic growth.

In rich medium (YEPA) at 30°C, the *rpl16a :: URA3* haploid spore clones grew with doubling times identical to wild-type haploids, i.e., 120 min. However, *rpl16b :: LEU2* spore clones grew significantly more slowly than wild-type cells, with a doubling time of 240 min. The *rpl16b* cells grew more slowly than *rpl16a* or wild-type cells in defined synthetic medium as well. The same results were obtained when the *rpl16a :: URA3* and *rpl16b :: LEU2* deletion-insertion alleles were introduced independently by direct transformation into either of two different haploid strains, JL8 (Fig. 3) and SC252. The phenotype of strains disrupted for *RPL16A* (*rpl16a :: URA3* and *rpl16a :: TRP1*) and for *RPL16B* (*rpl16b :: LEU2* and *rpl16b :: URA3*) was independent of the disruption marker used.

L16 is an essential yeast ribosomal protein

To determine whether L16 is an essential protein, we constructed a diploid strain (JW450) heterozygous for disruptions in both *RPL16* loci (*rpl16a :: URA3/RPL16A rpl16b :: LEU2/RPL16B*). Following sporulation of

JW450, asci were dissected and spore clones were analyzed. Gel transfer hybridization analysis verified that both the parental diploid and the haploid spore clone DNA contained the expected *RPL16* alleles. The observed ratio of parental ditype : nonparental ditype : tetratype tetrads of 1 : 1 : 4 was consistent with that expected for random segregation of two unlinked genes. This agrees with preliminary mapping data that indicate *RPL16A* is on either chromosome VII or XV and that *RPL16B* is on chromosome XVI (M. Moritz and M. Rotenberg, unpubl.). All *rpl16a :: URA3 RPL16B* (*Ura⁺Leu⁻*) haploid spore clones grew as fast as wild-type (*Ura⁻Leu⁻*) spore clones, whereas the slow-growth phenotype cosegregated with *RPL16A rpl16b :: LEU2* (*Ura⁻Leu⁺*) cells. No *Ura⁺Leu⁺* (*rpl16a :: URA3 rpl16b :: LEU2*) spore clones were recovered in cases where this genotype was predicted from the segregation pattern of the other *RPL16* alleles in the same ascus. This latter result is explained most simply if L16 is an essential ribosomal protein in yeast. Essentially the same results were obtained upon sporulation of diploid MM1402, which is heterozygous for the *rpl16a :: TRP1* and *rpl16b :: LEU2* mutations. The lethality was a direct consequence of the absence of L16 function, because viable spore clones containing chromosomal disruptions of *RPL16A* and *RPL16B* were obtained when either *RPL16A* or *RPL16B* was provided on a complementing plasmid. The fact that null mutations in either *RPL16* gene alone are not lethal indicates that both *RPL16A* and *RPL16B* are expressed and complement each other.

Figure 3. Effects of null mutations of the *RPL16* genes upon growth. The indicated strains were streaked on solid YEPA medium and incubated at 30°C for 60 hr. (AB) Wild-type haploid strain JL8; (*a⁻B*) haploid JL8Δ*l16a* of genotype *rpl16a :: URA3 RPL16B* derived from JL8 by transformation and replacement of *RPL16A*; (*Ab⁻*) haploid JL8Δ*l16b* of genotype *RPL16A rpl16b :: LEU2* derived from JL8 by transformation; (*AAb⁻*) *RPL16A RPL16A rpl16b :: LEU2* haploid derived from JL8Δ*l16b* by integration of one extra copy of *RPL16A* (in *YipL16A*) at the *ura3-52* locus; (*Ab⁻B*) *RPL16A rpl16b :: LEU2 RPL16B* haploid derived from JL8Δ*l16b* by integration of one extra copy of *RPL16B* (in *YipL16B*) at the *ura3-52* locus.

The RPL16B gene is expressed at twice the level of RPL16A

The more deleterious effect of the *rpl16b* mutations relative to the *rpl16a* mutations might be due to higher expression of the *RPL16B* gene in wild-type cells or to some specialized role performed by the L16b protein for which the L16a protein cannot substitute.

We measured the relative steady-state levels of *RPL16A* and *RPL16B* mRNA in wild-type haploid strains and in those containing a disruption allele of *RPL16A* or *RPL16B*. A 0.3-kb *HinfI* fragment from the protein-coding region of *RPL16A* was used as a probe for gel transfer hybridization analysis of RNA extracted from these strains. This *RPL16A* fragment is 99% homologous to the nucleotide sequence in the same region of *RPL16B* (J.L. Woolford, R. Last, M. Rotenberg, and A. Levy, in prep.) and hybridizes with equal efficiency to either *RPL16* mRNA. The amount of *RPL16* mRNA was quantitated by densitometry of several exposures of the hybridization filters and, in each case, was normalized to the amount of mRNA homologous to plasmid pY11-10, which carries a gene encoding an abundant yeast transcript. Data obtained by comparing the levels of *RPL16A* mRNA or *RPL16B* mRNA to each other in the disruption strains (Fig. 4, lanes 2 and 3) or to total *RPL16* mRNA in wild-type cells (Fig. 4) suggest that *RPL16B* is expressed at approximately twice the level of *RPL16A*. This interpretation is based on the assumption

Figure 4. The abundance of *RPL16* mRNA is decreased in haploids containing a null allele of *RPL16A* or *RPL16B*. Strains JL8, JL8Δ116a and JL8Δ116b were grown to a density of 2×10^7 to 4×10^7 cells/ml in defined synthetic medium. Total yeast RNA was extracted, and about 15 μg samples of each were fractionated on a 1.2% agarose-formaldehyde gel. RNAs were blotted to nitrocellulose and hybridized with both a ^{32}P -labeled 0.3-kb *RPL16A* *Hinf*I fragment and plasmid pY11-10. The *RPL16A* fragment is from a portion of the *RPL16A*-coding region that is 99% identical in nucleotide sequence to the equivalent region of *RPL16B* and hybridizes with equal efficiency to both species of *RPL16* transcript. The 11-10 RNA signal (Woolford and Rosbash 1979) indicates the relative amounts of total RNA loaded in each lane. The amount of *RPL16* mRNA relative to 11-10 RNA in each sample was determined by densitometry of several exposures of each hybridization filter. (Lane 1) JL8 (wild type); (lane 2) JL8Δ116a (*rpl16a* :: *URA3* *RPL16B*); (lane 3) JL8Δ116B (*RPL16A* *rpl16b* :: *LEU2*).

that there is no dosage compensation affecting either the rate of transcription or the stability of *RPL16* mRNA in strains containing disruptions of *RPL16A* or *RPL16B*. This assumption was proven to be correct, using hybridization probes specific for each *RPL16* mRNA [see below].

No dosage compensation of *RPL16* transcripts occurs in the *rpl16* mutants

We constructed two *RPL16A* and *RPL16B* gene-specific oligonucleotide probes complementary to a highly divergent region of the respective 3'-transcribed non-coding regions. These probes differ in 15 out of 19 nucleotides and hybridized to only their complementary RNAs. No *RPL16* mRNA was detected when the *RPL16A* oligonucleotide probe was hybridized to blots of RNA from the *rpl16a* mutant (Fig. 5, lane 2) or when the *RPL16B*-specific oligonucleotide was hybridized to RNA from the *rpl16b* null mutant (Fig. 5, lane 6). Furthermore, no transcripts differing in electrophoretic mo-

bility from the *RPL16* mRNA were detected in blots of RNA from wild-type cells or *rpl16* null mutants using either the gene-specific probes (Fig. 5) or the *RPL16A* *Hinf*I fragment homologous to both *RPL16* mRNAs (Fig. 4).

These probes were employed in a dot blot hybridization assay to measure the relative levels of *RPL16A* and *RPL16B* mRNAs in wild-type haploid cells. The *RPL16A*-specific probe hybridized to complementary denatured DNA with approximately twice the efficiency of the *RPL16B*-specific probe (Fig. 6), presumably because of its significantly greater G + C content. When the same DNA samples were then washed free of these probes and subsequently hybridized to the yeast *LEU2* gene, the signal intensities for the two sets of DNA samples were much more similar (Fig. 6). This confirmed that the different relative signal intensities observed for the two *RPL16* oligonucleotide probes were actually due to the differing hybridization efficiencies of these probes. When used to probe a dilution series of RNA extracted from wild-type strain JL8, the two *RPL16* oligonucleotide probes yielded hybridization signals of approximately equal intensity (Fig. 6). Taking into ac-

Figure 5. The deletion-insertion mutations *rpl16a* :: *URA3* and *rpl16b* :: *LEU2* are null alleles. Strains JL8, JL8Δ116a, and JL8Δ116b were grown to a density of 2×10^7 to 4×10^7 cells/ml in defined synthetic medium. Total yeast RNA was purified from these cells, subjected to electrophoresis on 1.2% agarose-formaldehyde gels, transferred to nitrocellulose, and probed with ^{32}P -labeled 19-mer oligonucleotides complementary to sequences within the 3' noncoding regions of *RPL16A* or *RPL16B*. (Lanes 1 and 4) RNA from JL8 (wild type); (lanes 2 and 5) RNA from JL8Δ116a (*rpl16a* :: *URA3* *RPL16B*); (lanes 3 and 6) RNA from JL8Δ116b (*RPL16A* *rpl16b* :: *LEU2*). Lanes 1–3 were probed with the 19-mer specific for *RPL16A*; lanes 4 through 6 were hybridized with the 19-mer specific for *RPL16B*.

Figure 6. Quantitation of *RPL16A* and *RPL16B* mRNA levels by dot blot hybridization. The indicated quantities of plasmids YEp13L16A and YEp13L16B containing the *RPL16A* and *RPL16B* genes, respectively, and the *LEU2* gene, and total RNA samples prepared from yeast strain JL8 were denatured and transferred to nitrocellulose sheets. DNA standards were included for determination of the relative hybridization efficiencies of ^{32}P -labeled probes. Oligonucleotides specific for *RPL16A* and *RPL16B* and a ^{32}P -labeled 2.2-kb *XhoI*–*SalI* fragment containing the yeast *LEU2* gene were hybridized to the filters.

count the differing hybridization efficiencies of the two probes, we conclude that there is approximately twice as much *RPL16B* mRNA as *RPL16A* mRNA in wild-type cells. This is consistent with our determination that there are one third and two thirds of wild-type amounts of *RPL16* mRNA in *rpl16a* and *rpl16b* null mutants, respectively (Fig. 4). Therefore, the synthesis and stability of mRNA encoded by either *RPL16* gene is apparently not affected by a null mutation in the other.

There is no functional distinction between the L16a and L16b proteins

We tested whether the difference in phenotype between *rpl16a* and *rpl16b* null mutants resulted from functional distinctions between the L16a and L16b proteins by asking whether an extra copy of *RPL16A* could suppress the growth deficiency produced by the *rpl16b* null mutation. *RPL16A*, cloned in the yeast integrating plasmid YIp5 (YIpL16A), was directed to integrate into *RPL16A* *rpl16b* :: *LEU2* yeast at the *ura3-52* locus by linearizing the plasmid within *URA3*. DNA gel blot hybridization analysis of uracil prototrophic transformants confirmed that a single copy was integrated at *ura3-52* and that the *RPL16A* and *rpl16b* :: *LEU2* alleles were also present (data not shown). One extra copy of *RPL16A* fully restored a wild-type growth rate to the *rpl16b* :: *LEU2* strain (Ab⁺A, Fig. 3). Unless *RPL16A* is overexpressed at the *ura3-52* locus due to a position effect, a total of two normally expressed copies of *RPL16A* are fully capable of replacing the function of *RPL16B*. Not surprisingly, a wild-type growth rate was also restored to the

rpl16b :: *LEU2* strain by integration of one copy of *RPL16B* at *ura3-52* (Ab⁺B, Fig. 3). The same results were obtained when extra copies of *RPL16A* or *RPL16B* were introduced on either the low copy centromere plasmid YCp50 or the high copy 2 μ m plasmid YEp24 (data not shown). We conclude that either of the two L16 proteins can perform the essential functions in vivo and that the *rpl16b* :: *LEU2* mutation is recessive, encoding no observably deleterious gene product.

rpl16b mutants have decreased pools of free 60S ribosomal subunits and form half-mer polyribosomes

We examined the effects of disruption of the *RPL16* genes and the presumed reduction of L16 protein levels upon ribosome biogenesis and function by comparing the levels of free ribosomal subunits, 80S monosomes, and polyribosomes in wild-type cells and *rpl16a* and *rpl16b* null mutants. Extracts of these cells were fractionated by sucrose velocity gradient centrifugation. A typical absorbance profile for a wild-type strain is shown in Figure 7A. There is a 2 : 1 ratio of absorbance for the peak of free 60S ribosomal subunits compared to the peak of free 40S subunits. Because the mass ratio of the two subunit particles is about 2 : 1, the absorbance ratio indicates that free 60S and 40S subunits are equimolar in wild-type cells. These wild-type cells contain an excess of 80S monosomes compared to free subunits. Distinct polyribosome peaks containing two to nine ribosomes can be observed. In contrast, the *rpl16b* cells contain a fourfold excess of free 40S subunits relative to 60S subunits, fewer 80S monosomes relative to free subunits,

and fewer larger polyribosomes than wild-type cells do (Fig. 7B). Most strikingly, there are discrete peaks sedimenting at positions in the gradient intermediate to those polyribosome peaks containing integral numbers of ribosomes (Fig. 7B, vertical arrows). These intermediate peaks are reminiscent of the half-mer peaks observed in polyribosome profiles of yeast treated with low

concentrations (10 $\mu\text{g/ml}$) of cycloheximide. Under these conditions, the initiation step of yeast protein synthesis is inhibited (Lin et al. 1966; Baliga et al. 1969; Cooper and Bossinger 1976; Helser et al. 1981). The *rpl16a* mutant that is depleted of one third of *RPL16* mRNA has a polyribosome profile intermediate to that of wild-type and *rpl16b* cells (Fig. 7C), the latter of which are depleted of two thirds of *RPL16* mRNA. There are roughly twice as many free 40S subunits as free 60S subunits in the *rpl16a* cells. Although the half-mer peaks are clearly visible in extracts of *rpl16a* cells, they are less pronounced than in *rpl16b* extracts. These biochemical defects in *rpl16* mutants are direct results of the *RPL16* disruption, because introduction of a single extra wild-type copy of either *RPL16A* or *RPL16B* restores the wild-type profile to *rpl16* mutant cells (data not shown).

Helser et al. (1981) showed that yeast half-mers consist of polyribosomes containing mRNA associated with one or more 80S ribosomes and a 43S preinitiation complex (derived from the 40S subunit and associated translation initiation factors). We suggest that in *rpl16* mutants deficient in L16 protein, 60S subunits fail to accumulate in quantities equimolar to 40S subunits. The excess 40S subunits may form 43S preinitiation complexes and attach to mRNAs to initiate protein synthesis but may then remain at the translation initiation site in the absence of a free 60S subunit with which to associate. In that case, the intermediate peaks containing putative half-mer polyribosomes would contain an excess of 40S subunits compared to 60S subunits. We have analyzed the ribosomal RNA present in each fraction of the polyribosome gradients from extracts of *rpl16b* cells to determine whether the putative half-mer fractions do indeed contain an excess of 40S subunits. The rRNA was quantitated by gel blot hybridization analysis using a DNA probe containing a portion of the yeast rDNA repeat that is complementary to 18S and 25S rRNA. As shown in Figure 8, fractions 8–11 contain predominantly 18S rRNA and fractions 13 and 14 contain mostly 25S rRNA, consistent with their containing free 40S and 60S subunits, respectively. Equimolar quantities of 18S and 25S rRNA can be seen in fractions 15 and 16 containing 80S monosomes (Fig. 8). Due to the difference in size of these rRNAs, the hybridization signal for 25S rRNA exceeds that for 18S rRNA when equimolar amounts are present and probed with comple-

Figure 7. Decrease in free 60S ribosomal subunit pool and accumulation of half-mer polyribosomes in *rpl16* mutant strains. (A) MM1401 (wild type); (B) MM1405 (*RPL16A rpl16b::LEU2*); (C) MM1404 (*rpl16a::TRP1 RPL16B*) cells (200 ml) were grown in defined synthetic medium to 1×10^8 to 2×10^8 cells/ml. Cells were harvested, and 50 A₂₆₀ units of lysate from each culture were analyzed on 7–47% sucrose gradients centrifuged for 4 hr at 27,000 rpm. The positions of 40S and 60S ribosomal subunits, 80S monosomes, and polyribosomes containing 2–10 ribosomes are indicated above the absorbance profiles. The vertical arrows indicate intermediate-sized polyribosomes, or half-mers.

Figure 8. Decreased ratio of 25S rRNA to 18S rRNA in half-mer polyribosome fractions isolated from *rpl16b* mutants. (Top) Polyribosome profile of an *rpl16b* mutant. Yeast strain MM1405 (*RPL16A rpl16b :: LEU2*) was grown to 1×10^8 to 2×10^8 cells/ml and harvested, and a lysate was prepared and subjected to sedimentation on a 7–47% sucrose gradient. The 40S and 60S ribosomal subunits, 80S monosomes, and polyribosomes associated with two to seven ribosomes are labeled above the absorbance profile of the gradient. Half-mer polyribosomes are denoted by vertical arrows. (Bottom) RNA gel blot hybridization analysis of 18S and 25S rRNAs in fractions of the *rpl16b* mutant polyribosome gradient. Fractions of 1.2 ml each were collected from the gradient as indicated. Each fraction was concentrated by ethanol precipitation. RNA was isolated and subjected to electrophoresis, transferred to nitrocellulose, and hybridized with ^{32}P -labeled DNA containing the yeast rRNA repeat unit to detect 18S and 25S rRNAs. Fractions from the polyribosome gradient are indicated above each gel lane. Differences in signal strengths among different fractions presumably reflect different recovery and loading of total RNA in each gel lane. Presumably there was no differential recovery of 18S vs. 25S rRNA.

mentary DNAs of equal specific activity. The ratio of 18S rRNA to 25S rRNA is clearly increased in fraction 17 (containing the putative '1 1/2-mer'), compared to fractions 15 and 16 (containing monosomes). These rRNA ratios were quantitated by densitometric scanning of several exposures of the autoradiograms of the RNA blots (data not shown). The putative 1 1/2-mer peak contained an approximately 1.5-fold higher ratio of 18S rRNA to 25S rRNA than the fractions containing monosomes or disomes. Likewise, we predict that the 2 1/2-mer fraction should have a 3 : 2 ratio of 40S to 60S subunits, unless multiple 40S subunits accumulate on these polysomes. We have been unable to resolve 2 1/2-mers from disomes or trisomes sufficiently well to test this hypothesis.

To demonstrate that the deficiency of 60S subunits in *rpl16* strains is not a result of the slow growth of these strains, we examined the polyribosome profile of wild-type cells constrained by a poor carbon source to grow with a doubling time equivalent to that of the *rpl16b* strain. The polyribosome profiles of these slow-growing

wild-type cells were identical to those obtained when wild-type cells were grown in rich medium (data not shown).

Discussion

We have constructed null mutations in the yeast *RPL16A* and *RPL16B* genes encoding ribosomal protein L16 to study the expression and function of this protein in ribosome biogenesis. Both copies of the *RPL16* genes are expressed and encode proteins with indistinguishable functions. Disruption of *RPL16B* results in a significant increase in the doubling time of haploid strains, whereas inactivation of *RPL16A* has no measurable effect upon growth rate. This is consistent with our finding that the steady-state level of *RPL16B* mRNA is approximately twice that of *RPL16A*. We do not know whether the approximately twofold difference in abundance of *RPL16A* and *RPL16B* mRNAs results from differential rates of transcription, differences in mRNA stability, or both. However, it is not due to differential mRNA splicing ef-

ficiencies, as the two *RPL16* genes are not interrupted by intervening sequences (J.L. Woolford, R.L. Last, M.O. Rotenberg, and A. Levy, in prep.). Although the arrangement of the tripartite consensus promoter motifs common to yeast ribosomal protein genes (Rotenberg and Woolford 1986; Larkin et al. 1987) differs between the two *RPL16* genes (see Fig. 7; Rotenberg and Woolford 1986), we have not been able to correlate these specific arrangements with levels of transcription of each gene. It seems unlikely that the *RPL16A* and *RPL16B* mRNA differ significantly in their translational efficiency, given our observation that cells containing two copies of *RPL16A* and the *rpl16b* null allele grow at the same rate as cells containing one copy of *RPL16B* and an *rpl16a* null allele.

The growth rates and molecular phenotypes of the *rpl16* mutants suggest that there is no regulatory response to a decrease in *RPL16* gene dosage manifest at the level of increased expression of the remaining *RPL16* gene. There is no dosage compensation of either *RPL16A* or *RPL16B* mRNA abundance in cells containing a null allele of the other gene. Because *rpl16b* mutants containing one-third the wild-type level of *RPL16B* mRNA grow more slowly than wild-type cells, we infer that the stability or translational efficiency of *RPL16* mRNA is not enhanced significantly in the *rpl16* mutant cells.

The failure to recover haploid spore clones containing null mutations in both *RPL16A* and *RPL16B* indicates that L16 is essential for spore germination. Recent experiments using a strain conditional for synthesis of L16 demonstrate that L16 is also necessary for mitotic growth (M. Moritz, unpubl.). Six other eukaryotic (yeast) ribosomal proteins have also been shown to be essential (Abovich et al. 1985; Fried et al. 1985; Kruse et al. 1985; A.G. Paulovich, J.C. Larkin, J.G. Anthony, E.R. Reynolds, and J.L. Woolford, in prep.). This is in marked contrast to the observation, that the absence of any one of at least 16 different ribosomal proteins from *Escherichia coli* is not a lethal event (reviewed in Dabbs 1985).

Why are there two functional copies of most yeast ribosomal protein genes? Although L16a and L16b differ by one amino acid, either protein is sufficient for wild-type growth when supplied in adequate amounts. Cells containing two thirds of wild-type levels of L16 protein (provided by one *RPL16B* gene or two *RPL16A* genes) grow at or near wild-type rates. Cells that contain one third of wild-type levels do not; therefore, there must be a minimum amount of L16 required that is somewhere between one third and two thirds of that which normally accumulates in wild-type cells. We cannot rule out the possibility that one form of L16 serves a specific function under certain physiological conditions not yet tested. However, the existence of functionally distinct subclasses of ribosomes derived from specific combinations of ribosomal proteins contributed by one or the other of each of the duplicate protein genes seems unlikely. Nevertheless, there is at least one precedent in *Plasmodium* for stage-specific ribosomes containing structurally distinct rRNAs (Gunderson et al. 1987).

We have shown that disruption of either *RPL16* gene

leads to excess 40S ribosomal subunits relative to 60S subunits. Ribosomal protein L16 must be necessary for the assembly of stable 60S subunits. In the absence of sufficient L16 protein with which to assemble, other 60S components may be synthesized but are not assembled and are then degraded. Under these conditions, 40S particles do accumulate and are apparently functional, as judged by binding to mRNA. Such turnover of excess molecules produced during ribosome biogenesis also occurs under several other circumstances. When the synthesis of ribosomal proteins is blocked, rRNA is transcribed but is not processed and is instead degraded (Shulman and Warner 1978; Stoyanova and Hadjiolov 1979). When rRNA synthesis or processing is blocked, ribosomal proteins are synthesized but degraded (Craig and Perry 1971; Gorenstein and Warner 1977; Tsurugi and Ogata 1977; Warner 1977; Jacobs et al. 1985; Pierandrei-Amaldi et al. 1985). Several yeast ribosomal proteins have been shown to be degraded when synthesized in excess of other ribosomal components (Abovich et al. 1985; Warner et al. 1985; El Baradi et al. 1986; Y.F. Tsay, J.R. Thompson, J.C. Larkin, M.O. Rotenberg, and J.L. Woolford, in prep.). We have also found that depletion of a 40S ribosomal subunit protein, rp59, results in a decrease in the pool of 40S subunits and accumulation of extra 60S subunits (A.G. Paulovich, J.C. Larkin, J.G. Anthony, E.R. Reynolds, and J.L. Woolford, in prep.). Similar results were obtained for rp51, another 40S subunit protein (Abovich et al. 1985). Accumulation of the pools of either 40S or 60S ribosomal subunits is apparently uncoupled.

Initiation of protein synthesis is slowed in *rpl16* mutants as a consequence of the deficit of functional 60S ribosomal subunits relative to 40S subunits. Half-mer polyribosomes accumulate in both *rpl16a* and *rpl16b* mutants, but to a greater extent in the latter. The average number of ribosomes associated with mRNAs is less in the *rpl16b* mutants than in wild-type strains. Presumably, this difference in the polyribosome size distribution is a result of the decreased rate of initiation of protein synthesis in the *rpl16b* mutants and results in the slower rate of growth of *rpl16b* strains than wild-type strains. The polyribosome size distribution and growth rate of *rpl16a* mutants are not distinguishable from those of wild-type cells, even though *RPL16* mRNA and 60S subunits are depleted by one-third and half-mer polyribosomes accumulate in *rpl16a* cells. This suggests that wild-type yeast contains an excess of both *RPL16* mRNA and functional 60S subunits under the growth conditions tested.

Materials and methods

Nomenclature

The duplicated yeast genes encoding the 60S ribosomal subunit protein L16 are designated *RPL16A* and *RPL16B*, based on standard nomenclature of yeast ribosomal proteins (Warner 1982). We previously denoted this protein rp39 and its genes *RP39A* and *RP39B* (Woolford et al. 1979; Teem et al. 1984; Rotenberg and Woolford 1986).

Plasmids, strains and media

Plasmid pY11-40HB contains a 3.0-kb yeast genomic fragment that includes the intact *RPL16B* gene, cloned between the *Hind*III and *Bam*HI sites of plasmid pBR322. pBR1078B is a derivative of pBR322 that contains the 1.2-kb *Hae*III fragment extending from 1.0 kb upstream of *RPL16A* to nucleotide +148 within the coding region of *RPL16A*. *Bam*HI linkers were added to this *Hae*III fragment, which was then inserted into the *Bam*HI site of pBR322 (Rotenberg and Woolford 1986). Yeast strains used in this study are listed in Table 1. Growth of *S. cerevisiae* was at 30°C in YEPD (1% yeast extract, 2% peptone, 2% dextrose), YEPG (1% yeast extract, 2% peptone, 3% glycerol), or defined synthetic medium with 2% dextrose as a carbon source [Jones and Lam 1973; Mackay and Manney 1974]. Yeast strains were constructed by standard genetic manipulations [Mortimer and Hawthorne 1969]. Matings between cells of the same mating-type (strains JL8Δ116a and JL8Δ116b) were obtained by selection, as described previously [MacKay and Manney 1974; Strathern et al. 1981].

Materials and enzymes

Two oligonucleotides (19-mers), 5' CACTGACTATACCGA-GACC 3' and 5' ATTTCTTCACAACTAATT 3', complementary to sequences within the 3'-noncoding regions of the *RPL16A* and *RPL16B* transcripts, respectively, were obtained from the University of Pittsburgh DNA Synthesis Facility. T4 DNA ligase and [α -³²P]ATP were obtained from New England Nuclear Corp. [γ -³²P]dCTP was purchased from ICN Biomedicals, Inc. Restriction endonucleases, nuclease BAL31, and calf alkaline phosphatase were purchased from Boehringer-Mannheim Biochemicals. *Bam*HI- and *Xho*I-linkers were obtained from New England Biolabs. T4 polynucleotide kinase was a generous gift of Dr. William McClure, Carnegie Mellon University. *E. coli* DNA polymerase I holoenzyme and Klenow fragment were kindly provided by Dr. William E. Brown, Carnegie Mellon University. DEAE-nitrocellulose paper (NA45) was purchased from Schleicher & Schuell.

DNA and RNA isolation and manipulation

Plasmid DNA, DNA restriction fragments, yeast genomic DNA, and yeast RNA were all purified by methods described previously [Rotenberg and Woolford 1986]. Restriction endonuclease digestions, BAL31 digestions, conversion of cohesive ends to blunt ends, and DNA ligations were also all performed as described [Woolford et al. 1979; Last et al. 1984; Rotenberg and Woolford 1986].

Isolation and construction of plasmid DNAs

A yeast genomic *Sau*3A library, cloned into plasmid YEp24 in *E. coli* [Carlson and Botstein 1982] was screened for the presence of plasmids carrying *RPL16A* by the colony hybridization procedure of Gergen et al. [1979]. Seven plasmids were identified in which the cloned yeast DNAs contained overlapping restriction patterns that matched those previously identified for the *RPL16A* genomic region [Woolford et al. 1979]. Therefore, only one plasmid, YEp24L16A-7, was selected for further analysis. *RPL16A* occurs on a 2.3-kb *Bam*HI fragment within the 10-kb genomic insert of this plasmid.

The *RPL16A* deletion-insertion plasmid p116aΔURA was constructed as follows. An *Xho*I linker insertion derivative of plasmid pBR1078B, pBR1078B + 87, was identified in which the *Xho*I linker was inserted between the endpoints of a deletion

Table 1. Yeast strains used in this study

Strain	Genotype
JW100	<i>MATa/MATa ade1/+ gal4/+ GAL80^S/+ leu2-3,112/leu2-3,112 ura3-52/ura3-52</i>
JL8	<i>MATa cry1 leu2-3,112 trp1 ura3-52</i>
JL8Δ116a	<i>MATa cry1 leu2-3,112 trp1 ura3-52 rpl16a :: URA3</i>
JL8Δ116b	<i>MATa cry1 leu2-3,112 trp1 ura3-52 rpl16b :: LEU2</i>
SC252	<i>MATa ade1 leu2-3,112 ura3-52</i>
JW450	<i>MATa/MATa cry1/cry1 leu2-3,112/leu2-3,112 trp1/trp1 ura3-52/ura3-52</i>
MM1401	<i>rpl16a :: URA3/+ rpl16b :: LEU2/+ MATa/MATa ade2-101/+ can1/+ his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1 lys2-801/lys2-801 trp1-Δ101/trp1-Δ101 ura3-52/ura3-52</i>
MM1402	<i>MATa/MATa ade2-101/+ can1/+ his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1 lys2-801/lys2-801 trp1-Δ101/trp1-Δ101 ura3-52/ura3-52 rpl16a :: TRP1/+ rpl16b :: LEU2/+</i>
MM1403	<i>MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ101 ura3-52</i>
MM1404	<i>MATa ade2-101 can1 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ101 ura3-52 rpl16a :: TRP1</i>
MM1405	<i>MATa his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ101 ura3-52 rpl16b :: LEU2</i>

from nucleotides +87 to +89 within codon 30 of *RPL16A* [Rotenberg and Woolford 1986]. *Xho*I linkers were added to the filled-in ends of the 1.1-kb *Hind*III *URA3* fragment of plasmid YEp24. This *URA3* fragment was inserted into the *Xho*I site of plasmid pBR1078B + 87 such that *URA3* is transcribed toward the 3' end of *RPL16A*. The *RPL16A* deletion-insertion mutation *rpl16a :: TRP1* was derived from plasmid pBR1078B + 87. This plasmid was digested with *Xho*I, and the 5' sticky ends of the *Xho*I sites were filled in. The 1.45-kb *TRP1 Eco*RI fragment was purified from plasmid pTC3 [Shaw and Olson 1984], and the sticky ends were filled in. The *TRP1* fragment was ligated into the flush-ended *Xho*I site of pBR1078B + 87 to create plasmid p116aΔTRP, such that *TRP1* is transcribed toward the 5' end of *RPL16A*.

An *RPL16B* deletion-insertion allele was constructed by first linearizing plasmid pY11-40HB at the unique *Kpn*I site near the 3' end of *RPL16B*. The linearized DNA was then treated with nuclease BAL31, as described by Maniatis et al. [1982], and synthetic *Xho*I linkers were attached to the resulting deletion endpoints. The 2.2-kb *Sal*I-*Xho*I fragment of plasmid YEp13 [Broach et al. 1979] containing *LEU2* was converted to an *Xho*I fragment by filling in and linker ligation and was inserted into the *Xho*I site of one of the BAL31 deletion derivatives of pY11-40HB. One resulting plasmid, p116bΔLEU, was chosen for use in further studies. In this construct, the 185bp of *RPL16B* between nucleotide +259 and +443 (codons 87–148) are deleted, and *LEU2* is inserted such that it is transcribed in the direction opposite that of *RPL16B*. A second *RPL16B* deletion-insertion allele was constructed by excising the *LEU2* fragment from p116bΔLEU and inserting in its place the 1.1-kb *Hind*III fragment encoding *URA3*. Prior to ligation, the *Xho*I ends of the opened plasmid and the *Hind*III ends of the *URA3* fragment were rendered flush through treatment with DNA polymerase I holoenzyme. In the resulting plasmid, p116bΔURA, both *Hin*-

dIII sites are regenerated. *URA3* is oriented in pl16bΔ*URA* such that its transcription is toward the 5' end of *RPL16B*.

Plasmids YEp24L16A-5, YEp13L16A, and YCp50L16A were constructed by inserting the 2.3-kb *RPL16A* *Bam*HI fragment of plasmid YEp24L16A-7 into the unique *Bam*HI sites of vectors YEp24 [Botstein et al. 1979], YEp13, and YCp50 [Kuo and Campbell 1983], respectively. Plasmids YEp24L16B, YEp13L16B, and YCp50L16B were similarly constructed by inserting the 3.0-kb *RPL16B* *Hind*III–*Bam*HI fragment of plasmid pY11-40HB into the *Bam*HI sites of vectors YEp24, YEp13, and YCp50. The *RPL16B* *Hind*III–*Bam*HI fragment was converted to a *Bam*HI fragment by linker addition prior to ligation. Integrating plasmids YipL16A and YipL16B were produced by excising the 2.2-kb *Eco*RI fragment carrying the 2 μm origin of DNA replication from plasmids YEp24L16A-5 and YEp24L16B, respectively.

Yeast transformation

Yeast was transformed by the lithium acetate method of Ito et al. (1983), using 2–5 μg of episomal DNAs. Integration of plasmids YipL16A and YipL16B was directed to the *ura3-52* locus of strain JL8Δ*16b* by the method of Orr-Weaver et al. (1981); 10 μg of each plasmid was linearized at the unique *Sma*I site within *URA3* prior to transformation. One-step gene replacements were performed by the method of Rothstein (1983), using 5 μg of linear DNA isolated from plasmids pl16aΔ*URA*, pl16aΔ*TRP*, pl16bΔ*LEU*, or pl16bΔ*URA* (e.g., see Figs. 1 and 2). The stability of all integrative transformants was assessed as described by Last et al. (1984).

Preparation of DNA and RNA dot blot samples

Samples (10 μg) of plasmids YEp13L16A and YEp13L16B were digested to completion with *Bam*HI and extracted once with phenol:CHCl₃ (1:1) and twice with CHCl₃: isoamyl alcohol (24:1). The DNAs were precipitated with ethanol at –80°C, collected by centrifugation, washed twice with 70% ethanol, dried, and suspended in TE to a final concentration of 50 ng/μl. Multiple aliquots of these samples were diluted with sterile water to a volume of 8.3 μl each, heated to 100°C for 10 min, and quick-chilled in an ice-water bath. An equal volume (8.3 μl) of 1.0 N NaOH was added, and the samples were incubated at room temperature for 20 min. All samples were neutralized with 0.5 volumes (8.3 μl) of buffer (1.0 M NaCl, 0.3 M sodium citrate, 0.5 M Tris–HCl (pH 8.0), and 1.0 N HCl); 5.0 μl aliquots of each were then applied to nitrocellulose filters that had been prepared previously, as described by Thomas (1980).

Total RNA, extracted from *S. cerevisiae* strain JL8, was diluted in 6% (vol/vol) formaldehyde to yield final RNA concentrations of 0.2, 0.4, 1.0, 2.0, and 3.0 μg/μl and a total volume of 25 μl for each sample. The samples were heated to 65°C for 5 min and quick-chilled in an ice-water bath then 5.0-μl aliquots of each were applied to nitrocellulose filters. After all DNA and RNA samples had been applied, the filters were permitted to air-dry for 15 min; they were then baked for 2 hr at 80°C in vacuo.

Nucleic acid electrophoresis, transfer, and hybridization

DNA and RNA were fractionated on 1.0 and 1.2% agarose gels, respectively, transferred to nitrocellulose, and hybridized with in vitro-labeled DNA probes, as described previously (Last et al. 1984) or as described below. Hybridization signal intensities were compared by densitometric analysis of several exposures of each hybridization filter and, when possible, by quantitation of a dilution series of each nucleic acid sample. These varia-

tions ensured that the measured signal intensities were within the linear range of sensitivity of both the film and the densitometer. Kodak XAR-5 film and the tungsten lamp of a Zeineh Soft Laser Scanning Densitometer were used for these measurements.

End-labeling of oligonucleotide probes

Each oligomer was end-labeled in a total reaction volume of 15 μl at 37°C for 30 min. Reaction mixtures contained 60 pmoles of oligonucleotide, 60 pmoles of [γ -³²P]ATP (7000 Ci/mmol), 1 × kinase buffer (pH 7.5) [Maniatis et al. 1982], and 10 units of T4 polynucleotide kinase. At the conclusion of the incubation period, reaction mixtures were extracted once with phenol:CHCl₃ (1:1) and twice with CHCl₃: isoamyl alcohol (24:1). Unreacted [γ -³²P]ATP was removed by subjecting each reaction mixture to chromatography through a 0.9-ml Bio-Gel P2 spin column.

Hybridizations with oligonucleotide probes

Prehybridization and hybridization buffers were identical. Each buffer was composed of 5 × SSC, 5 × Denhardt's solution [Maniatis et al. 1982], 0.5% SDS, 100 μg/ml salmon testes DNA, and 50 mM sodium phosphate, pH 7.1. Prehybridizations were for 1–2 hr at 37°C; hybridizations were for 16–20 hr at 37°C with 2–5 × 10⁵ cpm/ml of ³²P-end-labeled oligonucleotide probe. Filters were washed four times for 15 min at 42°C in 5 × SSC and 0.1% SDS, air-dried at room temperature, and subjected to autoradiography at –70°C, as described previously. If filters were to be reprobbed, they were first soaked in boiling water for 10 min to remove old probes.

Fractionation and characterization of polyribosomes

Selected yeast strains were grown to a density of 1.0–1.5 × 10⁸ cells/ml in 200 ml of YEPD or YEPG medium at 30°C. Polyribosomes were extracted and analyzed on 7–47% sucrose velocity gradients essentially as described by Baim et al. (1985) using an ISCO density gradient fractionator, model 640.

The presence and amounts of rRNA molecules in fractions collected from the polyribosome gradients were determined as follows. Two volumes of ice-cold ethanol were added to each fraction; RNA was precipitated at –20°C overnight and pelleted by centrifugation for 5 min at 16,000g. Each pellet was dissolved in 20 mM Tris–HCl (pH 8.0), 0.2 M NaCl, 3 mM EDTA, 0.1% diethylpyrocarbonate, and 1% SDS, and extracted with phenol for 3 min at 65°C. The RNA in the aqueous phase was ethanol precipitated, subjected to gel electrophoresis, and quantitated by gel transfer to nitrocellulose, hybridization, autoradiography, and densitometry, as described above.

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