Yeast Ribosomal Protein L24 Affects the Kinetics of Protein Synthesis and Ribosomal Protein L39 Improves Translational Accuracy, While Mutants Lacking Both Remain Viable†

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ABSTRACT: Four mutant strains from *Saccharomyces cerevisiae* were used to study ribosome structure and function. They included a strain carrying deletions of the two genes encoding ribosomal protein L24, a strain carrying a mutation *spb2* in the gene for ribosomal protein L39, a strain carrying a deletion of the gene for L39, and a mutant lacking both L24 and L39. The mutant lacking only L24 showed just 25% of the normal polyphenylalanine-synthesizing activity followed by a decrease in P-site binding, suggesting the possibility that protein L24 is involved in the kinetics of translation. Each of the two L39 mutants displayed a 4-fold increase of their error frequencies over the wild type. This was accompanied by a substantial increase in A-site binding, typical of error-prone mutants. The absence of L39 also increased sensitivity to paromomycin, decreased the ribosomal subunit ratio, and caused a cold-sensitive phenotype. Mutant cells lacking both ribosomal proteins remained viable. Their ribosomes showed reduced initial rates caused by the absence of L24 but a normal extent of polyphenylalanine synthesis and a substantial in vivo reduction in the amount of 80S ribosomes compared to wild type. Moreover, this mutant displayed decreased translational accuracy, hypersensitivity to the antibiotic paromomycin, and a cold-sensitive phenotype, all caused mainly by the deletion of L39. Protein L39 is the first protein of the 60S ribosomal subunit implicated in translational accuracy.

To understand the function of a complex biological system such as the ribosome, it is first necessary to know the role of its component parts. The recently published structures of the ribosome (*1*, *2*) and its subunits (*3*, *4*) will facilitate the determination of the function of the various ribosomal molecules. The functional roles that ribosomal proteins and rRNA play during the translation process are not well understood. With regard to ribosomal proteins, their conservation during evolution suggests that each is important in some aspect of organelle structure, function, regulation, or assembly (*5*, *6*).

The construction of mutants simultaneously lacking more than one ribosomal protein can aid in the determination of whether the proteins function cooperatively in ribosome assembly and activity. It has been shown that in *Escherichia coli*, surprisingly, one-third of the ribosomal protein genes are not essential (*7*). Moreover, a strain missing as many as four ribosomal proteins is viable, albeit barely (*7*). Nevertheless, several of these dispensable proteins play important roles in ribosome structure or function. For instance, L15 and L24 are necessary for the in vitro assembly of the *E. coli* large subunit. In vivo, mutants lacking L24 are greatly impaired in growth rate and 50S subunit assembly, but mutants lacking L15 are not. *E. coli* mutants lacking L27, which is located in close proximity to the peptidyltransferase center, grow very poorly (*8*).

Studies of yeast mutants lacking individual ribosomal proteins may help unravel eukaryotic ribosome function, and there is a very high degree of conservation between yeast and mammalian ribosomal proteins. In yeast, as in *E. coli*, most ribosomal proteins tested so far play essential roles. However, at least seven *Saccharomyces cerevisiae* ribosomal proteins have been found to be dispensable for ribosome activity (*9*−*12*). These include protein S31 [new nomenclature (*13*); formerly known as S37] and the acidic proteins P1A (YP1α), P1B (L44′ or YP1*β*), P2A (L44 or YP2α), and P2B (L45 or YP2*β*), as well as proteins L24 (L30) and L39 (L46).

Proteins L24 and L39 belong to the excess class of ribosomal proteins present only in eukaryotic and archaebacterial but not eubacterial ribosomes (*6*). Protein L24 is encoded by two functional genes which differ in only 35 of 467 nucleotides, leading to the conservative replacement of 5 of 155 amino acids. Disruption of both genes allows the cells to grow with a slightly longer doubling time than that of wild-type cells. Depletion of L24 has no serious effect on the assembly of the 60S subunit. Polysome profiles, however, suggest that its absence leads to the formation of stalled translation initiation complexes (*11*). Protein L24 is

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homologous to human L30 (*14*) and to HL21/22 from *Halobacterium marismortui* (*15*). Protein L39 is a 51 amino acid ribosomal protein, encoded by a single gene (*16*). It is homologous to rat liver L39 and to L46e, a basic ribosomal protein from *Sulfolobus solfataricus* (*17*). Strains lacking L39 are cold sensitive and have diminished levels of 60S subunits (*12*).

To determine the role of yeast ribosomal proteins L24 and L39, we employed a set of isogenic strains deficient in none, one, or both L24 and L39 ribosomal proteins. Moreover, in the case of L39, we carried out a comparative study of the strain lacking this protein and a strain carrying a mutation in the gene for L39. In all of these strains viability as well as ribosome-dependent functions such as rates of cell growth, protein synthesis, accuracy of translation, resistance to aminoglycoside antibiotics, P- and A-site binding, and cold sensitivity were examined.

MATERIALS AND METHODS

Media and Construction of Mutant Strains. A diploid (J809) that was heterozygous for a *URA3* disruption of *RPL24A* and a *HIS3* disruption of *RPL24B* was kindly provided by Dr. Jon Warner (Department of Cell Biology, Albert Einstein College of Medicine). This diploid was made (*11*) by disrupting the *RPL24* genes in a/α W303 diploid which was constructed from isogenic haploid parents and was homozygous for *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 and his3-11,15* (*18*). Meiotic progeny from J809 was obtained, and the presence of the *RPL24A::URA3* and *RPL24B::HIS3* disruptions was detected, respectively, by growth on media lacking uracil and histidine and by Southern hybridization. All segregants of this diploid were isogenic except for the presence or absence of the *RPL24* disruptions. Seven complete and seven incomplete (three spore) tetrads were analyzed. Eleven Ura^+His^+ segregants were obtained, six of them from complete tetrads. To disrupt the *SPB2* gene encoding the L39 ribosomal protein, a wild-type segregant 2D-J809 and a Ura^+His^+ segregant 2A-J809 lacking both genes for the L24 ribosomal protein were transformed with a *Bgl*II−*Hin*dIII fragment of a pAS128 plasmid (kindly provided by Dr. Alan Sachs, Department of Biochemistry, Stanford University Medical Center) containing the *LEU2* gene surrounded by the *SPB2* flanking regions. Southern blot analysis was used to confirm that the Leu^+ transformants of 2D-J809 and 2A-J809, named L1726 and L1725, respectively, contained only the disrupted *SPB2* and thus did not encode L39. Four L1726 and four L1725 isolates were used for further analyses.

Ribosomal protein L39 was studied also through use of strain YAS216 (*19*), which was isolated by Dr. Alan Sachs and kindly provided by Dr. Jon Warner. This strain contains mutation *spb2-1* in gene *SPB2*, which was shown to encode ribosomal protein L39 (*19*). The rest of its genotype is *his3-11,15 leu2-3,112 trp1-1 ura3-1*. It was compared to its isogenic wild-type strain YAS43. Mutation *spb2-1* is one of nine independent isolates at the *SPB2* locus, which is one of seven loci (*spb1*−*spb7*) in which extragenic cold-sensitive suppressors of a disruption of the gene for the poly(A)-binding protein, *PAB1*, were isolated. These suppressor mutations apparently arise from strong selective pressure caused when *PAB1* is deleted.

Unless otherwise indicated, all strains were grown at 30 °C in YPD (1% yeast extract, 2% peptone, 2% D-glucose).

Sensitivity to Paromomycin. Freshly grown yeast colonies were suspended in water, and 10-fold serial dilutions starting at 10^5 were spotted onto YPD media containing 0, 0.01, 0.02, 0.04, 0.08, 0.1, 0.25, 0.5, 1.0, and 2.0 mg/mL paromomycin. A concentration of 2.0 mg/mL is equivalent to 3.25 mM paromomycin. Growth was examined at 30 °C for 2, 3, 4, 5, and 6 days. Alternatively, the inhibition of growth of wild-type and mutant strains by paromomycin was also measured in YPD medium (*20*).

Preparation of a Yeast Cell-Free System for Translation in Vitro. Cells from wild-type or mutant strains of *S. cerevisiae* were grown to a density of 0.9 absorbance unit at 660 nm in YPD. S30 extracts were prepared according to Leibowitz and co-workers (*21*, *22*) as described recently (*23*) with some modifications. These include the exposure of the S30 extracts to 0.1 mM antibiotic puromycin in the presence of 0.1 mM GTP at 30 °C for 20 min to release the nascent polypeptide chains. The puromycin-treated crude S30 extract was then applied to a Sephadex G-10 column. Excluded fractions with the highest A_{260} were pooled and processed as described (*23*). They contained about 50 A_{260} ribosomes/mL and 6−10 mg of protein/mL.

To obtain ribosomes and soluble protein factors, the S30 extract was centrifuged for 3 h at 125000*g*. The pellet was resuspended in homogenization buffer [30 mM Hepes−KOH (pH 7.4), 100 mM potassium acetate, 2 mM magnesium acetate, and 2 mM dithiothreitol], and ribosomes were obtained after a final centrifugation at 10000*g* for 10 min to remove any insoluble material. To obtain soluble protein factors, two-thirds of the supernatant was subjected to 0−70% ammonium sulfate precipitation and centrifuged at 12000*g* for 10 min, and the pellet was resuspended in homogenization buffer, in which $[Mg^{2+}]$ was raised from 2 to 5 mM. Following dialysis in the same buffer, all aliquots containing ribosomes or soluble protein factors were flushed with nitrogen gas, quick-frozen in liquid nitrogen, and stored at −70 °C. With this method, from a 1 L cell culture, we obtained 1 mL of 200 A_{260} ribosomes/mL and 2 mL of 20 mg of protein/mL.

To prepare ribosomal subunits for hybridization experiments, the 125000*g* pellet was resuspended in a high-salt buffer containing 0.9 M KCl and 12 mM $MgCl_2$ and recentrifuged through a 15−40% linear sucrose gradient at 125000*g* for 6 h. The fractions containing each of the ribosomal subunits were pooled and subjected to a final centrifugation at 125000*g* for 16 h. The two pellets were resuspended in low-salt buffer and stored at −70 °C.

Translation of Poly(U) Templates in Vitro. This was carried out as described recently (*23*), using, per 0.1 mL, 25 μg of deacylated yeast tRNA and 0.3 nmol of [^{3}H]-phenylalanine and 0.23 nmol of L-leucine (for the incorporation of phenylalanine experiments) or 0.3 nmol of [^{3}H]leucine and 0.22 nmol of L-phenylalanine (for the misincorporation of leucine experiments). Translation mixtures also contained 1.5 A_{260} units of ribosomes, 25 μg of poly(U), and 100 μg of soluble protein factors. The accuracy of translation is expressed as the error frequency, i.e., the ratio of the incorporation of [^{3}H]leucine to the combined incorporation of [^{3}H]leucine plus [^{3}H]phenylalanine. It represents the number of errors per translated codon.

For time course measurements of polyphenylalanine synthesis, the translation assay was carried out using [^{3}H]-Phe-tRNA, prepared from a mixture of yeast tRNAs charged with 16 pmol of [^{3}H]Phe (250 000 cpm/A_{260} unit), essentially as described previously for the preparation of [^{3}H]Phe-tRNA from *E. coli* (*24*). Reaction mixtures were incubated at 30 °C for the indicated time intervals, and the reaction was stopped by adding an equal volume of 1 N KOH.

P-Site Binding. The reaction mixture (0.1 mL) contained 80 mM Tris-HCl, pH 7.4, 160 mM NH_4Cl, 11 mM Mg-$(CH_3COO)_2$, 6 mM β-mercaptoethanol, 0.4 mM GTP, and 2 mM spermidine (binding buffer), as well as 2.5 A_{260} units of ribosomes, 40 μg of poly(U), and 1.3 A_{260} units of yeast Ac-[^{3}H]Phe-tRNA prepared from [^{3}H]Phe-tRNA by acetylation (*24*). Reaction mixtures were incubated at 30 °C for 16 min, after which time the reaction was stopped by dilution with cold binding buffer. The ternary complex C formed, *N*-Ac-[^{3}H]Phe-tRNA·80S·poly(U), was immobilized on cellulose nitrate filters and washed three times with the same buffer, and its amount was determined by the radioactively labeled *N*-Ac-[^{3}H]Phe-tRNA in a liquid scintillation spectrometer.

A-Site Binding. To measure A-site binding, all P-sites had to be occupied first with deacylated tRNA. This was achieved by the indicator assay (*25*). Poly(U)-programmed ribosomes were first incubated for 30 min at 30 °C with increasing concentrations of a mixture of deacylated yeast tRNAs and then for 10 min with Ac-Phe-tRNA or Phe-tRNA as indicators at the specified concentrations. Then, puromycin at 1 mM was added, and a further incubation of the mixture for 16 min followed. The amount of Ac-Phe-puromycin or Phe-puromycin formed is a measure of the P-sites that are unoccupied by tRNA. At a molar ratio of 4:1 between $tRNA^{Phe}$ and ribosomes, no product was formed, and therefore, all P-sites were occupied by tRNA.

A-site binding was determined as follows: the reaction mixture (0.1 mL) was composed from the same binding buffer as that used for P-site binding except that it contained $Mg(CH_3COO)_2$ at a final concentration of 15 mM. It also contained 2.5 A_{260} units of ribosomes, 0.1 mg of soluble protein factors, and $tRNA^{Phe}$ and ribosomes at a molar ratio of 4:1. Following incubation at 30 °C for 30 min to fill all P-sites, 1.3 A_{260} units of yeast [^{3}H]Phe-tRNA was added. Reincubation followed for 10 min in order to form the [^{3}H]-Phe-tRNA·80S·poly(U) complex. The reaction was stopped by dilution in ice-cold binding buffer and application of the reaction mixture on cellulose nitrate filter disks. The radioactivity measured represents Phe-tRNA binding to the A-site.

Sucrose Gradient Analysis. This was performed as described by Warner et al. (*26*) with some modifications. To a 200 mL cell culture in mid-log phase was added 100 μg/mL cycloheximide. The culture was swirled rapidly and immediately poured over ice. The cells were collected by centrifugation and washed twice with 10 mM Hepes−KOH (pH 7.4), 100 mM NaCl, and 30 mM $MgCl_2$, containing 100 μg of cycloheximide and 200 μg of heparin per mL. The washed cells were resuspended in 1.2 mL of the same solution in a capped centrifuge tube and disrupted by mixing with glass beads and shaking by hand as described previously (*23*). To this mixture was added another 1.5 mL of solution, and the sample was spun twice at 5000*g* for 5 min. The supernatant was layered over a 15−40% (w/w) linear sucrose gradient in 50 mM Hepes−KOH (pH 7.4), 50 mM NH_4Cl, 12 mM $MgCl_2$, and 1 mM dithiothreitol and centrifuged for 5.5 h at 125000*g* and 4 °C in a Beckman SW41 rotor. The gradients were measured by UV absorption at 260 nm.

Preparation of Ribosomal Proteins. Puromycin-treated crude S30 extracts were centrifuged at 125000*g* for 3 h. The pellet was resuspended in 20 mM Tris-HCl (pH 7.4), 50 mM NH_4Cl, 12 mM $MgCl_2$, and 1 mM dithiothreitol and recentrifuged through a 15−40% linear sucrose gradient in the same buffer in a Beckman SW28 rotor at 125000*g* for 5.5 h. Under these conditions, the polysomes were converted to 80S ribosomes. These 80S ribosomes were pelleted either by centrifugation at 125000*g* for 14 h after a 1:1 dilution in the same buffer or by precipitation with 0.65 volume of absolute ethanol at −20 °C and centrifugation at 30000*g* for 20 min. Proteins were then extracted as described by Barritault et al. (*27*). The [Mg^{2+}] was raised to 0.2 M, 2 volumes of glacial acetic acid was added, and the mixture was left for 45 min. Precipitated RNA was removed by centrifugation at 5000*g* for 10 min. Five volumes of acetone was added to the supernatant, proteins were allowed to precipitate at −20 °C, and the pellet containing the ribosomal proteins was applied to 2-D PAGE.

Two-Dimensional Polyacrylamide Gel Electrophoresis (2-D PAGE). A total of 150 μg of protein was subjected to two-dimensional gel electrophoresis, which was run essentially as described previously (*28*).

Other Techniques. DNA probes were prepared by random priming, using [γ-^{32}P]dCTP (specific activity 3000 Ci/ mmol; Amersham Corp.). All yeast transformations were carried out according to Ito et al. (*29*).

RESULTS

Mutant Strains Carrying Three Disrupted 60S Ribosomal Protein Genes Encoding Ribosomal Proteins L24 and L39 Are Viable. The construction of isogenic strains bearing single and multiple disruptions of genes *RPL24A/RPL24B* and *SPB2* encoding ribosomal proteins L24 and L39, respectively, is described in Materials and Methods. By obtaining the L1725 isolates, we established that yeast cells simultaneously lacking all genes known to encode both the L24 and L39 ribosomal proteins are viable.

Strains lacking *RPL24A/RPL24B* genes were characterized by slightly reduced growth: the doubling time in YPD was 120 min, i.e., 30% lower than that of wild-type cells from strain 2D-J809, in agreement with an earlier observation (*11*). Mutants lacking *RPL24A* and *RPL24B* were also sensitive to paromomycin concentrations higher than 0.25 mg/mL (Figure 1). The reduction of growth rate and paromomycin sensitivity were significantly more extreme in strains lacking *SPB2*, and the effects were further exaggerated in segregants lacking the genes for both the L24 and L39 ribosomal proteins (Figure 1). Indeed, for L1726 and L1725 the doubling time was 180 and 240 min, respectively, and inhibition of growth was observed on media containing more than 0.08 and 0.06 mg/mL paromomycin, respectively.

Yeast cells carrying the mutation *spb2-1* had a doubling time of 160 min at 30 °C, i.e., nearly twice as long as that of the related wild-type strain YAS43 which was used as a

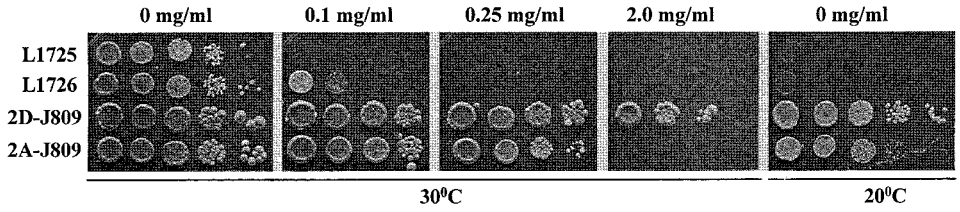

FIGURE 1: Effect of paromomycin and temperature on the growth of mutant strains. Freshly grown colonies of isogenic strains L1725 (-L24-L39), L1726 (-L39), 2D-J809 (wild-type), and 2A-J809 (-L24) were suspended in water, and 10-fold serial dilutions starting at 10^5 cells/mL were spotted onto YPD media containing the indicated concentrations of paromomycin. Plates were incubated for 4 days at the indicated temperature.

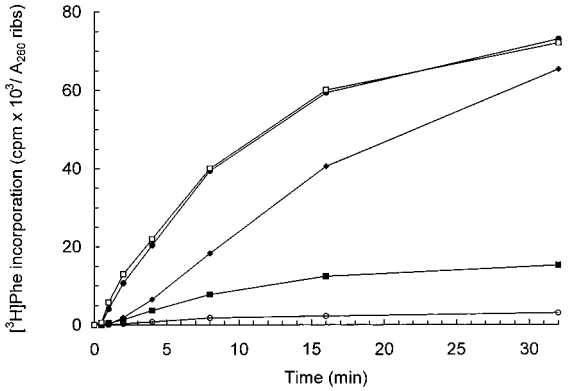

FIGURE 2: Time plots of [^{3}H]phenylalanine incorporation. This was carried out in the presence of soluble protein factors by ribosomes from the (●) 2D-J809 strain, (■) 2A-J809 strain, (□) L1726 strain, and (◆) L1725 strain. The [^{3}H]phenylalanine incorporation of the YAS216 (*spb2-1*) strain is omitted since it is identical to that of the L1726 strain. Wild-type ribosomes from the 2D-J809 strain without soluble protein factors were used as control (○). The specific activity of [^{3}H]phenylalanine was 33.6 Ci/mmol.

Table 1: Polyphenylalanine Formation for Hybrid Ribosomes from the Subunits of Wild-Type and Mutant Strains[a]

source of 60S subunit	genotype	source of 40S subunit	genotype	polyPhe (cpm)	% of control
2D-J809	wt	2D-J809	wt	102 800	100
2A-J809	-L24	2A-J809	-L24	26 000	25
L1726	-L39	L1726	-L39	99 800	97
L1725	-L24-L39	L1725	-L24-L39	91 000	89
2A-J809	-L24	2D-J809	wt	28 000	27
2D-J809	wt	2A-J809	-L24	91 000	89
L1726	-L39	2A-J809	-L24	95 200	93
L1725	-L24-L39	2A-J809	-L24	96 500	94
2D-J809	wt	L1726	-L39	93 000	90
2D-J809	wt	L1725	-L24-L39	94 000	91
YAS216	*spb2-1*	YAS216	*spb2-1*	101 750	99

[a] The total amount of subunits used in each experiment was 1.5 A_{260}/0.1 mL, and the ratio of 60S:40S was 2:1. The soluble protein fractions from each strain were interchangeable and displayed the same activity, showing that the mutations under study do not cause any defect outside the ribosome. The specific activity of [^{3}H]phenylalanine was 33.6 Ci/mmol. Polyphenylalanine formation of the wild-type 80S monosomes was 108 000 cpm or 106% of the control.

control. The growth curve of the latter was identical to that of 2D-J809. The growth curve of *spb2-1* was very similar to that of L1726.

Also, in agreement with previous observations (*12*, *19*), strains lacking L39 were cold sensitive; i.e., they did not grow when spotted on YPD at 20 °C, regardless of the presence of L24 (Figure 1).

Absence of Proteins L24 and L39 in Mutant Ribosomes. The presence or absence, respectively, of proteins L24, L39, or both was confirmed by two-dimensional polyacrylamide gel electrophoresis. Interestingly, there was also no spot corresponding to the L39 protein in the *spb2-1* mutant, suggesting that the *spb2-1* mutant is not just deficient in a functional product (*12*) but does not produce any stable L39.

Protein Synthesis Activity of Wild-Type and Mutant Ribosomes. Using in vitro poly(U)-dependent polyphenylalanine synthesis, we determined the activity of wild-type and mutant ribosomes.

All ribosomes were dependent on the presence of an appropriate amount of soluble protein factors. In their absence, the ribosomes were unable to polymerize phenylalanine (Figure 2, bottom line). As wild type, we routinely used ribosomes from strain 2D-J809, since they exhibited the same polyphenylalanine synthesis activity as well as error frequency as ribosomes from strain YAS43. As shown in Figure 2, mutant ribosomes lacking L24 formed fewer peptide bonds than wild-type ribosomes; both the rate and the extent of polyphenylalanine synthesis were decreased to about 25% of that of the control, despite the relatively small increase of the generation time. In contrast, the activity of ribosomes isolated from either the *spb2-1* or the L39 mutants was not significantly affected; both the rate and the extent of polyphenylalanine synthesis remained similar to those shown by the normal strain (Figure 2), despite the nearly 2-fold increase of the generation time caused by each of these two mutants. These data show that the presence of L39 does not affect polyphenylalanine synthesis. Interestingly, the extent of polyphenylalanine synthesis activity shown by the triple mutant was almost equal to that of the wild type.

To confirm which ribosomal subunit was responsible for the fluctuation in polyphenylalanine activity and to test whether other protein factors, such as the initiation factors, also played a role, we repeated the polyphenylalanine formation experiments using washed ribosomal subunits from the normal strain and from the mutant strains. Large ribosomal subunits were mixed with small ribosomal subunits and reassociated prior to addition to the reaction mixtures for poly(U) translation (Table 1). These subunits were obtained at high KCl concentration at which the initiation factors are removed. The homologous reconstituted ribosomes showed their full polyphenylalanine-synthesizing activity in the presence of the soluble protein fraction containing the elongation factors. This was similar to the activity exhibited by the respective native ribosomes. Thus, the ribosomal subunits were stable during isolation and reconstitution, and unlike the elongation factors, the presence of initiation factors is not required for phenylalanine polymerization. Furthermore, when both subunits came from the L24 mutant, polyphenylalanine formation was reduced to 25% of the normal (Table 1). When the 40S of the L24

Table 2: Error Frequencies in Vitro for Wild-Type and Mutant Ribosomes at 30 °C in the Absence or Presence of Paromomycin (PM)

strain	no. of expts	error frequencies ± SE	
		−PM	+50 μM PM
2D-J809	15	0.0024 ± 0.0005	0.0060 ± 0.0010
2A-J809	18	0.0034 ± 0.0007	0.0070 ± 0.0008
L1726	6	0.0095 ± 0.0011	0.0195 ± 0.0022
L1725	6	0.0110 ± 0.0015	0.0205 ± 0.0025
YAS43	6	0.0023 ± 0.0006	0.0057 ± 0.0012
YAS216	12	0.0086 ± 0.0010	0.0182 ± 0.0017

mutant was replaced by the 40S of wild type, the L39 mutant, or the triple mutant or by the 40S of the *spb2-1* mutant, the polyphenylalanine activity remained equally low, showing that the 60S subunit of the L24 mutant is responsible for this decrease. Conversely, substitution of this subunit by the 60S of wild type, the L39 mutant, the triple mutant, or *spb2-1* mutant restored polyphenylalanine formation to nearly 100%. Moreover, we checked the possibility that, in mutant strains in which the ratio 60S to 40S has been decreased, the excess 40S subunits may be inactivated by cytoplasmic enzymes (*30*). Such strains in our study are the two L39 mutants and the triple mutant, as will be shown from the ribosome profiles. However, no such deficiency in the activity of these 40S subunits was observed (Table 1).

Effect of Mutations on Translational Accuracy. The cell-free system also allowed measurement of the misincorporation of the near-cognate amino acid leucine with poly(U) as template. The accuracy of translation was determined by the error frequency. As shown in Table 2, the error frequency of the wild-type strain was 2.4 errors every 10^3 codons. This error rate is slightly higher than the 1.3 errors every 10^3 codons reported recently for a wild-type strain with a different genotype (*23*), but it is still within the range reported in the literature (*31*−*33*).

Four clear conclusions emerge from the results depicted in Table 2: first, the absence of L24 had essentially no effect on accuracy; second, the absence of L39, on the contrary, caused a 4-fold decrease in the accuracy of translation; third, the loss of accuracy shown by the triple mutant was caused almost exclusively by the absence of protein L39 and little or not at all by the concomitant absence of L24; fourth, the deletion of L39 and the *spb2-1* mutation had a nearly identical impact on the accuracy of translation.

The very slight increase in the error frequency of the triple mutant over the mutant lacking only L39 is fully accounted for by a similar insignificant increase in the L24 mutant over the wild type. We suggest that L39 is the first protein of the large ribosomal subunit implicated in the maintenance of translational accuracy.

Effect of Paromomycin on the Accuracy of Translation in the Presence of Mutants. Paromomycin is an error-inducing antibiotic which was also found to stimulate phenylalanine incorporation in wild-type and mutant ribosomes (*23*). Significantly, however, the increase in leucine misincorporation was even higher for each strain, including the wild type, than the respective increase in polyphenylalanine formation. A detailed account of the paromomycin effect on accuracy is shown in Table 2. Paromomycin at 50 μM increased the in vitro error frequency in all strains. The error frequency of ribosomes from the wild type increased by 0.0036, from 0.0024 to 0.0060, and that of ribosomes from the L24 mutants increased from 0.0034 to 0.0070. The two increases were identical, and this is further evidence that L24 plays no role in translational accuracy.

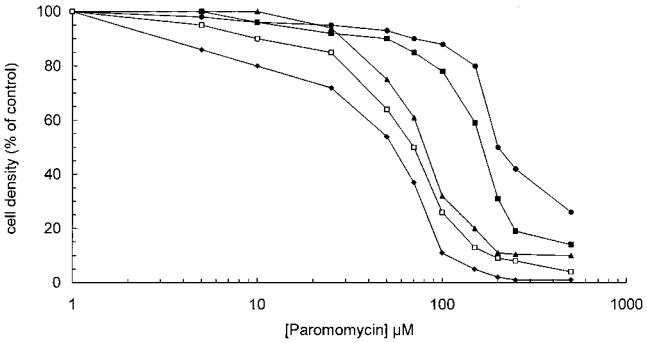

FIGURE 3: Inhibition of growth in the presence of paromomycin. Cells were grown in YPD at 30 °C to an A_{660} of 0.9, which was taken as 100%. Concentrations of paromomycin are as indicated. Curves: (●) 2D-J809 strain, (■) 2A-J809 strain, (▲) YAS216 strain, (□) L1726 strain, and (◆) L1725 strain.

In contrast to the above, the error frequencies of the L39 mutant and the triple mutant showed much higher increases from 0.0095 and 0.0110 to 0.0195 and 0.0205, respectively, in the presence of paromomycin. These are synergistic increases. If they were independent and additive, we would obtain an increase of 0.0036 + 0.0095 = 0.0131 rather than 0.0195 for the L39 mutant and 0.0036 + 0.0110 = 0.0146 rather than 0.0205 for the triple mutant. These differences in error frequencies, 0.0064 and 0.0059, respectively, indicate that paromomycin and the absence of L39 had a synergistic effect on the decrease of translational accuracy.

Strains Lacking L39 Are Hypersensitive to Paromomycin. The loss of translational accuracy has been linked to hypersensitivity of the mutant strains to antibiotics such as paromomycin. We tested whether the higher error frequencies shown by the L39, *spb2-1*, and triple mutants are accompanied by increased sensitivity of the cells toward paromomycin. The results are shown in Figure 3. The growth of the wild-type strain was inhibited by 50% at 200 μM paromomycin. Deletion of both L24 genes resulted in only a small increase of sensitivity of the cells toward paromomycin (165 μM for 50% inhibition). In contrast, the other three strains which showed increased error frequencies also displayed a significant increase in the sensitivity to paromomycin. Thus, 50% inhibition of growth was observed at only 70, 82 and 55 μM paromomycin for the L39, *spb2-1*, and triple mutant, respectively.

Ribosome Activity and P-Site Binding. Under the conditions described in Materials and Methods, Ac-Phe-tRNA is preferentially bound to the P-site. This was confirmed by the finding (results not shown) that the ribosome-bound Ac-Phe-tRNA was fully reactive toward puromycin (1 mM, 16 min).

The P-site binding capacities of the mutant strains under study were determined as the percent of the P-site binding capacity of the wild-type strain. The results are summarized in Table 3. The P-site binding of the L24 mutant was only 30% of that of the wild type and follows a similar decrease in polyphenylalanine-synthesizing activity.

A-Site Binding. Following the full occupation of the P-site by tRNA, we measured A-site binding by adding Phe-tRNA

Table 3: Binding Capacities of P- and A-Sites of Wild-Type and Mutant Ribosomes[a]

strain	no. of expts	P-site binding (% of control)	A-site binding (% of control)
2D-J809	7	100	100
2A-J809	7	30	93
L1726	7	95	162
L1725	7	86	173
YAS216	7	97	160

[a] For wild-type ribosomes, the amount of Ac-[^{3}H]Phe-tRNA bound to the P-site was 1.4 pmol/0.1 mL of reaction mixture, while the amount of [^{3}H]Phe-tRNA bound to the A-site was 2.0 pmol/0.1 mL of reaction mixture.

and measuring the radioactivity of cellulose nitrate filter disks as described in Materials and Methods. The results are expressed as the percent of the radioactivity contained in the complex Phe-tRNA·80S·poly(U) of the wild-type strain. It can be seen (Table 3) that the L24 mutant strain showed an A-site binding equal to that of the wild type. In contrast, A-site binding was higher in the L39, *spb2-1*, and triple mutant strains. As shown in a previous paragraph, the L39 and triple mutant strains are prone also to translational errors.

Effect of the Absence of L24 and L39 on Polysome Profiles. As expected from a previous report (*11*), the polysome profiles of the strain lacking L24 were defective, as they contained peaks corresponding to stalled translation initiation complexes (Figure 4B). These are probably 43S complexes, also known as half-mers, consisting of the 40S ribosomal subunit with attached initiation factors awaiting the addition of the 60S ribosomal subunit. They are stalled on the mRNA due either to a shortage of free 60S subunits or to defects in the available 60S subunits. Since the amount of 60S subunits was normal, it is suggested that the 60S subunits are defective and could not bind to the translation initiation machinery. Ribosome profiles also indicated a significant decrease in the amount of 80S ribosomes. This implies that L24 may be involved in 60S to 40S subunit interactions, either directly or through other factors.

The ribosome profile of cells containing either the *spb2-1* mutation or the deletion of L39 displayed also some 43S complexes as well as a significant decrease in the relative amount of the 60S ribosomal subunit compared to the 40S ribosomal subunit at 30 °C (Figure 4C). A slight decrease in the amount of 80S ribosomes was also observed.

The polysome profile of the triple mutant included apparently additive aberrations contributed by the absence of the two proteins (Figure 4D). Thus, the half-mers and the decrease in 80S monosomes contributed mainly by the absence of L24 were observed. Less marked was the decrease in the amount of 60S caused by the absence of L39, but this is explained by the fact that 60S subunits are supplied from the reduction of 80S caused by the deletion of L24. These characteristics of the polysome profiles agree well with the fact that, for optimal polyphenylalanine synthesis in vitro, the concentration of Mg^{2+} required is highest in the strains showing a decrease in the amount of 80S, i.e., in the mutant lacking L24 and in the triple mutant (results not shown). It is also interesting to note that a slight shift exists in the positions of the 80S and 60S peaks in the triple mutant, caused by the total absence of these two 60S ribosomal subunit proteins.

Absence of L39 Causes Increased Translational Infidelity at Low Temperature. The fact that the absence of protein L39 confers cold sensitivity to the respective strains was confirmed by the doubling times obtained at the near-restrictive temperature of 23 °C (Table 4a). The strains

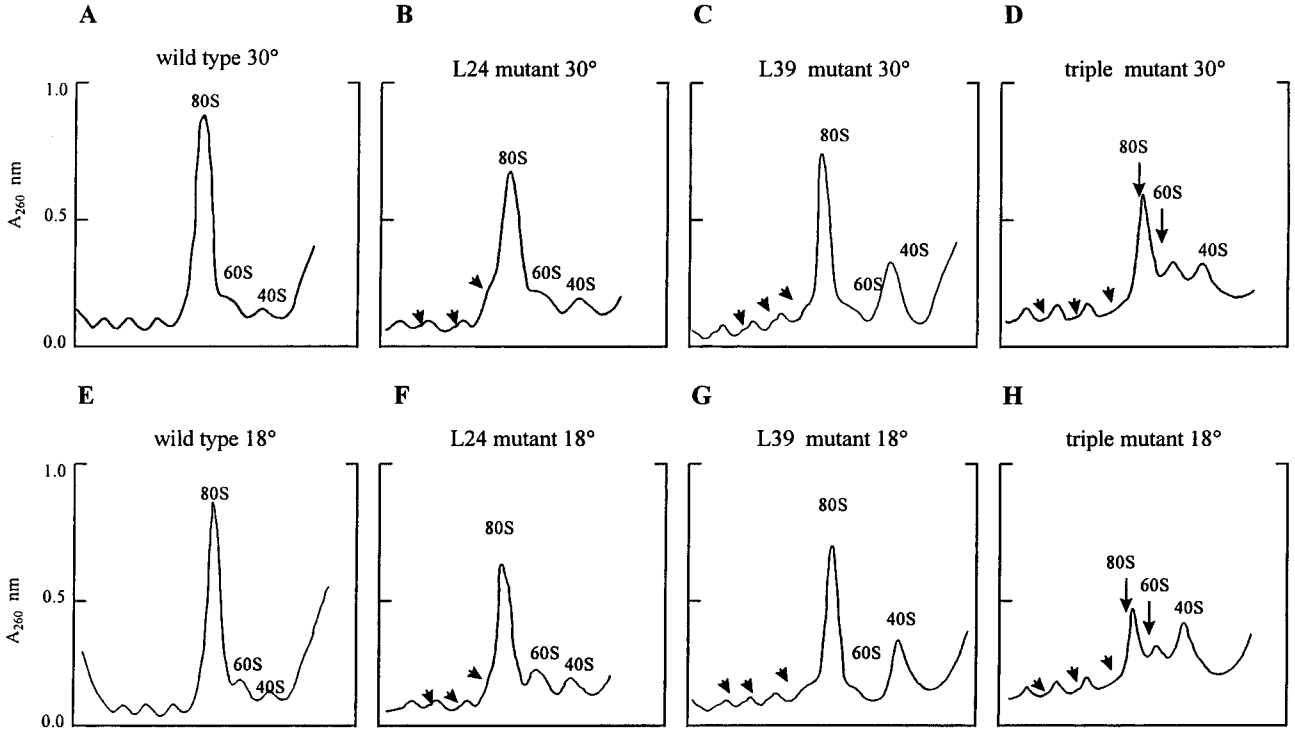

FIGURE 4: Polyribosome profiles at 30 °C (A−D) and 18 °C (E−H): (A, E) 2D-J809 strain, (B, F) 2A-J809 strain, (C, G) L1726 strain, and (D, H) L1725 strain. The profile of the YAS216 (*spb2-1*) strain is omitted since it is identical to that of L1726 at both 30 and 18 °C. Peaks representing half-mers are marked by short arrows. Note also the shift in the position of the monosomes and the large ribosomal subunits at 30 °C (D) and 18 °C (H). In this case the position of the normal 80S and 60S is marked by long arrows.

Table 4: Effect of Cold Sensitivity (a) on the Growth, (b) on the Sensitivity to Paromomycin, and (c) on the Accuracy of Translation[a]

strain	(a) doubling times (min), 23 °C	(b) [PM] (μM) for 50% inhibition, 23 °C	[PM] (μM)	(c) error freq ± SE, 18 °C	ratio
2D-J809	105	190	0	0.0034 ± 0.0006	1.0
			50	0.0047 ± 0.0008	1.4
2A-J809	148	135	0	0.0048 ± 0.0006	1.4
			50	0.0068 ± 0.0010	2.0
L1726	340	45	0	0.0400 ± 0.0038	11.7
			50	0.0795 ± 0.0054	23.4
L1725	485	39	0	0.0378 ± 0.0031	11.1
			50	0.0894 ± 0.0061	26.3
YAS216	320	52	0	0.0340 ± 0.0028	10.0
			50	0.0687 ± 0.0042	20.2

[a] Cells were grown at 30 °C and then shifted to 18 °C for 3 h, and ribosomes were isolated and assayed in vitro at 18 °C in the presence or absence of paromomycin.

lacking protein L39 showed increased hypersensitivity toward paromomycin at 23 °C compared to 30 °C (Table 4b). This effect was mainly the result of the absence of L39 and less of the absence of L24.

Finally, we tested the effect of cold sensitivity on polyphenylalanine formation and on the accuracy of translation. Cells were grown at 30 °C, shifted to 18 °C for 3 h, and then assayed at 18 °C. The amount of polyphenylalanine formed did not change significantly (not shown), while the accuracy of translation deteriorated enormously both in the absence and in the presence of paromomycin (Table 4c). Ribosomes from the triple mutant strain translated in the presence of 50 μM paromomycin in vitro with an error frequency of 89.4 amino acid misincorporations per 1000 codons and in the absence of paromomycin with an error frequency of 37.8 amino acid misincorporations per 1000 codons. Such high error frequencies may help explain the inhibition of cell growth at 18 °C. Again, this high error frequency was the additive result of the individual increases in error frequencies of the L24 and L39 mutants, and as shown in Table 4c, it was overwhelmingly the result of the absence of L39.

The cold-sensitive phenotype was reversed after cells, grown at 30 °C and shifted to 18 °C overnight, were brought back to 30 °C. Also, a 2-D PAGE of ribosomal proteins prepared from cells grown at 30 °C and shifted to 18 °C for 3 h (not shown) displayed no differences in the number or position of the proteins.

The effect of cold sensitivity on the polysome profiles of the various strains is shown in Figure 4E−H. There was a significant further decrease in the 80S ribosomes and in the 60S to 40S ratio especially of the triple mutant, while there was no substantial change in the profile of the mutant lacking L24 or the wild type.

DISCUSSION

The first major finding of the present work is that cells lacking three genes encoding two proteins, L24 and L39, of the large ribosomal subunit were viable and grew, albeit at a reduced rate (Figure 1). A similar case has been reported for a yeast quadruple-disruption strain, lacking all four ribosomal acidic proteins (*10*).

The concomitant absence of proteins L24 and L39 is, however, not without an effect on yeast ribosome activity as well as cell metabolism. Apart from a decrease in the cell growth rate, the most visible consequences are changes in the protein synthesis activity of the mutant ribosomes, a cold-sensitive phenotype, and a decrease in the accuracy of translation. The extent to which each of these effects can be attributed to one or the other protein was determined from the study of the individual mutants lacking L24 or L39, respectively.

There is evidence (*10*, *19*) supporting a role for 60S subunits in the control of translation initiation. The formation of stalled translation initiation complexes in the absence of L24 (Figure 4) accompanied by stability in the ratio of 60S to 40S subunits supports its involvement in the initiation of protein synthesis (*11*). Its absence also alters the protein−protein cross-linking patterns in the yeast 60S ribosomal subunit, and this alteration is involved in 60S to 40S subunit interactions. There was a substantial decrease in both the rate and the extent of protein synthesis compared to wild type (Figure 2). The decrease in the extent to 25% of the wild type can be explained by a corresponding decrease in P-site binding (Table 3). A similar result has been reported for *E. coli* ribosomal protein L1 (*34*). Mutants lacking L1 showed about 50% reduced capacity for in vitro protein synthesis accompanied by a reduction in P-site binding. From the hybridization experiments it became clear that the decrease in polyphenylalanine formation is specifically associated with the 60S subunit of the L24 mutant (Table 1). No major impact on other functions, such as the fidelity of translation, sensitivity to antibiotics, and sensitivity to temperature, could be attributed to this protein. It is, therefore, likely that the main role of protein L24 is in the kinetics of the process of protein synthesis. Protein L24 may belong to a class of ribosomal proteins that improves on an existing ribosomal function rather than adding a novel one.

Mutation *spb2-1*, on the other hand, suppresses the inhibition of translation initiation resulting from deletion of the poly(A)-binding protein gene, *PAB1* (*12*). This finding implies an involvement of protein L39 in the translation process, at a stage immediately prior to the initiation of translation and, therefore, not affected by polyphenylalanine synthesis. Indeed, Figure 2 shows there was no effect of the *spb2-1* mutation on polyphenylalanine formation. In addition, the *spb2-1* mutation decreased the amount of 60S ribosomal subunits (Figure 4C). Hence, the process in which L39 is likely to be involved is during formation of the large ribosomal subunit. Also, the decrease in 60S subunits is accompanied by an overproduction of 40S subunits. This represents an enormous waste of energy (*30*) compared to the L24 mutant, in which the ratio of 60S to 40S remains unaffected (Figure 4B). This drain of energy may contribute to the slow growth of the *spb2-1* mutant compared to the L24 mutant.

As shown by gel electrophoresis, the *spb2-1* mutation did not produce any stable L39. Further, we showed that, phenotypically, the deletion of L39 and mutation *spb2-1* are equivalent. The somewhat higher error frequencies and sensitivity to paromomycin displayed by the deletion mutant are due to the fact that the deletion of an entire gene is slightly more powerful than a point mutation.

The presence of L39 does not affect polyphenylalanine synthesis (Figure 2). Rather, the detection of the L39 effect on protein synthesis in vitro might require use of a natural message carrying a poly(A) tail instead of poly(U). In addition, the removal of L39 on top of that of L24 restored the extent of polyphenylalanine formation to wild-type levels, although the initial rates of this reaction were considerably lower than those of the wild type. This apparent suppression of the L24 effect indicates a possible participation of L39 in the process of protein synthesis.

Further study of the L39 and the *spb2-1* mutants unraveled some striking properties of protein L39. Most notable of these is that L39 is involved in the control of translational accuracy. In the absence of L39, the accuracy of translation decreased by a factor of 4 compared to wild type (Table 2). A 4-fold decrease of translational accuracy was also found in ribosomal protein S4 mutants (*35*). Protein S4 is an essential 40S protein that belongs to the accuracy center of the yeast ribosome. Moreover, as reflected by the noneffect of the L24 mutant on accuracy, the effect of L39 cannot be adequately described as a nonspecific effect caused by an impaired ribosome. Our results indicate that a protein of the large ribosomal subunit participates in the control of translational accuracy, probably through changes in the 60S ribosomal subunit.

The decoding process is controlled by the small ribosomal subunit. Recently, however, events occurring in the 30S decoding site were linked with those taking place in the 50S subunit (*1*). Thus, the possible participation of L39 and, therefore, of the 60S ribosomal subunit in translational accuracy is representative of the notion that the parts of the ribosome interact.

The notion that L39 affects the accuracy of translation was confirmed by the fact that the absence of L39 and paromomycin had a synergistic effect on mistranslation (Table 2). This hypersensitivity to paromomycin is typical of mutations decreasing translational accuracy. Similar cooperativity was observed recently between mutations in proteins S4 and S28, which actually belong to the yeast accuracy center, and paromomycin (*23*, *36*).

Besides the higher intrinsic misreading, cells carrying *spb2-1* or deletion of L39 displayed increased sensitivity to paromomycin in vivo. On the contrary, deletion of L24 had only a slight effect on paromomycin sensitivity (Figure 3).

Our results showed a large increase of A-site binding in the L39 and triple mutants compared to wild type (Table 3). An increase of A-site binding may arise from a higher affinity for accepting noncognate tRNAs and leads to a higher level of translational errors (*37*, *38*). Our results confirm this notion since the L39 and triple mutants are prone also to translational errors (Table 2). On the contrary, lack of L24 did not affect A-site binding.

Another important feature of protein L39 is that, in its absence, the mutant strain developed a cold-sensitive phenotype which was accompanied by a further drastic decrease of the accuracy of translation and hypersensitivity of this strain toward antibiotics such as paromomycin at the restrictive temperature (Table 4). Such a notion has been proposed for cycloheximide-resistant temperature-sensitive lethal mutations of *S. cerevisiae* (*39*). The cold-sensitive phenotype was reversed upon return to 30 °C, suggesting that the increase in translational errors to almost 1 every 10 codons at 18 °C (Table 4c) inhibited growth but did not cause cell death.

Protein synthesis per se excluded, the other ribosomal functions tested were additive. Also, they are overwhelmingly the result of the absence of L39 rather than that of L24. Thus, in the translational accuracy experiments (Table 2), the increase in the error frequencies of the triple mutant over the wild type (0.0086) is accounted for by the sum of the increases of the error frequencies of the individual mutants (0.0010 + 0.0071). Moreover, in the presence of paromomycin (Table 2), an increase of 0.0145 in the triple mutant over the wild type is equal to the sum of the increases of the individual mutants (0.0010 + 0.0135). Similarly, additive results were obtained from the experiments on the sensitivity to paromomycin in vivo (Figure 3), as well as from the cold-sensitivity experiments (Table 4 and Figure 4E−H).

In conclusion, one of the mutants under study (L39) has two phenotypes, cold sensitivity, and paromomycin sensitivity, and at least one of these can be explained by increased translational misreading. For this mutant none of the phenotypes is explained by impairment of a major step of translation such as polyphenylalanine synthesis. The other mutant (L24) is clearly defective in ribosome assembly with no obvious effect on translational accuracy.

Finally, we showed that ribosomal proteins, even excess ones present only in eukaryotic cells and not found in eubacteria, may have distinct and significant roles in both the assembly and the function of the eukaryotic ribosome. Specifically, protein L24 participates in the kinetics of translation, whereas protein L39 decreases translational accuracy probably through an altered 60S ribosomal subunit.

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