Yeast Ribosomal Protein Deletion Mutants Possess Altered Peptidyltransferase Activity and Different Sensitivity to Cycloheximide†

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ABSTRACT: The major function of the ribosome is its ability to catalyze formation of peptide bonds, and it is carried out by the ribosomal peptidyltransferase. Recent evidence suggests that the catalyst of peptide bond formation is the 23S rRNA of the large ribosomal subunit. We have developed an in vitro system for the determination of peptidyltransferase activity in yeast ribosomes. Using this system, a kinetic analysis of a model reaction for peptidyltransferase is described with Ac-Phe-tRNA as the peptidyl donor and puromycin as the acceptor. The Ac-Phe-tRNA−poly(U)−80S ribosome complex (complex C) was isolated and then reacted with excess puromycin to give Ac-Phe-puromycin. This reaction (puromycin reaction) followed first-order kinetics. At saturating concentrations of puromycin, the first-order rate constant (k_3) is identical to the catalytic rate constant (k_{cat}) of peptidyltransferase. This k_{cat} from wild-type yeast strains was equal to 2.18 min^{-1} at 30 °C. We now present for the first time kinetic evidence that yeast ribosomes lacking a particular protein of the 60S subunit may possess significantly altered peptide bond-forming ability. The k_{cat} of peptidyltransferase from mutants lacking ribosomal protein L24 was decreased 3-fold to 0.69 min^{-1}, whereas the k_{cat} from mutants lacking L39 was slightly increased to 3.05 min^{-1} and that from mutants lacking both proteins was 1.07 min^{-1}. These results suggest that the presence of ribosomal proteins L24 and, to a lesser extent, L39 is required for exhibition of the normal catalytic activity of the ribosome. Finally, the L24 or L39 mutants did not affect the rate or the extent of the translocation phase of protein synthesis. However, the absence of L24 caused increased resistance to cycloheximide, a translocation inhibitor. Translocation of Ac-Phe-tRNA from the A- to P-site was inhibited by 50% at 1.4 μM cycloheximide for the L24 mutant compared to 0.7 μM for the wild type.

We recently showed that proteins L24 and L39 of the large ribosomal subunit of *Saccharomyces cerevisiae* possess distinct functions, the former affecting the kinetics of protein synthesis and the latter improving translational accuracy (*1*). However, neither protein is essential for cell viability. Furthermore, their combined mutant lacking the two genes encoding L24 and the single gene encoding L39 is viable (*1*). They also belong to the excess class of ribosomal proteins present only in eukaryotic and archaebacterial but not eubacterial ribosomes (*2*, *3*). Thus, in addition to rRNA, ribosomal proteins, even nonessential ones, may also play an active role in ribosomal function.

Mutations in or deletions of ribosomal protein and rRNA genes are a useful tool with which to unravel ribosomal structure and function (*4*, *5*). In fact, studies of yeast mutants have divulged valuable information about several aspects of ribosomal function except about the main function of the ribosome, i.e., its ability to catalyze peptide bond formation. This is carried out by ribosomal peptidyltransferase, an enzymatic activity associated with the large ribosomal subunit. Recent biochemical and crystallographic evidence suggests that the ribosome is a ribozyme and its catalytic properties are carried out by an active site consisting entirely of rRNA (*6*, *7*). Moreover, in vivo mutational analysis of a single universally conserved nucleotide, A2451 (*Escherichia coli* numbering), within the central loop of domain V of 23S rRNA indicated that it has a pivotal role in ribosomal function (*8*). A mechanism is proposed in which this nucleotide serves as a general acid base during peptide bond formation (*8*). Within this framework, what do all the ribosomal proteins do? It seems that the ribosome is dependent on structural support from protein components; while substantially deproteinized large ribosomal subunits still carry out peptidyl transfer, complete deproteinization destroys this reactivity (*9*). Thus, it is likely that ribosomal proteins buttress, stabilize, and orient the otherwise floppy RNA into a specific, active structure (*10*).

In the present work, we have developed an in vitro system for the determination of peptidyltransferase activity in yeast ribosomes via a rate constant which is independent of concentration terms. Such a suitable system is the reaction between puromycin and a ribosomal complex bearing the donor substrate prebound; in this reaction single peptide bonds are formed, and the peptidyltransferase of each complex works only once. This reaction can be analyzed as a first-order reaction. At saturating concentrations of puro-

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mycin, the first-order rate constant is a measure of the catalytic rate constant (k_{cat}) of peptidyltransferase in the puromycin reaction.

We have used the puromycin reaction in the past as a model reaction for peptidyltransferase both in *E. coli* (*11*) and in rabbit reticulocytes (*12*). It is, however, worthwhile to make this reaction available in yeast too, not only because yeast is a widely studied eukaryote but also because there is a high degree of conservation between yeast and mammalian ribosomal components. This reaction is employed for the first time to determine the peptidyltransferase activity of yeast mutant ribosomes lacking one or more large ribosomal subunit proteins, specifically ribosomal proteins L24, L39, or both. Our results suggest that the presence of ribosomal protein L24 of the 60S subunit improves the catalytic properties of yeast peptidyltransferase.

Finally, the functional study of ribosomal proteins L24 and L39 was supplemented by determination of their effect on translocation, a major step of protein synthesis. Our results indicate that, while these mutants do not affect translocation, one of them, L24, shows increased resistance toward cycloheximide, an inhibitor of eukaryotic translocation.

MATERIALS AND METHODS

Media and Construction of Mutant Strains. The experiments described were performed using *S. cerevisiae* wild-type strain 2D-J809, strain 2A-J809 lacking the two genes encoding L24, strain L1726 lacking the single gene encoding L39, and triple mutant strain L1725 lacking both proteins. The construction of these strains, the media used, and confirmation of gene disruptions were all carried out as described recently (*1*).

Isolation of Ribosomes and the Fraction Containing the Soluble Protein Factors. 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 (1% yeast extract, 2% peptone, 2% D-glucose). Cells were broken and crude S30 extracts prepared by centrifugation at 30000*g* for 20 min as described previously (*13*), except that the homogenization buffer was a low-salt buffer containing 10 mM Tris-HCl (pH 7.4), 10 mM potassium chloride, 5 mM magnesium acetate, and 10 mM β-mercaptoethanol (buffer A) as well as 1 mM phenylmethanesulfonyl fluoride (*14*). Ribosomes and soluble protein factors were produced following centrifugation of the crude S30 extract for 3 h at 125000*g*. The pellet was resuspended in buffer A, and ribosomes were obtained after a final centrifugation at 10000*g* for 20 min to remove any insoluble material. To obtain soluble protein factors, two-thirds of the supernatant was subjected to 70% ammonium sulfate precipitation and centrifuged at 12000*g* for 10 min. The pellet was resuspended in buffer A and dialyzed in the same buffer. The S125 fraction provides the synthetases necessary for the preparation of Ac-[^{3}H]Phe-tRNA from yeast. All aliquots containing ribosomes or soluble protein factors were flushed with nitrogen gas, quick-frozen in liquid nitrogen, and stored at −70 °C. 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.

Alternatively, high-salt-washed ribosomes were prepared by resuspending the "unwashed" ribosomes, prepared as previously described, in a buffer containing 30 mM Tris-HCl, pH 7.4, 500 mM ammonium chloride, 10 mM potassium chloride, 100 mM magnesium acetate, 250 mM sucrose, and 5 mM β-mercaptoethanol (buffer B). Following mild shaking for 12 h at 4 °C, an equal volume of buffer A was added, and centrifugation followed at 125000*g* for 2 h, after which the pellet was resuspended in buffer A. After a further centrifugation at 10000*g* for 20 min, the washed ribosomes were distributed in appropriate aliquots and stored at −70 °C. The supernatant was subjected to 70% ammonium sulfate precipitation and centrifuged at 12000*g* for 10 min, and the pellet with the soluble protein factors was resuspended in buffer A containing 5% glycerol. Following dialysis in the same buffer, the aliquots were stored at −70 °C.

Preparation of Ac-[^{3}H]Phe-tRNA from Yeast. The peptidyltransferase activity assay, i.e., the puromycin reaction, was carried out using Ac-[^{3}H]Phe-tRNA as donor, prepared from a mixture of yeast tRNAs using yeast synthetases from the fraction containing the soluble protein factors, in a similar way to that described previously for Ac-[^{3}H]Phe-tRNA from *E. coli* (*11*). The final solution was 200 A_{260}/mL and contained 14.4 pmol of [^{3}H]Phe charged/1 A_{260} tRNA and 280 000 cpm incorporated/1 A_{260} tRNA.

Preparation and Isolation of Complex C. Complex C, i.e., the Ac-[^{3}H]Phe-tRNA−poly(U)−80S ribosome complex, was formed from unwashed or high-salt-washed ribosomes in a buffer containing 80 mM Tris-HCl, pH 7.4, 160 mM ammonium chloride, 11 mM magnesium acetate, 2 mM spermidine, and 6 mM β-mercaptoethanol (binding buffer), as well as 0.4 mM GTP, 30 A_{260}/mL 80S ribosomes, 0.4 mg/mL poly(U), and 13 A_{260}/mL Ac-[^{3}H]Phe-tRNA from yeast. When high-salt-washed ribosomes were used, 0.6 mg/mL of the fraction containing the initiation factors was added. For the experiments on the effect of magnesium on yeast peptidyltransferase activity, the concentration of Mg^{2+} in the binding buffer varied from 8 to 20 mM. Complex C was formed by incubation of the above binding mixture (0.2 mL) for 16 min at 30 °C. The reaction was stopped by placing the binding mixture in ice. The solution was immediately filtered through a cellulose nitrate filter disk under vacuum with three 4 mL portions of binding buffer, without allowing air to pass through the filter. These operations (filtering and washings) were carried out within 2 min. The disk was then immersed quickly into ice-cold binding buffer, cut in half, and used within 2 h. Alternatively, complex C was extracted off the disk in an active form according to a method published previously (*15*). Briefly, the filter disks containing the adsorbed complex C were subjected to gentle shaking for 30 min at 5 °C in binding buffer containing 0.05% Zwittergent 3-12 (ZW) (1.8 mL/whole disk). The extraction was stopped by removing the filters from the extraction mixture. Then the ZW extract containing complex C was kept in ice and used in the puromycin reaction.

Puromycin Reaction. The reaction between the Ac-[^{3}H]-Phe-tRNA bound in complex C and puromycin (puromycin reaction) was carried out under three different conditions.

Condition A: The puromycin reaction was carried out with complex C adsorbed on a cellulose nitrate filter disk (disk reaction) at 30 °C. Puromycin monohydrochloride (0.1 mL) at the desired concentrations was added in 0.9 mL of binding buffer. Where mentioned, the antibiotic sparsomycin was added at the specified concentrations. The puromycin reaction

was started by adding a half disk bearing complex C. At the time intervals indicated, the reaction was stopped with 1.0 mL of 1 N NaOH. If N_0 represents the total radioactivity of bound donor (e.g., Ac-[^{3}H]Phe-tRNA), the percentage (x) of the bound donor that was converted to product P (Ac-[^{3}H]Phe-puromycin) was calculated by dividing P by N_0 and multiplying by 100. Controls without puromycin were included in each experiment, and the values obtained were subtracted. Each percentage (x) was corrected, first, by dividing its value with factor A ($A = C/C_0$, where C and C_0 are the amounts of surviving complex C in binding buffer at the end of each incubation period and at zero time, respectively), and, second, with the extent factor α. The extent factor is determined when complex C is allowed to react completely, at any concentration of puromycin.

By the first correction (x/A), the parallel inactivation of complex C during the puromycin reaction is subtracted, while by the second correction the intervention of any species other than complex C is erased as if 100% of the bound Ac-[^{3}H]-Phe-tRNA were reactive toward puromycin. Thus, the experimental values of x were corrected by factor $A\alpha$, i.e., $x' = x/A\alpha$.

Condition B: The puromycin reaction was carried out with complex C in the ZW extract (reaction in solution). ZW extract (0.9 mL) was preincubated for 5 min at 30 °C. The reaction was started by adding puromycin dissolved in a buffer (0.1 mL), so that the final reaction mixture (1.0 mL) contained the appropriate concentrations of ions as well as the desired concentration of puromycin. At the time intervals indicated the puromycin reaction was stopped by the addition of 1.0 mL of 1.0 N NaOH. The corrected percentage ($x' = x/A\alpha$) of the ribosome-bound Ac-[^{3}H]Phe-tRNA that reacted with puromycin was determined as described under condition A.

Condition C: The puromycin reaction was carried out exactly as in condition B except that complex C was formed from high-salt-washed ribosomes and the fraction containing the initiation factors.

Resistance of Cells to Cycloheximide. Cells in the late log phase from wild-type or mutant cultures were inoculated into YPD medium which contained cycloheximide at increasing concentrations. When each strain without cycloheximide reached an optical density of 0.9 at 660 nm, the incubation was stopped, and the inhibition of growth of each strain was measured from the respective decrease in the optical density. In this way, the concentration of cycloheximide required for 50% inhibition of growth of each strain was determined.

In Vitro Translation in the Presence of Cycloheximide. The in vitro poly(U)-dependent translation assay was used (*13*) except that the reaction mixtures contained cycloheximide at the indicated concentrations. The increased resistance to cycloheximide of mutant compared to wild-type ribosomes was determined from the increase in the concentrations of cycloheximide needed for 50% inhibition of polyphenylalanine synthesis activity.

Translocation Assays. The reaction mixture for translocation (0.1 mL) was composed from the same binding buffer as that used for the formation of complex C, as well as 0.4 mM GTP, 25 A_{260}/mL of ribosomes, 0.4 mg/mL of poly(U), and tRNA^{Phe} at a ratio of 4:1 to ribosomes. At this ratio, all P-sites were occupied by tRNA (*1*). Following incubation for 30 min at 30 °C to fill all P-sites, 13 A_{260}/mL of yeast Ac-[^{3}H]Phe-tRNA were added, and $Mg(CH_3COO)_2$ was raised to 15 mM. Reincubation followed for 10 min at 30 °C in order to form a poly(U)-programmed ribosomal complex in which P-sites were filled with tRNA and A-sites with Ac-[^{3}H]Phe-tRNA.

The reaction was stopped by placing the binding mixture in ice. Filtering, washings, and extraction of the ribosomal complex into binding buffer containing 0.05% Zwittergent 3-12 (ZW) at 5 °C were carried out as described previously. ZW extract was preincubated for 2 min at 30 °C. Also, EF2 from rabbit reticulocyte or soluble protein factors (SPF) from yeast were preincubated with 1 mM GTP, 40 mM phosphocreatine, and 40 μg/mL creatine phosphokinase for 15 min at 37 °C. The translocation reaction was started when GTP-activated EF2 at 0.02 μM or SPF at 0.05 mg/mL was added to the pretranslocation ZW complex and allowed to react for the indicated time intervals. Single turnover translocation (EF2:active ribosomes = 7:1) was monitored by reaction with 2 mM puromycin ($3K_s$) for 4 min (7 half-lives) at 30 °C. The puromycin solution contained cycloheximide at a final concentration of 8 μM to ensure that no translocation takes place during the puromycin reaction.

Alternatively, to determine the sensitivity of the various strains toward cycloheximide, the antibiotic was added at the appropriate concentrations together with the SPF or EF2 fractions, the reaction was allowed to proceed for 1 min, and translocation was again monitored with the puromycin reaction.

Nonenzymatic translocation, i.e., translocation in the absence of elongation factors, was also checked and taken into account.

RESULTS

Ribosomes Active in the Puromycin Reaction. Complex C, prepared as described in Materials and Methods, contained about 18.7% of the input Ac-Phe-tRNA (2.6 A_{260} units or 37.4 pmol of Ac-Phe-tRNA), since only 18.7% of the input of radioactivity was adsorbed on the cellulose nitrate filter disk. Thus, complex C contained 7.0 pmol of Ac-Phe-tRNA. Assuming a 1:1 combination, this complex C also engages 6.5% of the ribosomes added (6.0 A_{260} units or 107.3 pmol).

Puromycin Reaction. The reaction to determine the activity of peptidyltransferase in vitro was carried out in two steps. In step 1, complex C, i.e., the ternary complex composed of the unwashed yeast ribosome, Ac-Phe-tRNA as the donor, and poly(U) as the message, was isolated on cellulose nitrate filter disks or desorbed therefrom into a 0.05% Zwittergent (ZW) solution. In step 2, complex C was allowed to react in a first-order reaction with excess puromycin (S) according to the scheme:

$$C + S \overset{K_s}{\rightleftharpoons} CS \xrightarrow{k_3} C' + P \qquad (1)$$

In this way, all reactions involved in the binding of the donor Ac-Phe-tRNA to the ribosomes (step 1) have been separated from peptide bond formation (step 2). It was thus possible to evaluate two distinct parameters of in vitro peptide bond formation (step 2): first, the final extent (or final degree) of the puromycin reaction, which determines the percentage of the total donor (N_0) that was bound at the ribosomal P-site and converted to product at infinitive time;

Table 1: Parallel Inactivation of Complex C in ZW Solution

exposure time (min)	% of surviving C[a]	
	2D-J809	L1725
0	100	100
1	99.90	98.93
2	99.48	98.62
3	99.05	98.31
4	98.74	98.00
8	98.21	97.39
16	96.50	94.52
32	92.24	90.49
64	86.57	84.60

[a] Complex C from wild-type strain 2D-J809 or from triple mutant strain L1725 was exposed for various time intervals at 30 °C. The surviving complex C was titrated by reaction with 2 mM puromycin for 4 min.

second, the activity of complex C as expressed by the first-order rate constant (k_3). This rate constant gives the reactivity of the P-site-bound donor Ac-Phe-tRNA and is equal to the catalytic rate constant (k_{cat}) of peptidyltransferase.

Activity of Complex C. Complex C reacted with puromycin under three different conditions. Under condition A, complex C containing unwashed ribosomes adsorbed on a cellulose nitrate filter disk reacted with puromycin. Under condition B, complex C containing unwashed ribosomes was first desorbed from the disk into a 0.05% ZW solution and then reacted with puromycin. Under condition C, complex C formed from high-salt-washed ribosomes and the fraction containing translation factors reacted also in ZW solution with puromycin. Our results showed that the cellulose nitrate filter disk affects the kinetics of the reaction, since under condition A the final extent was only 25% and the k_{obs} at 2 mM puromycin was 0.083 min $^{-1}$. Nevertheless, the reaction of Ac-Phe-tRNA with puromycin still followed pseudo-first-order kinetics (data not shown). In contrast, complex C in ZW solution allowed the determination of a much higher final extent and k_{obs}, regardless of whether complex C was formed from unwashed ribosomes (condition B) or from high-salt-washed ribosomes and initiation factors (condition C). In the former case, the k_{obs} at 2 mM puromycin was 1.61 min $^{-1}$ and the final extent was 62%, while in the latter case 1.52 min $^{-1}$ and 61%, respectively. Thus, we chose condition B and determined the activity of peptidyltransferase using complex C formed from unwashed ribosomes and desorbed into ZW solution prior to its reaction with puromycin.

First-Order Kinetic Analysis of Peptidyltransferase Activity. In the reaction described by eq 1, the product (P) is Ac-Phe-puromycin, and it was quantitated by extraction in ethyl acetate. The donor was converted to a form (tRNA) in which it could not participate in re-forming complex C. Thus, each catalytic center of peptidyltransferase could work only once. The reaction between the isolated complex C (C) and excess puromycin (S) proceeded as a pseudo-first-order reaction in which C was converted to C′ (eq 1). The parallel inactivation of complex C during the puromycin reaction was determined, and the amount (x) of Ac-Phe-tRNA that was converted to product was corrected with factor A (x/A). Complex C was remarkably stable since only 3.5% of the bound Ac-Phe-tRNA was dissociated after 16 min, a time interval longer than even the longest time interval used in the puromycin reaction (Table 1). The amount of donor that reacted with puromycin was also corrected with extent factor α. The differential rate equation of the first-order reaction (eq 2) leads to the integrated rate law given by eq 3, in which at each concentration of puromycin (S) the corrected values of $x' = x/A\alpha$ were obtained for various time intervals (t) and fitted in (*11*)

$$dp/dt = k_3[S][C_0 - P]/(K_s + [S]) \quad (2)$$

$$\ln[100/(100 - x')] = k_{obs}t \quad (3)$$

According to this analysis, the apparent rate constant k_{obs} is a function of [S], and it is given for each time point (t) by the equation

$$k_{obs} = k_3[S]/(K_s + [S]) \quad (4)$$

Figure 1A shows typical first-order time plots for Ac-[^{3}H]-Phe-puromycin formation in the presence of different puromycin concentrations. The reaction followed first-order kinetics up to more than 90% depletion over a wide range of puromycin concentrations. The slopes of these straight lines give the k_{obs} values at each puromycin concentration. Equation 4 predicts that a plot of the reciprocal of the experimental k_{obs} versus the reciprocal of the puromycin concentration (double-reciprocal plot) should be linear. The values of k_{obs} were fitted in eq 4 from which the double-reciprocal plot gave $k_3 = 2.18$ min $^{-1}$ and $K_s = 0.65$ mM (Figure 1B). By comparison, the reaction of the disk-adsorbed complex C with puromycin gave $k_3 = 0.16$ min^{-1} and $K_s = 1.25$ mM (data not shown).

Additional evidence showing that peptidyltransferase activity was monitored by this system was provided by the use of sparsomycin, a well-known inhibitor of both eukaryotic and prokaryotic peptide bond formation. Thus, at 2×10^{-4} M puromycin, the k_{obs} value of the reaction was 0.50 min $^{-1}$, whereas in the presence of 5×10^{-7} M sparsomycin this value was decreased to 0.23 min^{-1}.

Effect of Mg^{2+} on Yeast Peptidyltransferase Activity. There is a fine balance between the concentrations of the divalent ion (Mg^{2+}) and the monovalent ion (NH_4Cl or KCl) for full exhibition of *E. coli* peptidyltransferase activity in vitro (*11*, *16*). The results reported here for yeast peptidyltransferase were obtained with 11 mM Mg^{2+} and 160 mM NH_4Cl, both in the binding of the donor (step 1) and in peptide bond formation per se (step 2). To examine whether 11 mM was the optimal concentration of Mg^{2+}, we varied its concentration both in step 1 and in step 2 of the puromycin reaction from 8 to 20 mM, while keeping the concentration of NH_4Cl constant at 160 mM. Complex C formed in step 1 in the presence of 20 mM Mg^{2+} reacted subsequently in step 2 with puromycin in a complex reaction that was not first order, since the logarithmic time plot was not linear. To calculate the final extent, it was assumed that the reaction of the faster reacting species has reached the end point at a time comparable to the time needed by the standard reaction to reach its end point, i.e., 4 min at 2 mM puromycin. In the presence of 11 mM Mg^{2+} during binding, the k_{obs} at 2 mM puromycin was more than 2-fold higher than in the presence of 15 or 20 mM Mg^{2+} while the final extent remained the same (Table 2). By further varying the concentration of Mg^{2+}, we determined essentially two values for this k_{obs}, one around 1.60 min^{-1} and the other around 0.70 min^{-1} (data

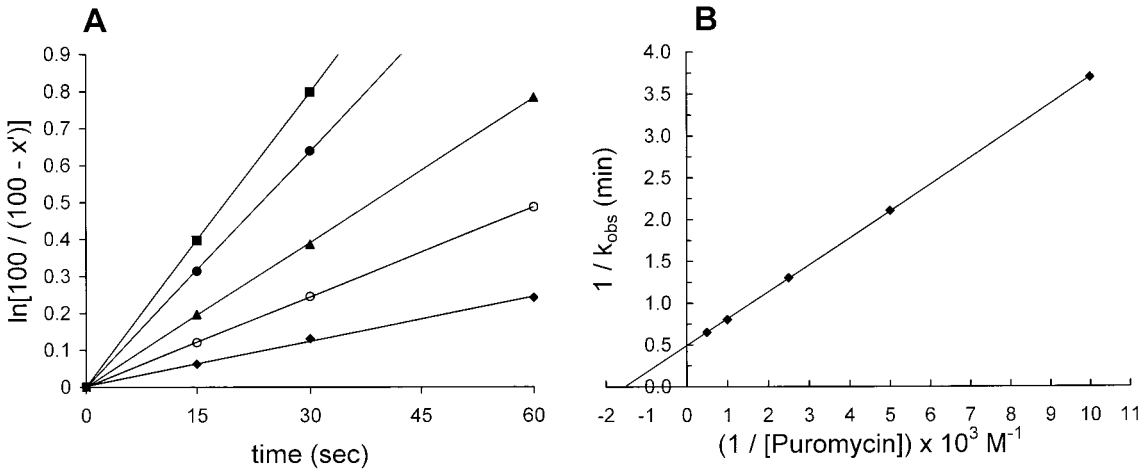

FIGURE 1: First-order analysis of the puromycin reaction. (A) First-order time plots for Ac-[^{3}H]Phe-puromycin formation. Complex C desorbed from the disk into a ZW solution reacted at the indicated time intervals with puromycin at the following concentrations: (■) 2 mM; (●) 1 mM; (▲) 0.4 mM; (○) 0.2 mM; (◆) 0.1 mM. (B) Double-reciprocal plot ($1/k_{obs}$ versus 1/[puromycin]) for Ac-[^{3}H]Phe-puromycin formation. The lines representing product formation at the various concentrations of puromycin have been obtained by the use of values (x) corrected by the application of the extent factor α.

Table 2: Effect of Various Concentrations of Mg^{2+} on the k_{obs} and the Final Extent of the Puromycin Reaction in ZW Solution

[Mg^{2+}] in step 1 (mM)	[Mg^{2+}] in step 2 (mM)	k_{obs}[a] (min^{-1})	final extent (%)
11	11	1.61 ± 0.11	62 ± 4
15	11	0.73 ± 0.09	57 ± 4
20	11	0.65 ± 0.06	58 ± 3
11	15	1.56 ± 0.12	61 ± 5
11	20	1.59 ± 0.11	57 ± 4

[a] The k_{obs} values were determined at 2 mM puromycin.

not shown). The same applies for all k_{obs}. In contrast, there is only one value for the final extent, around 60% (Table 2). Furthermore, changes in the concentration of Mg^{2+} during step 2 did not affect the properties of peptidyltransferase (Table 2). From the double-reciprocal plot of the various k_{obs} versus the concentrations of puromycin, the values of K_s and k_3 were calculated (data not shown). K_s was equal to 0.65 mM for concentrations of Mg^{2+} during step 1 between 8 and 20 mM, whereas there were essentially two values for k_3, one equal to 2.18 min^{-1} for concentrations of Mg^{2+} between 8 and 12 mM and a lower one, equal to 0.95 min^{-1} for concentrations of Mg^{2+} between 13 and 20 mM.

Deletions of Ribosomal Protein Genes Affect the Activity of Yeast Ribosomal Peptidyltransferase. In the experiments described in this paragraph, we determined for the first time the peptidyltransferase activity of yeast deletion mutants. We chose three previously studied deletion mutant strains, one lacking ribosomal protein L24, a second lacking L39, and a third lacking both proteins. Proteins L24 and L39 affect different parameters of protein synthesis (*1*). Similar to the wild-type complex C, complex C from the three mutant strains was tested for parallel inactivation during the puromycin reaction. As shown in Table 1, only 5.5% of Ac-Phe-tRNA was dissociated from complex C of the L1725 mutant after 16 min in ZW solution, an amount similar to that of the wild type. Nevertheless, the amount of donor that was converted to product was corrected by dividing its value with factor A as well as with extent factor α. The reaction of mutant complex C, formed in 11 mM Mg^{2+}, with puromycin followed also pseudo-first-order kinetics and the logarithmic

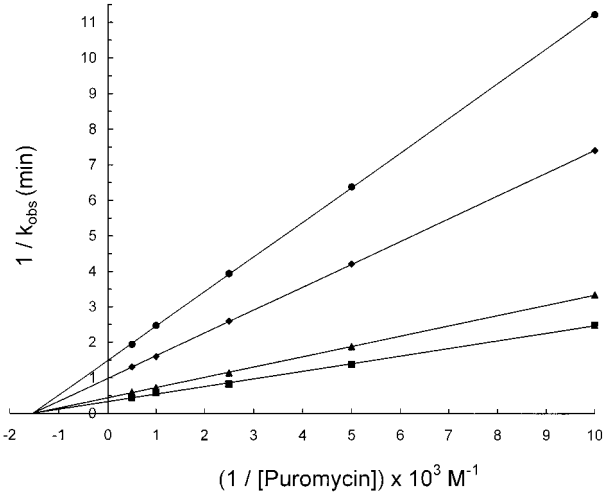

FIGURE 2: Double-reciprocal plots ($1/k_{obs}$ versus 1/[puromycin]) for the puromycin reaction of complex C formed with ribosomes from the (■) L39 mutant strain, (▲) wild-type strain, (◆) triple mutant strain, or (●) L24 mutant strain. From these plots, the K_s and k_3 values for each strain may be determined.

Table 3: Kinetic Parameters of Wild-Type and Deletion Mutant Ribosomes

strain	protein missing	complex C formed (cpm/50 μL)	final extent (%)	k_3 (min^{-1})	K_s (mM)	k_3/K_s (min^{-1} mM^{-1})
2D-J809	none	34 000	62 ± 4	2.18 ± 0.18	0.65	3.35
2A-J809	L24	14 600	53 ± 3	0.69 ± 0.09	0.64	1.08
L1726	L39	39 600	56 ± 5	3.05 ± 0.25	0.62	4.92
L1725	L24, L39	36 800	59 ± 6	1.07 ± 0.12	0.66	1.62

time plots were again linear (data not shown). Equation 4 predicts that double-reciprocal plots for each mutant strain should also be linear (Figure 2). From these plots it was calculated that the catalytic rate constants (k_3) for the L24, L39, and triple mutant strains are 0.69, 3.05, and 1.07 min^{-1}, respectively (Table 3). Thus, the mutant lacking L24 formed peptide bonds at one-third the rate of the wild-type strain (2.18 min^{-1}). In contrast, the mutant lacking L39 showed a small but significant increase of its k_3. Finally, the k_3 of the triple mutant strain was higher than the k_3 of the mutant lacking L24 but lower than the k_3 of the mutant lacking L39.

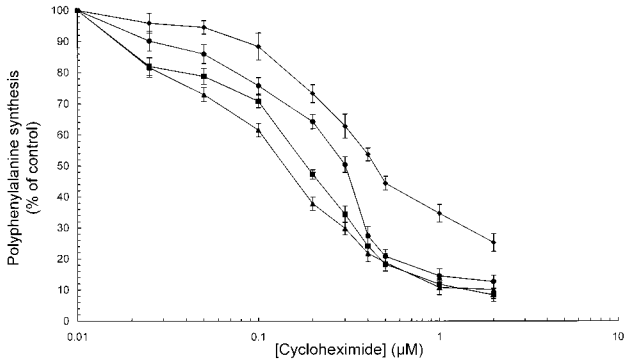

FIGURE 3: Inhibition of polyphenylalanine synthesis in the presence of cycloheximide. The extent of polyphenylalanine synthesis of each strain in the absence of antibiotic was taken as 100%. Plots: (▲) wild-type ribosomes; (■) L39 mutant ribosomes; (●) L24 mutant ribosomes; (◆) triple mutant ribosomes.

The lack of interaction between the two proteins, suggested by the absence of a synergistic effect, may be explained by recent crystallographic data (*6*) that the *Haloarcula marismortui* homologues of L24 and L39 are relatively far apart in the ribosome.

The equilibrium constant K_s remained roughly the same for the wild type and the three mutants (Figure 2).

Table 3 summarizes the amounts of donor bound to complex C per 50 μL of binding mixture, the k_3 values, the K_s values, the extents of the puromycin reaction, and ratios k_3/K_s of the various deletion mutants and compares them to the respective values of the wild-type strain. It is worth noting that ribosomes from the L24 mutant bound less donor Ac-Phe-tRNA, and the extent of the bound donor that reacted with puromycin was somewhat reduced. Thus, not only is the term N_0 (see Materials and Methods) lower, but the percent of N_0 that reacted with puromycin (C_0) is also reduced. Finally, the ratio k_3/K_s, which is a measure of the activity status of peptidyltransferase, was significantly decreased to 1.08 min^{-1} mM^{-1} for L24 mutant ribosomes compared to 3.35 min^{-1} mM^{-1} for wild-type and 4.92 min^{-1} mM^{-1} for L39 mutant ribosomes.

Increased Resistance of L24 Mutants to Cycloheximide. It has been suggested that mutations conferring resistance to cycloheximide exhibit a wide range of phenotypes (*17*). Cycloheximide is an inhibitor of polypeptide chain translocation. We examined the effect of cycloheximide on the growth of cells as well as on ribosome function in wild-type and mutant strains.

The concentration of cycloheximide required for 50% inhibition of growth was 12 μg/L for wild-type cells, 28 μg/L for L24 mutant cells, 15 μg/L for L39 mutant cells, and 35 μg/L for triple mutant cells. Thus in the absence of protein L24 yeast cells become over twice as resistant to cycloheximide as either wild-type or L39 mutant cells.

Next, we examined the activity of wild-type and mutant ribosomes in the presence of cycloheximide. The concentration of cycloheximide required for 50% inhibition of polyphenylalanine synthesis was estimated at 0.14 μM for wild-type ribosomes, 0.30 μM for L24 mutant ribosomes, 0.18 μM for L39 mutant ribosomes, and 0.44 μM for triple mutant ribosomes (Figure 3). These results are in agreement with those reported for the inhibition of growth of the cells and show that ribosomes from the two strains lacking L24 exhibit increased resistance to cycloheximide. In contrast, ribosomes from the strain lacking L39 exhibit sensitivity toward cycloheximide similar to that of wild-type ribosomes.

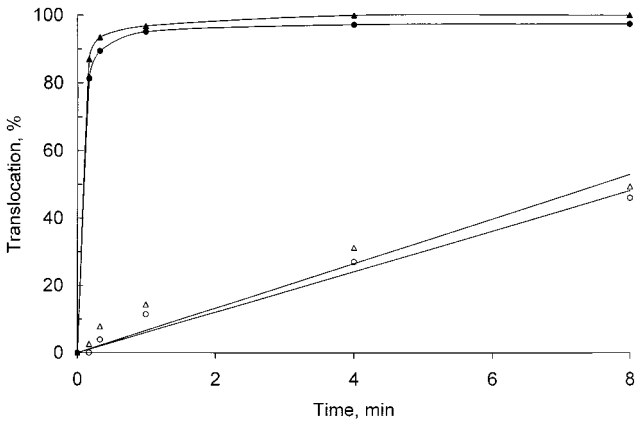

FIGURE 4: Time course of single turnover translocation: wild-type ribosomes (▲) in the presence of 0.02 μM EF2 or (△) in its absence and L24 mutant ribosomes (●) in the presence of 0.02 μM EF2 or (○) in its absence. 100% indicates the extent of translocation of the wild-type strain with EF2. Translocation was determined by titration with 2 mM puromycin for 4 min as described in Materials and Methods.

Translocation Experiments. Translocation was titrated with 2 mM puromycin for 4 min at 30 °C. It was found that high concentrations of cycloheximide, while inhibiting translocation, did not affect the puromycin reaction (results not shown). Thus, cycloheximide was used for two purposes: first, prior to the puromycin reaction, at several concentrations, with the objective to determine the concentration(s) at which translocation of the wild-type or the L24 mutant strain is inhibited, and second, during the puromycin reaction, at high concentrations, to prevent translocation during the titration process.

Under conditions of single turnover, either EF2 (0.02 μM) or the fraction containing the soluble protein factors (SPF) promoted rapid translocation that was complete within 20 s in both strains as measured by the puromycin reaction (Figure 4). The puromycin reaction can detect changes in a reaction which takes place in a time period of $\geq$5 s.

Our results, corrected by the extent factors 0.62 and 0.53 of the puromycin reaction for the wild type and the L24 mutant, respectively, showed that elongation factor-dependent translocation from the A- to P-site of the L24 mutant was 97.4% of that of the wild type (Figure 4). These results show that translocation was equally efficient for the two strains.

In the absence of elongation factors, translocation was slower and proceeded in a time-dependent manner (Figure 4).

To test the resistance of each strain toward cycloheximide, we added this antibiotic in descending concentrations together with elongation factors for the specified time intervals and titrated the amount of Ac-Phe-tRNA translocated from the A- to P-site with the puromycin reaction (Figure 5). The results showed that translocation was inhibited by 50% at 0.7 μM cycloheximide in the wild-type strain and at 1.4 μM cycloheximide in the L24 mutant strain. Consequently, the L24 mutant strain was shown to be more resistant to this antibiotic. These results are in agreement to those reported earlier.

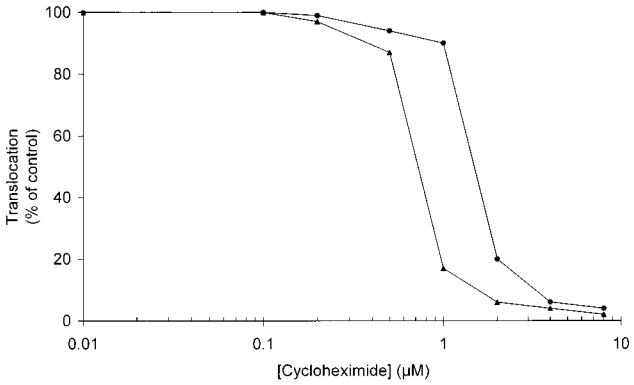

FIGURE 5: Inhibition of EF2-dependent translocation in the presence of cycloheximide. The percentage of the P-site translocated Ac-Phe-tRNA was titrated with 2 mM puromycin for 4 min at 30 °C. The extent of translocation in the absence of antibiotic was taken as 100%. Plots: (▲) wild-type ribosomes; (●) L24 mutant ribosomes.

DISCUSSION

New insights into the function of yeast ribosomes can be gained by the use of an in vitro method such as the puromycin reaction. Recent crystallographic and biochemical evidence that A2451 of 23S rRNA is responsible for the catalytic activity of the ribosome (*6*, *7*) can now be supplemented by this reaction, and an important parameter of protein synthesis, i.e., peptide bond formation, can be evaluated. Using this reaction, we were able to determine for the first time the true effect of the deletion of one or more ribosomal protein genes on peptide bond formation. Moreover, the puromycin reaction may be used in order to explore the mechanisms of action of several antibiotics and other ligands on ribosomal function and provide information about antibiotic binding sites on the ribosome.

The puromycin reaction was carried out as a pseudo-first-order reaction since puromycin (S) reacted in great excess over complex C (C) and formed a single peptide bond in Ac-Phe-puromycin. The reactivity of the donor, which is part of complex C, is expressed through an apparent rate constant, k_{obs} (eq 2), and not through the initial velocity of the reaction. The value of k_{obs} is independent of the initial concentration of the donor but still depends on the concentration of puromycin. Additional evidence that this method measures peptide bond formation was provided by the fact that this k_{obs} was substantially decreased in the presence of sparsomycin, a peptidyltransferase inhibitor. The maximal value (k_3) of the k_{obs}, at saturating concentrations of puromycin, is also a measure of the activity of peptidyltransferase expressed as its catalytic rate constant (k_{cat}). Thus, both the k_3 and the k_3/K_s ratio are independent of the puromycin concentration as well as of the population of active ribosomes. Therefore, k_3 would be the same regardless of whether the percentage of active ribosomes was higher or lower than the 6.5% reported here.

It has been suggested that, in general, enzymes display different kinetic constants when immobilized on solid supports (*18*). It may even be argued that complex C free in solution is closer to the natural conditions of the cell. The ratio k_3/K_s is an accurate measure of the activity status of peptidyltransferase, since it determines the specificity of complex C toward puromycin, in analogy to the analysis of the single substrate enzyme reactions (*19*). Thus, the ratio k_3/K_s is 3.35 min^{-1} mM^{-1} for the ZW reaction compared to 0.128 min^{-1} mM^{-1} for the disk reaction. Therefore, yeast peptidyltransferase is 26.2 times more active when complex C is free in solution. By comparison, *E. coli* and rabbit reticulocyte peptidyltransferase were also more active in ZW solution, albeit by a factor of only 3 (*12*, *15*). It is possible that the more marked change of yeast peptidyltransferase activity between the two media is a closer reflection of the properties of eukaryotic peptidyltransferase in general.

As shown in Table 2 the activity status of yeast peptidyltransferase, similar to that of *E. coli* peptidyltransferase (*20*), is dependent on the concentration of the divalent ion, Mg^{2+}, during the formation of complex C (step 1). Complex C assumes its fully active conformation when it is formed in the presence of Mg^{2+} between 8 and 12 mM. A similar range of Mg^{2+} concentrations for fully active complex C has been reported in *E. coli* too (*20*). Since Mg^{2+} was found to be present in the peptidyltransferase center of archaebacteria (*6*), our finding suggests that the Mg^{2+} binding site is conserved throughout prokaryotes and eukaryotes.

It has now been confirmed that rRNA is the main contributor of peptidyltransferase activity and that ribosomal proteins play, at best, supporting roles (*6*, *7*, *9*). The proteins are dispersed throughout the structure and mostly concentrated on the ribosome's surface, but they are largely absent from the regions that are of primary functional significance to protein synthesis: the small ribosomal subunit interface and the peptidyltransferase active site. The primary role of most proteins appears to be stabilization of the 3D structure of this rRNA (*6*, *21*, *22*). It is possible that their effect may be exerted through allosteric interactions. We reported recently (*1*) that L24 mutants show substantially decreased polyphenylalanine synthesis. In this work we provide evidence in terms of a catalytic rate constant that in the absence of L24 the ribosome forms in vitro less than one-third the number of peptide bonds it forms in its presence in a specified period of time (Figure 2 and Table 3). It is therefore suggested that ribosomal protein L24 activates the *S. cerevisiae* peptidyltransferase.

Recent evidence suggests that the *H. marismortui* L24e ribosomal protein, homologous to yeast L24 protein, is involved in protein−protein interactions and forms a protein cluster composed of L3, L6, L13, L14, and L24e. This cluster is found close to the factor binding site. Although none of the proteins belongs to the peptidyltransferase center, the nonglobular extensions of proteins L2, L3, L4, and L10e penetrate deeply into domain V of 23S rRNA and approach the active site (*7*). Until very recently L2 and L3 were thought to be involved in the peptidyltransferase reaction (*23*). It is possible that L24e, which interacts with L3, might help to stabilize the positioning of ribosome-bound tRNAs. Such a notion explains our finding that yeast L24 enhances the peptidyltransferase activity, but it is not absolutely essential for it.

Furthermore, the absence of L24 caused increased resistance to cycloheximide, a translocation of protein synthesis inhibitor (Figure 5). We were unable to detect significant differences in the rate or the extent of translocation between the wild type and L24 mutant (Figure 4). However, the absence of L24 caused also increased resistance to cyclo-

heximide during polyphenylalanine synthesis (Figure 3) or cell growth. None of these effects were exhibited by the L39 mutant. These results indicate that ribosomal protein L24 does not affect elongation factor EF2 activity but it may participate in the binding site of cycloheximide on the ribosome. The mechanism of action of cycloheximide is not yet fully understood, although it is known to bind to the large subunit of the 80S ribosome with 1:1 stoichiometry (*24*). Thus, the exact nature of the involvement of L24 in the binding site of the antibiotic must probably await the precise structural analysis of the 80S ribosome. Mutations in two other ribosomal proteins, L41 and L29, were also shown to affect cycloheximide resistance (*24*, *25*). Subsequently, it was suggested that these two proteins may also play a central role in forming the cycloheximide binding site on the 60S subunit (*24*).

Protein L39 was recently shown to be essential for the maintenance of translational accuracy; its absence caused a loss of translational accuracy (*1*). The present finding that the k_3 of mutants lacking L39 was increased to 3.05 min $^{-1}$ from 2.18 min $^{-1}$ for the wild type (Figure 2 and Table 3) supports the notion that ribosomal proteins may possess more than one ribosomal function. The effect of L39 on yeast ribosomal function may be explained by the finding that its *H. marismortui* homologue is the only nonglobular protein of this organism and helps to form part of the wall of the polypeptide exit tunnel together with the nonglobular regions of proteins L4 and L22. Most of the surface of the polypeptide exit tunnel is formed by domains I−V of 23S rRNA. Thus, an allosteric effect of L39 transmitted along this tunnel is possible. While rRNA is principally responsible for the basic activities of the ribosome in translation, ribosomal proteins are assigned auxiliary roles. It is possible that proteins bound to rRNA enable the ribosome to carry out translation quickly and precisely (*26*).

In conclusion, our results contribute to the notion that the ribosome is a dynamic structure. Its parts move constantly and interact with each other. We showed recently (*1*) that L39, a ribosomal protein of the 60S subunit, affects translational accuracy, an activity normally associated with the 40S subunit. In the present paper we showed that some ribosomal proteins such as L24 and, to a lesser extent, L39 affect peptidyltransferase activity, an activity recently shown to be associated in archaebacteria with 23S rRNA.

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