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An accuracy center in the ribosome conserved over 2 billion years

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ABSTRACT **The accuracy of translation in *Escherichia coli* is profoundly influenced by three interacting ribosomal proteins, S12, S4, and S5. Mutations at lysine-42 of S12, originally isolated as causing resistance to streptomycin, increase accuracy. Countervailing "ribosomal ambiguity mutations" (*ram*) in S4 or S5 decrease accuracy. In the eukaryotic ribosome of *Saccharomyces cerevisiae*, mutations in *SUP46* and *SUP44*, encoding the proteins equivalent to S4 and S5, lead to omnipotent suppression—i.e., to less accurate translation. The evolution of ribosomal protein S12 can be traced, by comparison with archaebacteria and *Tetrahymena*, to S28 of *S. cerevisiae*, even though the two proteins share only very limited regions of homology. However, one region that has been conserved contains a lysine residue whose mutation leads to increased accuracy in *E. coli*. We have introduced into S28 of yeast the same amino acid substitutions that led to the original streptomycin-resistant mutations in *E. coli*. We find that they have a profound effect on the accuracy of translation and interact with *SUP44* and *SUP46*, just as predicted from the *E. coli* model. Thus, the interplay of these three proteins to provide the optimal level of accuracy of translation has been conserved during the 2 billion years of evolution that separate *E. coli* from *S. cerevisiae*.**

In the selection of amino acyl-tRNAs during translation, there is a balance between rate and accuracy (reviewed in ref. 1). That this balance can be influenced by ribosomal proteins was clearly demonstrated by the work of Gorini, who showed that ribosomal protein mutations conferring resistance in *Escherichia coli* to the aminoglycoside streptomycin caused hyperaccurate translation (2). These mutations were found frequently in ribosomal protein S12 (3) and occurred at specific residues (4). Later work established that mutations in ribosomal proteins S4 and S5 (i.e., *ram* mutations, for ribosomal ambiguity) could cause a loss of translational accuracy and increased sensitivity to streptomycin (5–8). More recently, Noller's group (9, 10) established that mutations in S12, S4, and S5 can result in alterations in rRNA structure. They hypothesize that these proteins affect a structural equilibrium in the rRNA that influences the accuracy of translation.

Many mutations affecting translational accuracy in the eukaryote *Saccharomyces cerevisiae* have been described (11). *SUP44* and *SUP46*, two "omnipotent suppressor" mutations that suppress all three classes of nonsense mutations and that cause increased sensitivity to the aminoglycoside paromomycin, have phenotypes similar to the *ram* mutations of *E. coli* (12–14). *SUP44* and *SUP46* encode ribosomal proteins S4 (15) and S13 (16–18), the evolutionary homologs of the *E. coli* S5 and S4, respectively. While many hyperaccurate "antisuppressor" mutations have been described in yeast, none have been shown to occur in ribosomal protein genes (11). Alterations in seven ribosomal proteins have been linked to increases or decreases of translational accuracy in *Podospora anserina*, but the primary sequences of the proteins remain unknown (19). In summary, while individual components of the ribosome that are implicated in accuracy have been identified, no functional interactions within the eukaryotic ribosome that influence accuracy have been established.

We have described the cloning of the two genes encoding ribosomal protein S28 of *S. cerevisiae* (20). S28 is related, albeit very distantly, to S12 of *E. coli*. The region of greatest amino acid conservation includes the residue known to be altered in most bacterial mutations that cause resistance to streptomycin. We now ask whether such mutations in S28 of *S. cerevisiae* affect accuracy as they do in S12 of *E. coli*. In particular, can we predict mutations that counteract the phenotype of the omnipotent suppressor mutants, as the mutations that cause resistance to streptomycin counteract the phenotype of the *ram* mutations?

Several site-directed mutations in the gene encoding S28 were found to reduce the drug sensitivity associated with *SUP44* and *SUP46* mutations just as the mutations conferring streptomycin resistance reduced the drug sensitivity of *ram* mutants. Surprisingly, while most mutations in *E. coli*'s S12 have been associated with increased accuracy and drug resistance, one mutation in S28 reduced accuracy and decreased drug resistance. Nevertheless, the importance of S28 in maintaining translational accuracy and its interaction with the eukaryotic equivalents of S4 and S5 have been conserved throughout 2 billion years of evolution.

MATERIALS AND METHODS

Strains. Classical yeast genetic methods were used to construct strains containing both the suppressible *met8-1* (amber) and *leu2-1* (ochre) alleles and the *trp1-a, lys2-BBΔ, his3-Δ1* and *ura3-52* alleles required for disruption of *RPS28A* or *RPS28B* and plasmid selection.

The nonrevertible allele *lys2-BBΔ* was constructed by utilizing the pop-in/pop-out allele replacement method (21) with a *URA3* YIp plasmid (pRAA9) carrying a 2.6-kilobase (kb) *Bgl* II–*Bam*HI deletion within the *LYS2* gene.

Diploid L-1460 (**a**/α *met8-1/met8-1 leu2-1/leu2-1 trp1-a/trp1-a lys2-BBΔ/lys2-BBΔ his3-Δ1/his3-Δ1 ura3-52/ura3-52 SUP44/sup44 rps28b::LYS2/RPS28B*) was made by disrupting one allele of *RPS28B* with *LYS2* as described (20).

Tetrad analysis was used to couple the *SUP46* and *rps28a::TRP1* alleles (20) in strain SL-999 (**a**/α *met8-1/met8-1 leu2-1/leu2-1 trp1-a/trp1-a lys2-BBΔ/lys2-BBΔ his3-Δ1/his3-Δ1 ura3-52/ura3-52 ilv1-1/ILV1 SUP46/sup46 rps28a::TRP1/RPS28A*).

Plasmids. In plasmid pS28A-wt, where wt = wild type, the *Eco*RI–*Pvu* II fragment containing RPS28A was cloned into the *Eco*RI and *Sma* I sites of the multiple cloning site in plasmid pRS313 (22) followed by deletion of the multiple cloning sites between *Cla* I and *Eco*RI. Plasmids pS28A-N, pS28A-Q, pS28A-R, and pS28A-T were identical to pS28A-wt except for replacement of Lys-62 by asparagine,

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glutamine, arginine, and threonine, respectively, by site-directed mutagenesis. Site-directed mutations were generated by using a modified polymerase chain reaction procedure (23). Each mutant was sequenced to establish the accuracy of the mutagenesis.

The *URA3*-selectable 2-μm-based plasmids used to quantitate suppression contain the *lacZ* gene fused to the galactose-inducible *GAL1* promoter and the *GAL1* coding sequence for the first 29 amino acids (gift of A. Hinnebusch and G. Fabian of the National Institutes of Health). The control vector (pFB14), called here pLacZ-WT, contains a serine codon at the fusion junction and expresses *lacZ* at high levels when the *GAL1* promoter is induced. The plasmid used to assay suppression (pLacZ-UGA) is identical to pFB14, except for a UGA codon rather than a serine codon at the fusion junction. This nonsense codon must be suppressed for expression of functional β-galactosidase.

Translation Suppression and Paromomycin Sensitivity Tests. Strains to be tested were suspended at equivalent concentrations in sterile water. An aliquot of each cell suspension was placed on the indicated plates, and growth was analyzed for 3–6 days.

To determine levels of omnipotent suppression, cells auxotrophic for methionine or leucine were tested on plates lacking either one or both of the required nutrients. To determine the level of paromomycin sensitivity, cells were tested on plates containing between 0.1 and 1.0 mg of paromomycin (gift of Warner/Lambert Pharmaceutical Division) per ml.

β-Galactosidase Expression Assay to Quantitate Levels of Suppression. A Δ*RPS28B* haploid strain was cotransformed with a plasmid carrying either the wild-type *RPS28A* gene or the Lys-62 → Arg mutant gene, along with a *GAL1*-inducible *lacZ* construct with or without an in-frame UGA nonsense codon (see *Results*). Several samples of each transformant were grown in -his/-ura medium containing 3% (vol/vol) glycerol, 1% (vol/vol) ethanol, and 2% galactose. β-Galactose activity was assayed by using a permeabilized cell method (24).

RESULTS

Having cloned the *S. cerevisiae* gene encoding ribosomal protein S28 and observed that its sequence is apparently related to the *E. coli* ribosomal protein S12 (20), we now wish to ask whether its role in establishing the accuracy of translation and its interaction with the *S. cerevisiae* equivalents of *E. coli* ribosomal proteins S4 and S5 have been maintained during the evolutionary divergence of eukaryotes and prokaryotes. Although the divergence of primary sequence between the two genes has been extensive, one short region is substantially conserved:

```
                              42
                              |
E. coli S12        . . . TTPKKPNSALRKVCRVRL . . . .
                              62
                              |
S. cerevisiae S28  . . . IESKQPNSAIRKCVRVQL . . . .
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Most of the *E. coli* streptomycin-resistant/-dependent mutations occurred at residue Lys-42 (4). Reasoning that Lys-62 of *S. cerevisiae* is the equivalent, we introduced a similar set of mutations into the *S. cerevisiae* ribosomal protein S28—i.e., Lys-62 → Arg, Thr, Gln, or Asn. We then substituted the mutant gene for one of the wild-type genes in the wild-type strain and in *SUP44*- and *SUP46*-containing strains.

Reduced translational accuracy was detected by increased suppression of nonsense alleles in *met8-1* (amber) and *leu2-1* (ochre) and by increased sensitivity to the aminoglycoside antibiotic paromomycin. *SUP44* and *SUP46* strains showed all three phenotypes. The mutations introduced into S28, by analogy to the situation in *E. coli*, should lead to increased accuracy—i.e., to reduced sensitivity to paromomycin and to an antisuppressor effect against the *SUP44* and *SUP46* alleles.

The data are presented largely in Figs. 1 and 2, which represent tetrads dissected from diploids heterozygous for *SUP44* or *SUP46* and for a deletion of one of the *RPS28* genes. Cells derived from each spore of the tetrad were transformed with centromere-based (single copy) plasmids (22) carrying either no gene (pRS313) or wild-type or mutant alleles of *RPS28A*. Except in a few cases, the phenotypes of the mutant alleles of *RPS28* were apparent only when one of the chromosomal RPS28 genes had been deleted, presumably leading to a higher concentration of mutant ribosomes.

Most Mutant Alleles of RPS28A Reduce Paromomycin Sensitivity. Fig. 1 represents the interaction of *SUP46* (S13) with S28. In the fourth line of Fig. 1*A*, one can see that in the presence of both wild-type genes for S28, *SUP46* causes sufficient suppression of both the amber *met8-1* allele and the ochre *leu2-1* allele to permit growth and that this suppression is independent of the S28 gene carried on the plasmid. However, when one of the genomic S28 genes is disrupted (second line in Fig. 1*A*), the presence of the Lys-62 → Gln or Thr alleles of S28 largely counteracts the effect of *SUP46*. Similarly line 2 of Fig. 1*B* shows that the presence of the Lys-62 → Asn or Gln or Thr alleles of S28 reduce the sensitivity to paromomycin caused by the *SUP46* omnipotent suppressor. However, note that this is the case only in cells in which one of the endogenous *RPS28* genes has been disrupted (compare lines 2 and 4 in Fig. 1*A*). It is not clear whether this is because only one-third of the ribosomes will have both a SUP46 protein and a mutant S28 protein or because the mutant protein competes poorly for assembly into the ribosome.

Fig. 2 represents a similar analysis of *SUP44*(S4) and S28. While in this case, the mutants of S28 do not counteract the *SUP44*-mediated suppression of the *met8-1* amber allele, the Lys-62 → Thr or Asn alleles reduce the sensitivity to paromomycin (Fig. 2*B*, line 4). This is even more apparent in Fig.

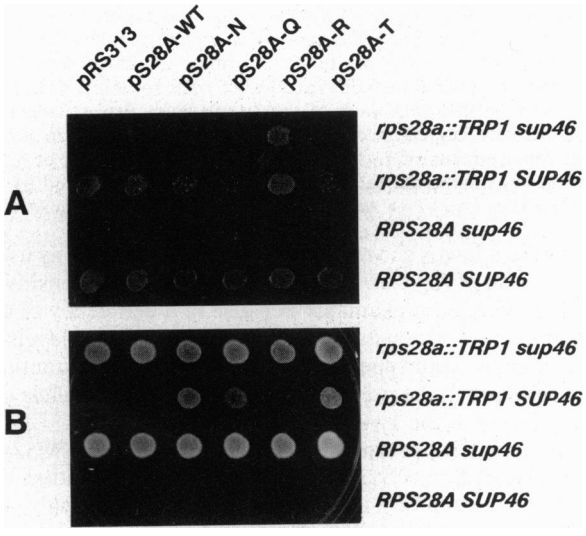

FIG. 1. Interaction of *SUP46* and various alleles of *RPS28A*. Four spores of a diploid heterozygous for *SUP46* and for the deletion of *RPS28A* were transformed with pRS313 alone or carrying several mutant alleles at position 62 of S28. (*A*) Each was spotted on a plate lacking methionine and leucine to assess growth due to suppression of the amber allele of *met8-1* and the ochre allele of *leu2-1*. (*B*) Each was spotted on a plate supplemented with 0.3 mg of paromomycin per ml to determine antibiotic sensitivity. Plates were incubated for 6 days at 30°C.

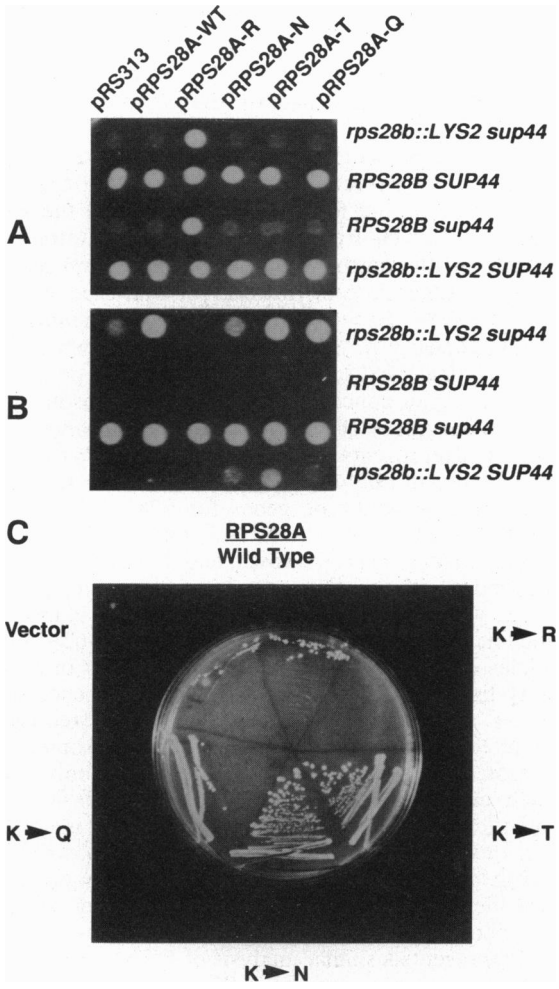

FIG. 2. Interaction of *SUP44* and various alleles of *RPS28A*. Four spores of a diploid heterozygous for *SUP44* and for the deletion of *RPS28B* were transformed with pRS313 alone or carrying several mutant alleles at position 62 of S28. (*A*) Each was spotted on a plate lacking methionine to assess growth due to suppression of the amber allele of *met8-1*. (*B*) Each was spotted on a plate supplemented with 0.2 mg of paromomycin per ml to determine antibiotic sensitivity. Plates were incubated for 3 days at 30°C. (*C*) The rps28b::Lys2SUP44 strain transformed with each of the six plasmids was streaked for single colonies on medium containing 0.1 mg of paromomycin per ml. While replacements of Lys-62 with asparagine, threonine, or glutamine clearly counteract the paromomycin sensitivity caused by the *SUP44* allele, Lys-62 → Arg causes strikingly increased sensitivity.

2*C*, where a lower concentration of paromomycin was used. The deletion of *RPS28B* itself causes an increased sensitivity to paromomycin, presumably because of a deficiency of 40S subunits (25). The resistance is restored to wild-type levels by addition of the wild-type gene, but cells become substantially more resistant in the presence of the Lys-62 → Gln allele and even more with the Lys-62 → Thr or Asn alleles.

Omnipotent Suppression by One Mutant Allele of *RPS28A*. In the case of *E. coli* S12, most of the mutants identified lead to increased accuracy of translation (26). Surprisingly, the mutation Lys-62 → Arg in the *S. cerevisiae* S28 leads to substantially decreased accuracy. This mutation acts as an omnipotent suppressor, suppressing both the amber allele *met8-1* and the ochre allele *leu2-1* (Fig. 1*A*, line 1 and Fig. 2*A*, line 1) as well as the amber allele *ilv1-1* (data not shown). Hence, the suppressor can act on a variety of nonsense codons in different contexts. In this case, the suppression of nonsense mutations occurs even when both wild-type genes are present (Fig. 2*A*, line 3). The mutation also leads to substantially increased sensitivity to paromomycin (Figs. 2 *B* and *C*).

Table 1. Amber suppression by the Lys-62 → Arg allele of S28

	β-Galactosidase activity	
	pLacZ-WT	pLacZ-UGA
S28 (wt)	17,000 ± 1300	83 ± 40
S28 (K-62 → R)	8,500 ± 1000	1600 ± 110

A strain with the genotype *RPS28A/rps28b::LYS2* was transformed with pRS313 carrying either the wild type (wt) or the Lys-62 → Arg allele of *RPS28A* and a plasmid containing the *LacZ* gene of *E. coli* under control of the *GAL1* promoter. The *LacZ* gene was either intact (wt) or carried an amber codon (UGA). Three independent transformants were grown as described in text. The cells were harvested, and β-galactosidase activity was measured in arbitrary units (method 2 of ref. 24).

The suppression of a UGA nonsense codon by the Lys-62 → Arg allele of S28 was quantitated by using constructs containing *LacZ* fused to the *GAL1* promoter. Table 1 shows that the background level of nonsense suppression in the presence of wild-type S28 is approximately 0.5%. However, in the presence of the Lys-62 → Arg allele of S28, the level of suppression approaches 20%. This is a remarkable degree of suppression considering that only a portion of the ribosomes carry the mutant protein.

DISCUSSION

The data presented above show clearly that ribosomal protein S28 of *S. cerevisiae* is involved in the accuracy of translation. This is evident because mutations in S28 either can increase accuracy (e.g., Lys-62 → Thr, Gln, or Asn) or can decrease accuracy (e.g., Lys-62 → Arg). It is interesting to note that Eustice *et al.* (spot c in figure 2*B* of ref. 27) observed that the presence of the omnipotent suppressor mutation *SUP35* alters the electrophoretic mobility of a protein that appears to be S28. Thus, S28 is implicated in the accuracy of translation of the basis of two distinct experimental approaches.

The extensive genetic and biochemical analysis of the *E. coli* ribosome has revealed a great deal about the arrangement of ribosomal proteins within the ribosome, about their interaction with each other and with the ribosomal RNA, and in many cases about their functional role. One of the most telling examples is that the accuracy of selection of the incoming aminoacyl-tRNA depends in great part on the functional interaction of 16S rRNA with three neighboring (28) ribosomal proteins: S4, S5, and S12 (3, 6, 8, 9). There is little likelihood that any eukaryotic ribosome will receive the attention devoted to the *E. coli* ribosome. Therefore, it is important to understand the confidence with which we can extrapolate the structure–function information obtained about the *E. coli* ribosome to the ribosomes of eukaryotic cells. Comparison of the sequences of the ribosomal RNAs of many organisms has led to the conclusion that there has been a great deal of conservation of secondary, and probably tertiary, structure during evolution (29). A recent report showed that a portion of yeast 25S RNA can substitute for the homologous region in *E. coli* 23S RNA (30). Comparisons of ribosomal protein structures, in which we do not have the advantage of compensatory base substitutions, are more obscure. In many instances the evolution of ribosomal proteins between eukaryotic and prokaryotic cells has been so great that one can only relate them through proteins of the archaebacteria (31, 32). Since few of the ribosomal proteins have a defined functional role in translation, the problem is even more difficult. Only rarely have functional homologies been observed (e.g., *E. coli* L23 and *S. cerevisiae* L25 bind to the homologous site on the large ribosomal RNA; ref. 33). However, an important advance is the demonstration that

ribosomal proteins S13 (16–18) and S4 (15) of *S. cerevisiae* are related to S4 and S5, respectively, of prokaryotic ribosomes. Mutations in S13 (*SUP46*) or S4 (*SUP44*) can also lead to reduced accuracy, with a phenotype of omnipotent suppression.

We show above that S28 of *S. cerevisiae* is the functional equivalent of S12 of *E. coli*, even though its sequence homology is limited. Furthermore, Figs. 1 and 2 show that S28 interacts with S4 and S13 to determine the accuracy of translation just as S12 does with S4 and S5 in *E. coli*. Therefore, this functional interaction has been maintained through the more than 2 billion years that separate *E. coli* and *S. cerevisiae* (29). These results suggest that the secondary and tertiary structures of these three proteins has remained largely unchanged in spite of the extensive alteration in primary structure. Perhaps more important, these results give us confidence that we can extrapolate from the structure of the *E. coli* ribosome to that of eukaryotes.

One substitution (Lys-62 → Arg in *S. cerevisiae* S28, Lys-42 → Arg in prokaryotic S12) demonstrates an interesting polymorphism. This was originally described as a streptomycin-resistance mutation leading to a minor increase in the accuracy of translation (2, 4). More recently it was found to have a modest suppressive phenotype in both *E. coli* (34) and Salmonella (35), but only in certain genetic backgrounds. On the other hand, this mutation in *S. cerevisiae* S28 leads to a striking decrease in accuracy (Figs. 1 and 2; Table 1). Indeed it is a strong omnipotent suppressor. We suggest that the accuracy of uncoding is highly sensitive to the microenvironment of this site.

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