Mutations of highly conserved bases in the peptidyltransferase center induce compensatory rearrangements in yeast ribosomes

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ABSTRACT

Molecular dynamics simulation identified three highly conserved rRNA bases in the large subunit of the ribosome that form a three-dimensional (3D) “gate” that induces pausing of the aa-tRNA acceptor stem during accommodation into the A-site. A nearby fourth base contacting the “tryptophan finger” of yeast protein L3, which is involved in the coordinating elongation factor recruitment to the ribosome with peptidyltransfer, is also implicated in this process. To better understand the functional importance of these bases, single base substitutions as well as deletions at all four positions were constructed and expressed as the sole forms of ribosomes in yeast *Saccharomyces cerevisiae*. None of the mutants had strong effects on cell growth, translational fidelity, or on the interactions between ribosomes and tRNAs. However, the mutants did promote strong effects on cell growth in the presence of translational inhibitors, and differences in viability between yeast and *Escherichia coli* mutants at homologous positions suggest new targets for antibacterial therapeutics. Mutant ribosomes also promoted changes in 25S rRNA structure, all localized to the core of peptidyltransferase center (i.e., the proto-ribosome area). We suggest that a certain degree of structural plasticity is built into the ribosome, enabling it to ensure accurate translation of the genetic code while providing it with the flexibility to adapt and evolve.

Keywords: ribosome; translation; fidelity; structure; rRNA

INTRODUCTION

Polypeptide chain synthesis proceeds through the universally conserved repetitive three-step elongation cycle performed by the ribosome: decoding, peptidyl transfer, and translocation. In eukaryotic ribosomes, the process begins when the aa-tRNA•eEF1A•GTP ternary complex is delivered to the ribosomal decoding center through base-pairing of the tRNA anticodon with the mRNA codon. After decoding and subsequent GTP hydrolysis, elongation factor eEF1A dissociates, and aa-tRNA accommodates into the A-site of the peptidyltransferase center (PTC). During the spontaneous peptidyl transfer reaction, the peptide chain from peptidyl-tRNA positioned in the ribosomal P-site is transferred onto the amino acid of the aa-tRNA in the ribosomal A-site. Subsequently, elongation factor eEF2

is recruited to the ribosome, and the A-site and P-site tRNAs adopt their hybrid states. This is followed by GTP hydrolysis, which allows translocation of the ribosome in the 3' direction by one codon, repositioning the deacylated tRNA fully into the E-site and the peptidyl-tRNA into the P-site. After eEF2 dissociation, the empty A-site is ready to accept a new incoming aa-tRNA, thus renewing the cycle. The efficiency and accuracy of protein synthesis depend on how efficiently these steps along the elongation cycle are coordinated by the ribosome. It is not surprising that the ribosome undergoes many dynamic inter- and intramolecular interactions and acquires different conformations throughout the course of this process.

Accommodation of the aa-tRNA into the PTC of the large ribosomal subunit (LSU) has been suggested to be the rate-limiting step in peptide bond formation (Gromadski and Rodnina 2004). During this process, aa-tRNA has to move from the A/T to the A/A conformation, swinging the aa-tRNA 3'-end from the periphery of the ribosome deep into the PTC in the ribosomal core. Although static images obtained by cryo-EM and X-ray crystallography allow comparison of the aa-tRNA structure in the A/T and A/A

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conformations (for review, see Frank et al. 2005), the precise structural transformations occurring in the ribosome–tRNA complexes during accommodation remain unclear. In an effort to address this problem, molecular dynamics simulations were used to identify a new functional region of the large ribosomal subunit, the aa-tRNA accommodation corridor, suggesting that 18 universally conserved nucleotides interact with the aa-tRNA during accommodation (Sanbonmatsu et al. 2005). Three nucleotides in the LSU rRNA—U2861, C2925, and C2942 (unless otherwise specified, *Saccharomyces cerevisiae* numbering is used throughout the text and corresponding *Escherichia coli* bases are listed in Table 1)—were specifically proposed to form two obstructions or “gates,” each of which was proposed to cause the aa-tRNA 3′-end to pause inside of the accommodation corridor before it enters the PTC. However, a recent study using molecular dynamics simulations and FRET approaches led to the suggestion that the accommodation process is stochastic in nature and that aa-tRNA 3′-ends may accommodate into the PTC by multiple pathways (Whitford et al. 2010). Regardless, the high degree of conservation of the rRNA bases that form the aa-tRNA accommodation corridor suggests that they are functionally involved in the accommodation mechanism, making them attractive candidates for targeted mutagenesis and functional analyses.

In a prior study using *E. coli*, the U2492, C2556, and C2573 (yeast U2861, C2925, and C2942) accommodation gate bases were identified among other functionally im-

portant nucleotides by systematic selection of functional sequences by enforced replacement (SSER) (Sato et al. 2006). In a similar study, C2556 (yeast C2925) was also identified and proposed to be actively involved in translation (Blanchard and Puglisi 2001; Yassin and Mankin 2007). The *E. coli* 23S rRNA base U2492 (yeast U2861) is listed among the ribosome accuracy mutations in domain V (O'Connor and Dahlberg 1995). Positioned next to the gate base (C2942), A2941 makes a contact with W255 of protein L3, which closely approaches the PTC and helps to coordinate structure/function of the accommodation corridor in yeast (Meskauskas and Dinman 2007). This nucleotide was also shown to participate in structural rearrangements caused by PTC-specific antibiotics (Davidovich et al. 2008). In addition, mutation A2572U (yeast A2941U) promoted resistance to the PTC antibiotic valnemulin in *Mycobacterium smegmatis* (Long et al. 2009). In *E. coli*, various mutations at positions A2572 and C2573 (yeast A2941 and C2942) did not affect aa-tRNA accommodation but promoted decreased rates of RF2-dependent peptidyl-tRNA hydrolysis (Burakovsky et al. 2010). However, the three “gate bases,” as well as A2572 (yeast A2941), changed their conformations in response to different functionally important ribosomal mutations, suggesting that they are a part of the functional communication network within the ribosome (Beringer et al. 2005; Meskauskas and Dinman 2007; Petrov et al. 2008). In the present study, mutants of each of the three gate base positions, as well as at the L3 contacting position A2941, were expressed as the sole forms of 25S rRNAs in yeast cells. The effects of these mutants were assayed using genetic, biochemical, and rRNA chemical protection approaches. Even though viable alleles displayed very strong resistance/sensitivity effects to translational inhibitors and some of them affected translational fidelity, they did not promote dramatic changes on aa-tRNA affinities to ribosomal A-sites and P-sites. However, these mutants promoted local changes in the structure of 25S rRNA, i.e., in the vicinity of the introduced mutations, in the core of the peptidyltransferase center, and at the entrance of the accommodation corridor, which manifested themselves as increases in apparent rates of peptidyltransfer. This suggests that rRNA in these functional regions of the ribosome formed compensatory rearrangements in response to single base substitutions at targeted positions. This further supports the idea that the ribosome is a robust machine (Burakovsky et al. 2010) and can adjust its structure to preserve function.

RESULTS

Generation of rRNA mutants at the “gate” base positions and A2941-L3 W255 contact

The universally conserved LSU bases U2861, C2925, and C2942 (*E. coli* U2492, C2556, C2573, respectively; note that

TABLE 1. Comparative numbering and conservation frequency (in three domains and two organelles) of rRNA nucleobases discussed in the current study

<i>S. cerevisiae</i>	<i>E. coli</i>	% Conservation ^a
A2398	G2056	68.32
A2401	A2059	99.41
A2819	A2450	99.76
A2820	A2451	100.00
C2821	C2452	100.00
U2829	U2460	98.19
U2859	G2490	81.40
U2860	U2491	92.51
U2861*	U2492	98.79
U2862	U2493	97.71
U2875	U2506	99.76
G2898	G2529	97.46
A2902	U2533	39.88
C2925*	C2556	100.00
A2941*	A2572	96.21
C2942*	C2573	100.00
U2954	U2585	99.49
A2971	A2602	99.46

Asterisks denote bases of yeast 25S rRNA targeted for mutagenesis in the current study.

^aValues taken from the Comparative RNA website and Project (<http://www.rna.cccb.utexas.edu/>).

E. coli bases are in parentheses throughout) were previously proposed to form a three-dimensional (3D) gate in the aa-tRNA accommodation corridor and promote proper positioning of the aminoacyl-ends of aa-tRNAs in the ribosomal A-site (Sanbonmatsu et al. 2005). In addition, A2941 (A2572) interacts with W255 of protein L3, which closely approaches the peptidyltransferase center and helps to coordinate the function of both the PTC and the aa-tRNA accommodation corridor (Meskauskas and Dinman 2007). These are mapped onto the local 25S rRNA structure in Figure 1. In this study, all possible base substitutions as well as deletions of these four bases were created and expressed as the sole forms of 25S rRNA in yeast cells. Using the two-step plasmid replacement method (Rakauskaite and Dinman 2008), seven viable strains expressing pure mutant ribosomes were isolated (Table 2). All base substitutions—i.e., U2861A, U2861C, and U2861G—were viable at position U2861, which is one of the two partners in the first “gate.” At position C2925, the other partner in the first gate, all base substitutions were lethal when expressed as the sole form of 25S rRNA. In the second gate base, only C2942U was viable; C2942A and C2942G were not. In the L3-W255 interacting position, all three mutants (A2941C, A2941G, A2941U) were viable. All deletions of any of the four targeted bases were lethal. These data and comparisons to mutagenesis studies in *E. coli* and *M. smegmatis* are summarized in Table 2.

None of the viable mutants appeared to promote obvious effects on cell growth at physiological conditions, i.e., at 30°C, or at the lower temperature of 20°C. Only the C2942U and A2941G alleles promoted slight growth defects at elevated temperature, 37°C (Fig. 2). All of the viable mutants were also able to maintain the *S. cerevisiae* killer virus, which is sensitive to functional changes of translational machinery (for review, see Harger et al. 2002; data not shown). In contrast, pharmacogenetic assays revealed

FIGURE 1. The aa-tRNA accommodation corridor modeled onto the yeast 25S rRNA. (Gray) rRNA; (red) mutagenized bases in H89-92; (blue) L3 protein. Nucleotide A2820 (2451 in *E. coli*) indicates the core of the peptidyltransferase center.

TABLE 2. Viability of corresponding rRNA mutations

Mutation/viability					
<i>S. cerevisiae</i>		<i>E. coli</i>		<i>M. smegmatis</i>	
U2861A	+ ^a	U2492A	+ ^b		
U2861C	+ ^a	U2492C	+ ^b		
U2861G	+ ^a	U2492G	+ ^b		
U2861Δ	— ^a				
C2925A	— ^a				
C2925G	— ^a				
C2925U	— ^a	C2556U	+ ^c		
C2925Δ	— ^a	C2556Δ	+ ^d		
A2941C	+ ^a	A2572C	+ ^e		
A2941G	+ ^a				
A2941U	+ ^a	A2572U	+ ^e	A2572U	+ ^f
A2941Δ	— ^a	A2572Δ	— ^e		
C2942A	— ^a	C2573A	+ ^e		
C2942G	— ^a	C2573G	+ ^e		
C2942U	+ ^a	C2573U	+ ^e		
C2942Δ	— ^a	C2573Δ	— ^e		

(+) Viable allele; (—) inviable allele.

^aThis study.

^bO'Connor and Dahlberg 1995.

^cBlanchard and Puglisi 2001.

^dYassin and Mankin 2007.

^eBurakovsky et al. 2010.

^fLong et al. 2009.

that ribosomal inhibitors had strong effects on all of the mutants, suggesting that ribosome function in these mutants was altered. Anisomycin is a competitive inhibitor of aa-tRNA binding to the ribosomal A-site (Grollman 1967). All mutants of position A2941 displayed strong anisomycin resistance, while U2861G also promoted resistance to this drug, albeit to a lesser degree. Three of the anisomycin-resistant mutants—U2861G, A2941G, and A2941U—were also hypersensitive to sparsomycin, an indicator of altered P-site function (Hansen et al. 2003). Although U2861A did not affect cell growth in the presence of anisomycin, it was hypersensitive to sparsomycin. Cellular response to paromomycin is indicative of problems related to the ribosomal decoding center (for review, see Ogle et al. 2003). All but one (A2941C) of the viable mutants were also hypersensitive to paromomycin (Fig. 2).

Effects on translational fidelity

An in vivo dual-luciferase system was employed to monitor translational fidelity in yeast cells (Harger and Dinman 2003; Plant et al. 2007). Maintenance of translational reading frame was assayed using programmed ribosomal frame-shifting (PRF) reporters harboring the L-A virus-derived −1 PRF signal or the Ty1-derived +1 PRF signal. Levels of −1 PRF in the seven mutants ranged between 0.77-fold (A2941U) and 1.4-fold (C2942U) of wild type (Fig. 3A). However, these changes were not sufficient to impair

FIGURE 2. Effects of rRNA mutants on growth at different temperatures in the presence of translational inhibitors. Tenfold serial dilutions (10^4 – 10^0) of yeast cells were spotted on YPAD medium and grown at the indicated temperatures. Alternatively, cells were spotted on YPAD plates containing the indicated concentrations of antibiotics and grown at 30 °C.

maintenance of the yeast Killer virus (Dinman and Wickner 1992; data not shown). Similarly, none of the mutants exerted strong effects on +1 PRF (Fig. 3B). In contrast, allele-specific effects on tRNA misincorporation were noted. The U2861C and U2861G mutants promoted increased rates of UAA codon read-through (1.76-fold and 2.09-fold, respectively) (Fig. 3C), while the A2941G and A2941U alleles promoted an ~50% reduction in the ribosome's ability to misincorporate the Arg-tRNA^{UCU} at a near-cognate AGC Ser codon as compared to wild type (Fig. 3D). None of the mutants affected misreading of a non-cognate (UCC) codon (Fig. 3E).

tRNA binding and peptidyltransfer effects

The strong resistance to anisomycin and hypersensitivity to sparsomycin by selected mutants prompted us to examine their effects on binding of aa-tRNA and Ac-aa-tRNA to the ribosomal A-sites and P-sites, respectively. Although anisomycin is a competitive aa-tRNA inhibitor for the ribosomal A-site (Grollman 1967), unexpectedly most of the mutants did not significantly affect aa-tRNA K_D values. The exception was A2941C, which promoted a 60% increase in K_D for aa-tRNA. This correlated with the strong anisomycin resistance displayed by this allele (Figs. 4A,B, 2). Sparsomycin stabilizes peptidyl-tRNA in the P-site and further interferes with peptidyl transfer (Pestka 1977).

Similar to A-site effects, we did not observe significant changes in Ac-aa-tRNA binding to the ribosomal P-site. While the U2861C mutant promoted a small increase (40%) in Ac-aa-tRNA K_D , the overall P-site binding data did not correlate with sparsomycin effects (Figs. 4C,D, 2). Apparent rates of peptidyl-transfer (K_{obs}) were assayed using the puromycin single turnover reaction. Interestingly, all three substitutions at the L3 contacting position A2941 promoted increased rates of K_{obs} : these values were 1.6-fold, 2.0-fold, and 2.4-fold higher than the wild type for the A2941C, A2941G, and A2941U mutants, respectively (Fig. 4E,F).

rRNA mutations cause local structural changes in the peptidyltransferase center

Puromycin-treated salt-washed ribosomes isolated from selected mutants were probed for rRNA structural changes both local to the sites of mutation as well as in major functional centers of the ribosome including the PTC, the Sarcin/Ricin loop, along the entire tRNA accommodation corridor, in the vicinity of the P-loop, and in Helix 39, which stabilizes the first gate base U2861 (U2492). Apoptate ribosomes were modified with either 1M7 using selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE) technique (Merino et al. 2005) or dimethylsulfate (DMS) (Rodriguez-Fonseca et al. 1995). Examination of the primer extension products showed that alterations of the rRNA structure clustered in the PTC and along the aa-tRNA accommodation corridor (Figs. 5, 6).

The mutations at the first gate position, U2861, caused deprotection of neighboring bases in Helix 89 (U2829, U2859–62, *E. coli* U2460, and G2490–U2493, respectively) when 2'-OH riboses were probed with 1M7, indicating localized changes in 25S rRNA structure. In addition, three bases in the catalytic core of the PTC—A2819–20 (A2450 and A2451) and U2875 (U2506)—were hyperprotected in

FIGURE 3. Effects of rRNA mutants on translational fidelity. Translational fidelity assays were performed using standard dual luciferase reporters with cells harvested at mid-log phase. (A) Programmed –1 ribosomal frameshifting. (B) Programmed +1 ribosomal frameshifting. (C) Read-through of a UAA nonsense codon. (D) Misincorporation of Arg-tRNA^{UCU} at near-cognate AGC codon. (E) Misincorporation of Arg-tRNA^{UCU} at non-cognate UCC codon. Error bars denote standard error.

FIGURE 4. Effects of rRNA mutations on ribosome biochemistry. (A) Enzymatic steady-state binding isotherms of [^{14}C]Phe-tRNA to ribosomal A-sites. (B) K_D values calculated from data in panel A. Nonlinear regression analysis of binding data was performed with GraphPad Prism 4.0 software (GraphPad Prism Software), applying one site saturation with ligand depletion model. (C) Steady-state binding isotherms of N-Ac[^{14}C]Phe-tRNA to ribosomal P-sites. (D) K_D values calculated from data in panel C. Curves were analyzed as in panel B. (E) First-order exponentially fitted curves of peptidyltransferase reactions performed using N-Ac[^{14}C]Phe-tRNA pre-bound to ribosomal P-sites and puromycin. (F) K_{obs} values for peptidyltransferase activities at saturating 10 mM puromycin concentration calculated from data shown in panel E. A first-order exponential equation was applied, and values of K_{obs} were calculated using Graphpad Prism 4.0 software (GraphPad Prism Software).

U2861A ribosomes (Fig. 5A). Importantly, the base-specific probe DMS did not reveal changes in the protection patterns of A2819-20, suggesting that only 2'-OH ribose groups were mislocated in the mutant ribosomes (Fig. 5B). The sole viable allele at the second gate base position, C2942U, also promoted rRNA rearrangements, although to a lesser extent (Fig. 5C). Similar to the changes caused by the first gate mutations, C2942U substitution destabilized Helix 89 at the first gate position, U2861, suggesting that two bases involved in the formation of the 3D gate in the aa-tRNA accommodation corridor are structurally coordinated. U2875 (U2506) was also hyperprotected in 1M7 modified ribosomes, while A2902 (U2533) was deprotected in DMS modified C2942U ribosomes relative to wild-type controls. The greatest changes in 25S rRNA structure were observed in ribosomes harboring mutations of the L3-W255 contacting A2941 (A2572) (Fig. 5D). A2819-20 (A2450 and A2451) in the PTC were hyperprotected from 1M7 modification but were not affected by DMS modifi-

cation. Similarly, U2875 (U2506) at the center of the PTC was hyperprotected in these mutants. Helix 89 at the first gate was destabilized, as suggested by increased reactivities of nucleotides U2861-62 (U2492, U2493). The A2941 mutants also caused changes in the loop of Helix 91 (deprotection of G2898 [G2529] and hyperprotection of A2902 [U2533]), positioned at the entrance of the accommodation corridor.

The strong anisomycin resistance phenotypes of many of the mutants were suggestive of rRNA rearrangements in the anisomycin-binding pocket. When anisomycin is bound to the ribosome, it forms a hydrogen bond with C2821 (C2452) and causes protection of specific bases in the peptidyltransferase center from chemical modification (Rodriguez-Fonseca et al. 1995). Comparison of DMS modification patterns between empty ribosomes and ribosomes loaded with anisomycin showed that the "gate" mutants did not cause any changes in rRNA protection patterns (data not shown). However, the mutations at position A2941 (A2572) caused pronounced effects in the chemical protection patterns of anisomycin-specific nucleotides (Fig. 5E). In empty ribosomes, C2821 (C2452) was protected by the A2941C substitution and deprotected by A2941G, and A2941U. This nucleotide was equally well protected in ribosomes loaded with anisomycin. Another anisomycin-specific

nucleotide, A2401 (A2059), became deprotected in mutants A2941G and A2941U only after binding of anisomycin. Overall, the A2941G and A2941U alleles caused similar anisomycin-induced DMS protection patterns, while the A2941C allele was distinctly different. This correlated with the intensity of anisomycin resistance: A2941C conferred very strong anisomycin resistance, while this phenotype was weaker in both the A2941G and A2941U mutants (Fig. 2). A2398 (G2056) was also protected by the A2941G and A2941U alleles, but it did not respond to anisomycin binding.

DISCUSSION

Structural plasticity enables ribosomes to optimize the many changes in macromolecular interactions that occur during the dynamic rearrangements of rRNAs and protein throughout the course of the translation elongation cycle. Accommodation of aa-tRNA into the PTC immediately follows tRNA decoding and involves one of the most dramatic

FIGURE 5. Effects of rRNA mutations on 25S rRNA structure. Ribosomes were chemically probed with either 1M7 or DMS. Changes in 25S rRNA structure caused by mutations of U2861 (A,B), C2942 (C), and A2941 (D) were revealed by reverse transcriptase primer extensions as described in Materials and Methods. Control reactions were performed with unmodified ribosomes. (E) Comparison of DMS modification patterns of empty, puromycin-treated ribosomes (H_2O samples) and ribosomes loaded with anisomycin between the wild-type and A2941 mutant ribosomes.

conformational shifts occurring during translation elongation—transition of the aa-tRNA from initial bent A/T state to the “classical,” fully accommodated A/A state. Although the precise mechanism of accommodation re-

mains unclear, a number of the LSU rRNA bases have been implicated in this process (Sanbonmatsu et al. 2005). The present study used a reverse molecular genetics approach to examine the functional importance of the three “gate”

positions in the aa-tRNA accommodation corridor in the yeast ribosome. In addition, we targeted A2941 (A2572), which contacts W255 of ribosomal protein L3, because it was previously proposed to help position and coordinate the aa-tRNA accommodation process (Meskauskas and Dinman 2007). Despite the high degree of evolutionary conservation of these four rRNA bases (Table 1), seven of the 12 possible allelic substitutions were viable as the sole forms of 25S rRNA in yeast and did not confer major growth defects. Only C2925 (C2556), which is a part of the first gate, was refractory to changes in identity. Interestingly, this nucleotide in bacteria has less stringent qualities: Deletion of this base was viable although

FIGURE 6. Summary of rRNA structural changes caused by rRNA mutations. Bases showing changes in chemical protection patterns are mapped onto the 25S rRNA secondary structure (A), and into the three-dimensional structure (B).

highly deleterious (Yassin and Mankin 2007), and the C2556U (yeast C2925U) substitution was also viable in *E. coli* (Blanchard and Puglisi 2001). NMR studies suggested that this nucleotide is conformationally flexible and is functionally involved in the mechanism of translation (Blanchard and Puglisi 2001). Similarly, the C2942U allele of the second gate position was the only viable mutation at this position in yeast, but all three possible base substitutions were viable and did not show significant growth phenotypes in bacteria (Burakovsky et al. 2010). These observations suggest that, despite the high degree of evolutionary conservation of these nucleotides, certain processes are differentially fine-tuned in eukaryotic as compared to prokaryotic ribosomes. In addition, a recent study suggested that peptide bond formation and not accommodation may actually be the rate-limiting step in the translation elongation cycle (Johansson et al. 2011). The observations that mutation of critical bases in the tRNA accommodation corridor did not affect accommodation rates (Burakovsky et al. 2010), coupled with the observations in the present study, support this notion.

Mutations of important rRNA bases typically cause structural perturbations in functionally and/or structurally related ribosomal RNA regions that can be identified by chemical protection methods. The most complete information is obtained when both base and sugar modifications are used simultaneously. DMS, kethoxal, and CMCT identify misplacements of the nucleobases (Christiansen et al. 1990), while 1M7 used in SHAPE technology probes ribose 2'-OH groups, where reactivity of the 2'-OH corresponds to unpaired nucleobases and correlates with RNA secondary structure (Merino et al. 2005). In the present study, rRNA structural changes caused by the viable "gate" alleles as well as mutants of the A2941/L3 contact were generally confined to the local rRNA structure. Specifically, the structural changes were all localized to the core of the PTC, in the vicinity of the first gate, and near the entrance of the accommodation corridor. Several substitutions at all three "viable" positions caused deprotection of rRNA bases in the vicinity of the first gate in Helix 89. Additionally, mutations at the first gate caused deprotection of U2829 (U2460), also known as ribosome accuracy position (O'Connor and Dahlberg 1995), located at the opposite side of the mutagenized base U2861. We suggest that H89 can flex around U2861 (U2429) in response to incoming aa-tRNA, or acquire alternative conformations, thus providing alternate paths for accommodation of different aa-tRNAs (Whitford et al. 2010). In addition, two nucleotides identified in the present study (yeast G2898 and A2902, *E. coli* G2529 and U2533) located in the distal loop of H91, which forms the entrance of the accommodation corridor, may also participate in this process. Interestingly, C2942U located at the second gate promoted deprotection of U2861 (U2492) in the first gate, suggesting that the two gate bases are structurally coordinated. Three mutations—U2861A,

A2941C, and A2941G—caused protection of the A2820 (A2451) when modified with 1M7. However, the DMS modification patterns of this base did not change, showing that conformational displacement occurred only at the 2'-OH group of A2820 but not in the base itself. Repositioning of the 2'-hydroxyl of the 2820 is particularly interesting, since this group is the only functional element of rRNA that is essential for peptidyltransfer. Specifically, in atomic mutagenesis experiments, removal of the 2'-OH group virtually eliminated peptidyltransferase activity both with puromycin and native tRNA substrates. These findings engendered the hypothesis that the 2'-OH of the A2451 (yeast A2820) makes a hydrogen bond with the A76 ribose of peptidyl-tRNA, thus properly positioning the P-site tRNA in the PTC, consistent with the model of "substrate-assisted catalysis" (Erlacher et al. 2005, 2006; Lang et al. 2008). The finding that none of the three mutations that caused protection of A2820 showed reduced rates of peptidyltransfer as monitored by the puromycin reaction, despite their ability to readjust the 2'-OH of this base (Fig. 4E,F), suggests that a certain degree of structural plasticity by its 2'-OH group can compensate for ribosomal structural changes and thus preserve peptidyltransferase function. In bacterial ribosomes, mutation of the inner-shell nucleotide U2506 (yeast U2874) promoted a twofold to fourfold reduction of RF1-mediated peptide release, and mutations of A2572 (yeast A2941, the L3 contact) and C2573 (yeast C2942, the second gate) were shown to interfere with peptide release at the termination step of protein synthesis (Youngman et al. 2004; Burakovsky et al. 2010). In yeast ribosomes, the protection of A2820 (A2451) observed for one of the first gate alleles and two of the L3 contacting alleles correlated with protection of the universally conserved inner-shell nucleotide U2875 (U2506). In addition, the C2942U second gate mutant promoted protection of U2875 (U2506) without affecting A2820 (A2451). Although in vitro assays using bacterial ribosomes enable precise monitoring of ribosome/release factor interactions by capitalizing on the ability of a Shine-Dalgarno sequence to precisely position a termination codon in the decoding center, eukaryotic ribosomes lack this advantage, thus preventing efficient and accurate biochemical measurement of this parameter. However, paromomycin hypersensitivity and the structure probing data suggest that all three positions (yeast U2861, A2941, and C2942; *E. coli* U2492, A2572, and C2573, respectively) are also likely involved in this process in yeast ribosomes.

Similar to the effects caused by rRNA mutations, binding of antibiotics to the ribosome also promotes rRNA remodeling and severely interferes with protein synthesis. In the present study, specific rRNA mutants conferred strong phenotypic effects in the presence of translational inhibitors (Fig. 2). Despite the fact that biochemical assays did not reveal any serious defects in tRNA binding (Fig. 4), rRNA structure-probing experiments comparing empty ribosomes

with ribosomes loaded with anisomycin identified strong effects at anisomycin-specific bases, which may explain the observed anisomycin resistance of the A2942 mutants (Fig. 5). In particular, C2821 and A2401 (C2452 and A2059), which normally are protected upon anisomycin binding in yeast ribosomes (Rodriguez-Fonseca et al. 1995), had differential DMS modification effects. In empty ribosomes, nucleobase C2821 (C2452), which makes a hydrogen bond with anisomycin, was hyperprotected in A2941C ribosomes and deprotected in A2941G and A2941U ribosomes (Figs. 5E, 6). However, structural movement of C2821 did not seem to affect binding of the antibiotic: C2821 was equally well protected in ribosomes loaded with anisomycin (Figs. 5E, 6). In contrast to C2821, A2401 was differently protected from the chemical attack only in the presence of anisomycin. It seems likely that while misplacement of C2821 in A2941 mutant ribosomes occurred in two different ways, one explanation is that the hydrogen bond between the C2821 and anisomycin may have been disrupted in both cases, thus altering the anisomycin-binding site and causing resistance to this drug. Overall, these results suggest that structural readjustments at functionally essential sites allow ribosomes to functionally tolerate certain changes at highly conserved nucleotides.

In *E. coli* 23S rRNA, the first gate base U2492 (yeast U2861) is known as the “ribosome fidelity position,” so defined because all three base substitutions negatively affected cellular growth, increased ribosome misreading rates on all nonsense codons, and caused increased rates of +1 and –1 frameshifting (O’Connor and Dahlberg 1995). In sharp contrast, yeast cells accepted all three possible substitutions without compromising growth rates or translational fidelity. Only the U2861C and U2861G mutants promoted a twofold increase in UAA codon read-through (Fig. 3), suggesting that this position may be involved in quality control of translation termination in yeast. These findings suggest the identification of functional differences between bacterial and eukaryotic ribosomes that may be pharmacologically exploited. Mutations of nucleobase A2941 (A2572) reduced rates of near-cognate Arg-tRNA misincorporation, which inversely correlated with the rates of the puromycin reaction (Figs. 3D, 4E,F). Even though these effects were small, it seems unlikely that mutant ribosomes were able to increase both translational fidelity (i.e., the proofreading step preceding accommodation) and the speed of peptidyltransferase reaction. However, increased rates of peptidyltransfer suggest that mutant ribosomes may be locked in the “PTC activated” state (Schmeing et al. 2005). This could be possible for the A2941C and A2941G mutants, since both caused protection of the U2875 (U2506), which, according to the “induced fit” model, undergoes specific movement and reorients the ester group of the peptidyl-tRNA (Schmeing et al. 2005). Alternatively, the general conformational distortions observed in the PTC, specifically involving U2875, could have had the effect of opening up

the PTC, increasing rates of diffusion of puromycin, a much smaller substrate than aa-tRNA, to the ribosomal A-site, and thus causing the observed apparent increase in peptidyltransferase activity. Similar stimulation of the puromycin reaction was previously observed with the ribose-abasic ribosomes at U2506 (yeast U2875) in *Thermus aquaticus*, suggesting that the removal of the uracil allowed more rapid diffusion of puromycin into the PTC (Erlacher et al. 2006).

Although the ribosome is an extremely complex nucleoprotein organelle, its main function, transpeptidation, is ensured by the “pocket-like” rRNA structure of the PTC. It is composed exclusively of RNA and has twofold symmetry formed by rRNA domain V (Bashan et al. 2003). The extremely high degree of sequence and structural conservation of this region has engendered the proposition that this region constitutes the “proto-ribosome” (for review, see Polacek and Mankin 2005; Davidovich et al. 2009; Belousoff et al. 2010; Fox 2010). Moreover, in vitro experiments selecting for the ribozymes capable of transpeptidation selected sequences that resembled the PTC (Zhang and Cech 1997). This suggests that from its beginning ~3.5 billion years ago, the process of natural selection identified and preserved those primary rRNA sequences that best optimized the topology of the PTC in order to ensure translational efficiency and fidelity. The rRNA mutations presented in this study targeted universally or highly conserved bases in the PTC region. In response, it appears that the ribosome is capable of accepting these base replacements with only minor effects on general translational accuracy, aa-tRNA binding, and peptidyltransferase activity. It is interesting that these mutations triggered structural rearrangements exclusively in the proto-ribosome area (Bashan et al. 2003; Bokov and Steinberg 2009). This suggests that, even though the ribosome has had eons to maximize its performance, it has retained a certain degree of structural and functional plasticity to allow it to adapt to new selective pressures, thus ensuring the accuracy of translational processes in an ever-changing world.

MATERIALS AND METHODS

Strains, media, reagents, molecular methods

The *E. coli* DH5 α strain was used to amplify plasmids. For genetic, biochemical, and structural assays, yeast rRNA mutants were generated using strain NOY1049 (Wai et al. 2000), kindly provided by Dr. M. Nomura. For Killer (K⁺) virus maintenance assays, yeast rRNA mutants were made in the JD1314 (K⁺) strain background as previously described (Rakauskaitė and Dinman 2006). Yeast media were prepared as described (Wickner and Leibowitz 1976). Mutations in rDNA encoding regions were introduced on plasmids pJD694 (pTET) and pJD180.Trp (Rakauskaitė and Dinman 2008). Oligonucleotides for site-directed mutagenesis were purchased from Integrated DNA Technologies and the Quick-Change Lightning Mutagenesis Kit from Agilent Technologies.

Oligonucleotide design and reaction conditions were as recommended by the manufacturer. All mutations were confirmed by sequencing. Mutagenized plasmids were transformed into yeast strains NOY1049 and JD1314 using the alkaline cation method (Ito et al. 1983). Yeast cells expressing mutant rRNAs were made using the two-step plasmid replacement method and selected as described in Rakauskaitė and Dinman (2008). Expression of mutant ribosomes as a pure ribosome population was confirmed by RT-PCR as previously described (Rakauskaitė and Dinman 2006).

Genetic assays

General growth defects were monitored by dilution spot assays in which 2.5 μ L of five sets of 10-fold dilutions were spotted on the YPAD medium yielding a range of spots from 10^4 to 10^0 CFU/spot. Plates were incubated at 20, 30, and 37°C. For pharmacogenetic assays, YPAD plates with indicated concentrations of drugs were used and incubated at 30°C. Translation fidelity assays for -1 PRF, $+1$ PRF, UAA nonsense suppression, near-cognate aa-tRNA misincorporation, and non-cognate aa-tRNA misincorporation were performed using the dual luciferase reporter constructs pYDL-LA, pYDL-Ty1, pYDL-UAA, R218S(AGC), and R218S(TCC), respectively (Harger and Dinman 2003, 2004; Plant et al. 2007). Between four and 12 readings were taken for each sample until data were normally distributed to enable statistical analysis both within and between experiments (Jacobs and Dinman 2004).

Aminoacyl-tRNA binding assays, puromycin reaction, rRNA chemical protection, and molecular visualization

Yeast phenylalanyl-tRNA was purchased from Sigma, charged with [14 C]-Phe or Ac-[14 C]Phe, and purified by HPLC as previously described (Triana-Alonso et al. 2000). Ribosomes were isolated and tRNA-binding studies as well as puromycin reactions were carried out as described (Meskauskas and Dinman 2010). For SHAPE chemical protection, 50 pmol of ribosomes was pre-incubated in 200 μ L of buffer (80 mM Tris-HCl at pH 7.4, 100 mM NaCl, 15 mM Mg(CH₃COO)₂, 6 mM β -mercaptoethanol) for 10 min at 30°C. The mixture was divided into two parts of 100 μ L each (control and modification samples), and 10 μ L of DMSO or 10 μ L of 1M7 (60 mM in DMSO) was added to the control and modification tubes, respectively. Reactions were incubated for 10 min at 30°C, and ribosomes were precipitated with 250 μ L of ethanol. rRNAs were extracted and primer extension analyses using Superscript III reverse transcriptase (Invitrogen) were performed as described (Meskauskas and Dinman 2010). For DMS chemical modification, ribosomes were pre-incubated with a 400-fold molar excess of anisomycin or buffer and probed with DMS, followed by extraction of the rRNAs and primer extension (Rodriguez-Fonseca et al. 1995). Helices H37–H42 and H84–H97 were analyzed on SHAPE-modified ribosomes, and helices H73–H74 and H88–H93 were analyzed on DMS-modified ribosomes. Modification effects were confirmed with two independent ribosome isolations. The cryo-electron microscopy reconstruction of *Thermomyces laniginosus* modeled with *S. cerevisiae* rRNA and ribosomal proteins (Taylor et al. 2009) were visualized using PyMOL (DeLano Scientific LLC).

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