A flexible loop in yeast ribosomal protein L11 coordinates P-site tRNA binding

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ABSTRACT

High-resolution structures reveal that yeast ribosomal protein L11 and its bacterial/archael homologs called L5 contain a highly conserved, basically charged internal loop that interacts with the peptidyl-transfer RNA (tRNA) T-loop. We call this the L11 ‘P-site loop’. Chemical protection of wild-type ribosome shows that the P-site loop is inherently flexible, i.e. it is extended into the ribosomal P-site when this is unoccupied by tRNA, while it is retracted into the terminal loop of 25S rRNA Helix 84 when the P-site is occupied. To further analyze the function of this structure, a series of mutants within the P-site loop were created and analyzed. A mutant that favors interaction of the P-site loop with the terminal loop of Helix 84 promoted increased affinity for peptidyl-tRNA, while another that favors its extension into the ribosomal P-site had the opposite effect. The two mutants also had opposing effects on binding of aa-tRNA to the ribosomal A-site, and downstream functional effects were observed on translational fidelity, drug resistance/hypersensitivity, virus maintenance and overall cell growth. These analyses suggest that the L11 P-site loop normally helps to optimize ribosome function by monitoring the occupancy status of the ribosomal P-site.

INTRODUCTION

Over the past decade, atomic resolution ribosome structures have revealed the locations of critical elements. However, these static images do not reveal the dynamic movements within this complex macromolecule. The ribosome must coordinate multiple activities between spatially and functionally different sites in two subunits. These include three transfer RNA (tRNA)-binding sites, the peptidyltransferase and decoding centers and the elongation factor interacting regions. Events occurring in these regions must be carefully coordinated to assure rapid and accurate decoding of messenger RNAs (mRNAs). Current efforts in the field are focusing on determining the mechanisms by which these functional centers synchronize their actions and communicate with each other.

The eukaryotic ribosome contains nearly 80 intrinsic proteins. The high degree of similarity across species, from the primary amino acid sequences to their tertiary structures, suggests conserved functional roles beyond serving as mere scaffolding for the rRNAs. Ribosomal protein L11 of Saccharomyces cerevisiae is an essential, highly conserved component of the 60S subunit (in bacteria and archaea, the homologous protein is named L5; the yeast nomenclature is used throughout this text to minimize confusion). At the primary amino acid sequence level, L11 is well conserved among eukaryotes (~67–97% identity), while bacterial and archaeal L5 proteins are less well conserved (27–55% identical) (Supplementary Figure S1A). L11 is uniquely positioned at the interface between the large subunit central protuberance (Figure 1A and 1B) and the head of the small subunit (Figure 1A) (1–5). In the small subunit, the head region undergoes significant rotational movement relative to the central protuberance between the pre- and post-translocational states (6), and the protein–protein interactions between L11 and S18 (S13 in bacteria and archaea) on the small subunit (the B1b and B1c intersubunit bridges) undergo the largest intersubunit structural rearrangements between these two states (7–9). These observations suggest that L11 may play a central role as an informational conduit between the two subunits. Detailed analysis of X-ray crystallographic and cryo-EM structures (Figure 1B) reveals that the concave surface of the β-sheet portion of L11 interacts with specific nucleotides in the minor groove of 23S rRNA helix 84 (10). L11 also makes contacts with the helix III and loop C regions of 5S rRNA; these connections have been hypothesized to help stabilize 5S rRNA interactions and may participate in an information signal transmission...
network linking functional centers within the ribosome (1,11). Importantly, the B1b and B1c intersubunit bridges with S18 are the only protein–protein interactions between the two subunits (2,3,5,8). Analyses of these structures indicate that contacts involving L11 and S18 through the B1b and B1c bridges break and rearrange after eEF-2 binding and ribosome ratcheting, controlled in part by differentially charged amino acid side chains between the two proteins (2,4,5,7,9,12). An internal loop of L11 that we denote the ‘L11 P-site loop’, which is roughly formed by amino acid residues 48–68, also directly contacts the T-loop of the peptidyl-tRNA in the P-site through tRNA nucleotide 56 (3–5). At the level of primary amino acid sequence, the P-site loop is highly conserved among eukaryotes (85–100% identity), while it is less well conserved among bacteria and archaea (42–57% identity) (Supplementary Figure S1B). At the biochemical level, however, the P-site loop is significantly more homogeneous, containing a large number of well-aligned charged and aromatic amino acids. In particular, A50, F57, R60 and I65 (yeast numbering) are universally conserved. An alignment of the P-site loop structures from yeast, Haloarcula marismortui, Thermus thermophilus and Escherichia coli reveals that the P-site loop is extremely well conserved at the structural level (Supplementary Figure S1C).

In yeast, L11 is encoded by the paralogous genes RPL11A and RPL11B located on chromosomes 16 and 7, respectively (13). The 19-kDa proteins are 174 amino acids long and are identical except for an alanine (L11A) to threonine (L11B) difference at the third amino acid position. Analysis of L11 in the late 1980s (a.k.a. L16) showed that expression of either isoform was sufficient for cell viability (14). However, when expressed as the sole form of L11, RPL11A mRNA transcripts accumulated to only 33–40% of wild-type levels as compared to cells expressing both isogenes, while RPL11B mRNAs accumulated to 60–66%. Expression of either isogene alone also affected 60S subunit assembly: a strain expressing only L11B grew at wild-type rates but synthesized fewer 60S subunits than wild-type cells (although apparently not below a threshold necessary for wild-type growth rates), while strains expressing only L11A grew more slowly than wild-type, and synthesized only 33–40% of wild-type levels of total L11 and 60S subunits (14). A random mutagenesis screen of RPL11B for cold-sensitive mutants identified alleles that promoted 25S pre-rRNA processing and initiation defects (15). The specific mutants identified in that study were S34P, S41P, S97F, A98V, S119C and G135D. In Arabidopsis, divergent 5′ untranslated regions (UTRs) between the two isogenes were found to result in differential expression among plant tissues (16). In addition to its function as a ribosomal protein, L11 has been implicated in p53 activation through its interactions with HDM2 in the nucleus of human fibroblast cells (17), and mutant forms of L11 have been linked to Diamond–Blackfan anemia in humans (18).

Although the structural information suggests that L11 should play a significant role in translation, functional analyses of the protein in this role have not been

Figure 1. Localization of L11 within the ribosome. (A) Image of the yeast ribosome. The large subunit is colored green, and the small subunit is pink. L11 (cyan) is located in the central protuberance of the large subunit where it interacts with 5S rRNA, Helix 84 of 25S rRNA, the T-loop of the peptidyl-tRNA, and the small subunit protein S18 via the B1b and B1c intersubunit bridges. (B) Close-up view of L11 and neighboring structures. Amino acids of L11’s P-site loop targeted for mutation (R51-R61) are colored deep blue. (C) L11P-site loop amino acids, mutations analyzed in the current study, and their viabilities as the sole form of L11. Y52* represents multiple mutations: Δ, A, R, E, S, I, Q, N, H and F. Ribosomal structures generated in PyMol using yeast cryo-EM (4) with tRNAs from T. thermophilus (5).
performed. In this report, a series of mutants were generated using a reverse genetics approach to parse the role of the L11 P-site loop. Detailed biochemical and structural analyses focused on two multi-amino acid mutants with opposing effects on rRNA structure and tRNA binding. We propose that prior to peptidyltransfer, the presence of peptidyl-tRNA in the large subunit P-site positions the L11 P-site loop to interact with the Helix 84 of the large subunit rRNA. After peptidyltransfer, spontaneous translocation of the deacylated tRNA to the large subunit E-site allows the L11 P-site loop to extend into the P-site, breaking contact with Helix 84. By this model, we hypothesize that the L11 P-site loop functions locally as a sensor of the occupancy status of the ribosomal P-site.

MATERIALS AND METHODS

Strains, plasmids and media

Restriction enzymes were obtained from Promega (Madison, WI, USA), MBI Fermentas (Vilnius, Lithuania) and Roche Applied Science (Indianapolis, IN, USA). The QuikChange XL II site-directed specific mutagenesis kit was purchased from Stratagene (La Jolla, CA, USA). DNA sequencing was performed by Genewiz (Germantown, MD, USA). Escherichia coli DH5α was used to amplify plasmid DNA. Transformation of yeast and E. coli and were performed as previously described (19). YPAD, SD and 4.7 MB plasmids for testing the killer phenotype were as previously described (19). YPAD, SD and 4.7 MB plasmids previously described for the native 5′RPL11B UTR regions (1228 bp and 485 bp, respectively) were purified by agarose gel electrophoresis. This 2238-bp fragment was ligated into BamHI digested plasmid pYCP50L11B URA3. Using flanking BamHI RPL11B was isolated from yeast strain PSY2088 (MATα rpl11a::HIS3 rpl11b::HIS3 ura3-52 leu2A1 trp1A63 his3Δ200 + YCpL11B URA3), an rpl11a/rpl11b gene deletion strain in which L11 is supplied by a URA3-CEN6 based PRL11B clone, was a generous gift from Dr. Pamela Silver (21). The L-A and M1 viruses were introduced into PSY2088 by cytoplasmic mixing (cyctoduction) through nonproductive mating with JD758 [MATα kar1-1 arg1 (L-AHN M1)]. Transformation of yeast and E. coli and were performed as previously described (19). YPAD, SD and 4.7 MB plasmids for testing the killer phenotype were as previously reported (19). Plasmids for expression of dual luciferase reporters were described previously (20).

Saccharomyces cerevisiae strain PSY2088 (MATα rpl11a::HIS3 rpl11b::HIS3 ura3-52 leu2A1 trp1A63 his3Δ200 + YCpL11B URA3), an rpl11a/rpl11b gene deletion strain in which L11 is supplied by a URA3-CEN6 based PRL11B clone, was a generous gift from Dr. Pamela Silver (21). The L-A and M1 viruses were introduced into PSY2088 by cytoplasmic mixing (cyctoduction) through nonproductive mating with JD758 [MATα kar1-1 arg1 (L-AHN M1)] to produce the Killer+ strain JD1313 as previously described (19). Wild-type PRL11B was isolated from yeast strain PSY2088 plasmid (pYCP50L11B URA3). Using flanking BamHI restriction sites, a 2.2-kb fragment of DNA containing both the 525-bp wild-type PRL11B ORF plus the native 5′ and 3′ UTR regions (1228 bp and 485 bp, respectively) was purified by agarose gel electrophoresis. This 2238-bp fragment was ligated into BamHI digested pRS314, a low copy TRP1-selectable plasmid (purchased from ATCC, Manassas, VA, USA) (22) to create pRS314L11B-TRP1. This plasmid served as the template for generation of rpl11B mutants by site directed mutagenesis using the primers listed in Supplementary Table S1. Wild-type and mutant pRS314L11B-TRP1 clones were transformed into JD1313, selected for growth on –trp medium, and cells having lost the URA3-based plasmid were identified by their ability to grow in the presence of 5-fluoroorotic acid (5-FOA) (23).

Cell growth and drug resistance/sensitivity phenotypes

The effects of temperature and translational inhibitors were assessed by standard 10-fold dilution spot assays. Yeast were grown in H-tryptophan synthetic deletion (SD) media (–Trp) to mid log phase. OD₅₉₅ values were obtained, and cells were serially diluted 10-fold from 10⁵ to 1 CFU per 2.2 μl and spotted on –Trp plates. Growth was monitored at 20°C, 30°C and 37°C, and pharmacogenetic assays utilized 2 mg/ml paromomycin, 40 μg/ml anisomycin or 30 μg/ml sparsomycin incubated at 30°C for 3–5 days. Killer virus assays were performed as previously described (19).

Translational fidelity assays

The dual luciferase reporter plasmid pYDL-control, pYDL-LA, pYDL-Ty1, pYDL-UAA (20) and pYDL-AGC₂₁₈ (24) were employed to quantitatively monitor programmed –1 ribosomal frameshifting, programmed +1 ribosomal frameshifting, suppression of a UAA codon and suppression of an AGC serine codon in place of an AGA arginine codon in the firefly luciferase catalytic site respectively. In this study, the reporters were housed in LEU2-based reporters: the 0 frame dual luciferase reporter was pJD419, the L-A dsRNA virus –1 PRF containing reporter was pJD420 and the Ty1 containing +1 PRF reporter was pJD421. Cells were grown overnight in 5-ml volumes of –leu synthetic depletion media to mid log phase (A₅₉₅ = 0.8–1.5). Cells were washed, resuspended in lysis buffer (1X PBS pH 7.4, 1 mM PMSF) and lysed using 0.5-mm glass beads with a vortex mixer for 3–5 min at 4°C. Lysates were clarified by centrifugation for 5 min. at 8000 r.p.m. at 4°C. Samples were maintained on ice, and 5 μl of clarified lysate was added to 50 μl of pre- aliquoted Promega LARII reagent, mixed by pipetting, and read in a TD20/20 luminometer. Immediately upon completion of this read, 50 μl of Promega Stop and Glo buffer was added to the tube, pipetted to mix and read again. This was repeated 6–12 times per strain per reporter depending on the consistency of the data. Frameshifting rates were determined by taking the ratio of firefly to Renilla luciferases for each sample, and then taking the ratio of the average ratios of the 0 frame samples to that of test reporter ratios to obtain the rates for both –1 and +1 PRF. These results were then analyzed by t-test to determine statistical significance compared to wild-type levels as previously described (25). Prior to determining rates of UAA readthrough (nonsense suppression), strains were cured of the endogenous yeast prion [PSI+] by daily serial passage of cells in -trp liquid media containing 5 mM guanidine hydrochloride for 10 days. Rates of nonsense suppression were determined as previously described (20) using the LEU2-selectable 0-frame control pJD419 and in-frame UAA containing reporter pJD702. Missense reporters were based on URA3 plasmids previously described for the sense reporter (20) and for the firefly luciferase 218 arginine codon (AGA) to serine (AGC) missense reporter plasmid pYDL-AGC (24). Methodologies were the same as those for other dual luciferase assays described above.
Ribosome preparation

Cells were grown overnight in a 30°C shaker in 500 ml of YPAD media to mid-log phase (OD595 0.8–1.5), cooled to 4°C for 1 h to allow ribosomes to run off of transcripts while remaining tightly coupled. Cells were harvested by centrifugation and washed three times with 40 ml 0.9% KCl solution. Cell pellets were stored at −80°C until needed, at which time they were thawed and resuspended in 1 ml binding buffer (10 mM Tris–HCl pH 7.5, 10 mM MgCl2, 60 mM NH4Cl, 2 mM DTT, 1 mM PMSF) per gram of cells. Cells were lysed with a 1:1 vol of Zirconian beads (BioSpec, Bartlesville, OK, USA) and disrupted using two 2-min pulses of a minibead beater. Lysates were clarified by centrifugation at 20 000 r.p.m. (50 000 g) using an MSL-50 rotor at 4°C for 25 min.

Ribosomes were chromatographically purified using Sulfolink beads (Pierce, Rockford, IL, USA) as previously described (26), and eluted from the resin in 8 ml of elution buffer (10 mM Tris–HCl pH 7.5, 10 mM MgCl2, 500 mM KCl, 2 mM DTT, 0.5 mg/ml heparin). Eluted ribosomes were treated with 2 mM puromycin and 1 mM GTP for 30 min at 30°C and were layered on top of a 22-ml glycerol cushion [50 mM HEPES-KOH pH 7.6, 10 mM Mg(CH3COO)2, 50 mM NH4Cl, 1 mM DTT, 25% glycerol] and pelleted by centrifugation at 30 000 r.p.m. at 4°C for 18–20 h. Pellets were washed with 1 ml of storage buffer [50 mM HEPES-KOH pH 7.6, 10 mM Mg(CH3COO)2, 50 mM NH4Cl, 1 mM DTT, 25% glycerol], and resuspended in 200–400 μl of storage buffer. Concentrations were determined spectrophotometrically (1 OD260 = 20 pmol ribosomes). The salt-washed ribosomes were aliquoted and stored at −80°C for up to 3 months. Ribosomal rRNA quality was checked on 1.3% agarose gels and rRNA to protein ratios were monitored by determining OD260 to OD280 ratios. Polysome profiles were obtained by sucrose density gradient centrifugation as previously described (27).

Assays of ribosome/tRNA interactions

[14C]Phe-tRNAph and Ac-[14C]Phe-tRNAph species were generated and purified by high-performance liquid chromatography (HPLC) as previously described (28). Salt-washed ribosomes were thawed and activated at 30°C for 15 min. To monitor binding of [14C]Phe-tRNAph to the A-site, 125 pmol of ribosomes were pre-incubated at 30°C for 30 min in 8 ml of A-Site Binding Buffer [80 mM Tris pH 7.4, 160 mM NH4Cl, 15 mM Mg(CH3COO)2, 2 mM spermidine, 6 mM β-mercaptoethanol containing 1.33 mM GTP, 0.25 mg polyU, 51 μg soluble binding factors generated as described (29) and 0.1 mg uncharged tRNAph. Subsequently, 12.5 pmol of ribosomes were added to 2-fold serial dilutions of [14C]tRNAph to a total volume of 30 μl and incubated at 30°C for 35 min. Samples were passed through Millipore (Billerica, MA, USA) nitrocellulose filters using a multi-sample vacuum manifold, filters were washed three times with 3 ml of A-site binding buffer and amounts of bound [14C]Phe-tRNAph were determined by scintillation counting. Background values (no ribosome controls) were subtracted from all samples. All strains were tested at least twice in triplicate. Kd values were calculated using GraphPad Prism Software fitted to 1-site binding with ligand depletion formula. To examine binding of Ac-[14C]Phe-tRNAph to the P-site, 150 pmol of ribosomes were resuspended in 150 μl of P-Site Binding Buffer [80 mM Tris pH 7.4, 160 mM NH4Cl, 11 mM Mg(CH3COO)2, 2 mM spermidine, 6 mM β-m] plus 0.25 mg polyU. Ribosomes (17 pmol) were added to 2-fold serial dilutions of Ac-[14C]Phe-tRNAph to a total volume of 30 μl and incubated at 30°C for 40 min. Filter binding assays and determination of steady-state Kd values were performed as described above. Multiple turnover peptidyltransfer reactions utilized 250 pmol of ribosomes pre-loaded with Ac-Phe-tRNAph by incubating for 40 min at 30°C with 256 pmol of Ac-[14C]Phe-tRNAph in 300 μl of binding buffer (BB) [80 mM Tris pH 7.4, 160 mM NH4Cl, 11 mM Mg(CH3COO)2, 2 mM spermidine, 6 mM β-mercaptoethanol] including 0.17 mg of polyuridine. Puromycin was added to each 100-ml reaction to a final concentration of 25 mM, samples were incubated at 30°C and 10 μl samples were taken at 2-, 5-, 10-, 20-, 40- and 60-min time points, added to 90 μl binding buffer plus 100 μl of 1 M NaOH to stop the puromycin reactions. Two 5-μl aliquots were taken from each sample and added directly to 5 ml of scintillation fluid to determine total possible counts. Peptidylpuromycin products were extracted into 400 μl of ethyl acetate, half of which were added to 5 ml of scintillation fluid for counting. Data were plotted by counts per minute versus the percentage of N-Ac-[14C]Phe-tRNAph reacted. Kobs was determined by plotting first-order time plots using the formula: ln[100/(100 – x)] where x is the percentage of tRNA reacted. The first three time points yielded straight lines, the slopes of which are equal to the Kobs values.

rRNA structural analysis using selective 2'-hydroxyl acylation analyzed by primer extension

Empty, polyU loaded, or polyU and deacetylated P-site tRNA loaded salt-washed ribosomes (50 pmol) were resuspended in 200 μl of selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE) buffer [80 mM Tris–HCl pH 7.4, 100 mM NaCl 15 mM Mg(CHCOOH)3] and incubated for 10 min at 30°C. Samples were split, and 10 μl of dimethyl sulfoxide (DMSO) was added to half of the samples, while 10 μl of 60 mM 1M7 was added to the other half. Samples were incubated at 30°C for 20 min. Ribosomes were precipitated by the addition of 275 μl of ice-cold 100% ethanol and stored at −20°C for 1–2 h. Ribosomes were pelleted by centrifugation at 10 000 r.p.m. for 2 min and resuspended in lysis buffer and rRNAs were isolated using an Ambion (Austin, TX, USA) RNeaQeous®-Micro RNA isolation kit. Optical densities were taken at 260 nm and 280 nm to monitor the quantity and quality of RNA, and samples were resuspended at a concentration of 1 μg rRNA/7 μl in pure water. HPLC purified oligonucleotide primers purchased from IDT (Coralville,
IA, USA) are listed in Supplementary Table 2. Oligonucleotides were resuspended to 25 pmol/μl, 3’ end labeled with γ[32P]ATP with T4 polynucleotide kinase (Roche, Indianapolis, IN, USA), and purified from free radiolabeled nucleotide by passage through a MicroSpin G-25 column (GE Healthcare, Piscataway, NJ, USA). Annealing reactions utilized 1 μg of modified rRNAs and 3 μl of labeled oligonucleotide heated at 80°C for 1 min, followed by a 4–7-min incubation at 5–10°C below the Tm of each oligonucleotide. Annealed rRNA/primers (2 μl each) were added to 3 μl of cold enzyme mix [0.25 μl 10 mM dNTP, 0.25 μl 100 mM DTT, 1 μl 5X Superscript III Buffer, 0.25 μl Superscript III (Invitrogen Life Technologies, Carlsbad, CA, USA), 1.25 μl H2O]. For sequencing samples, an additional 1 μl of each ddNTP was added to each C, T, A, G, sample, respectively. Primer extension reactions were performed at 52°C for 25 min, with potential 2-min-long extensions preceding the 52°C at lower temperatures depending on the individual Tm values of the primers. Denaturing RNA loading dye (2 μl) was added to each sample, heated to 94°C for 2.5 min, and samples were resolved through 6% urea-acrylamide denaturing gels. Gels were dried and radiolabeled samples were visualized by phosphorimagery.

### Computational analyses and ribosome structure visualization

The published structures for the 70S ribosome from *E. coli* [PDB accession numbers: 2AVY, 2AW4; (2)], as well as yeast 80S structures from yeast (1SII, 1S1H, 3JYV, 3JYW, 3JYX; (4,12)] were used in the analysis of this work and the generation of figures. Published *T. thermophilus* 70S subunits containing A-site, P-site and E-site Phe-tRNA were also employed (1G1X, (5). All structures were visualized and manipulated using MacPyMol software (30).

### RESULTS

#### Generation and initial characterization of *rpl11b* alleles

The visualization of a single salient loop of L11 interacting with peptidyl-tRNA indicated that it might play a vital role in sensing peptidyl-tRNA occupancy status and transmitting this information to other functional centers of the ribosome. As cells expressing *RPL11B* alone were healthier than those solely expressing *RPL11A*, genetic manipulations began with the yeast *rpl11A*/*rpl11B* double knockout strain JD1313 expressing wild-type (WT) *RPL11B* from a low-copy, *URA3*-selectable episomal plasmid (pPRPL11B-URA3). Oligonucleotide site-directed mutagenesis was used to construct a series of mutants, each containing changes of 1, 4 or 10 sequential amino acids (Figure 1C). Stretches of amino acids from arginine 51 to arginine 61 in the L11 P-site loop were targeted for site-directed mutagenesis expressed from a low-copy, *TRP1*-selectable episomal plasmid under control of the endogenous *RPL11B* promoter (pPRPL11B-TRP1). After transformation and selection on SD medium lacking tryptophan (–trp), cells expressing only mutant *rpl11B* alleles were identified by their ability to grow on SD–trp medium containing 5-flouroorotic acid (5-FOA). Three of the multiple substitution mutants were inviable as the sole forms of L11B. These were R51YTVRTFGIR60→alanine (i.e. 51-60A); deletion of residues 51-60 (51-60Δ); and F57GIR60→alanine (57-60A). Viable mutants, R51YTV54→alanine (51-4A), V54RTF57→alanine (54-7A), R51A, Y52* (mutations including Δ, A, R, E, S, I, Q, N, H and F), F57A and R61A were rescued from yeast into *E. coli*, and the mutations were confirmed by DNA sequencing.

#### The L11 P-site loop mutants confer temperature- and drug-specific growth phenotypes

Standard 10-fold dilution spot assays were performed multiple times to measure the effects of the L11 P-site loop mutants on cell growth across a range of temperatures (30°C, 20°C and 37°C) (Figure 2A). Growth at 30°C represents the ideal temperature, and thus all other conditions were compared to this baseline. At 30°C, the 51-4A mutation conferred depressed growth while 54-7A...
displayed roughly wild-type growth. Cold sensitivity was assessed at 20°C and both mutants grew at wild-type rates. S1-4A showed enhanced growth at 37°C relative to itself at 30°C, while mutant S4-7A was similar to wild type. R51A grew at wild-type rates at 30°C and 37°C but showed enhanced growth at 20°C. The Y52* mutants displayed mutant-specific effects on growth rates at 30°C, but did not confer significant phenotypes at either 20°C or 37°C. F57A had wild-type growth rates at all temperatures, while R61A showed depressed growth at 30°C, which was rescued at 37°C.

Small molecule inhibitors of protein translation are useful probes for identifying changes in ribosome function. This study utilized three such molecules: paromomycin, anisomycin and sparsomycin. The effects of all three drugs were monitored using dilution spot assays at 30°C on SD-trp media containing various drug concentrations. Paromomycin is an aminoglycoside antibiotic that increases translational error rates by artificially stabilizing codon:anticodon interactions at the decoding center in the small ribosomal subunit (31). As compared to their intrinsic growth in the absence of drug, both the S1-4A and S4-7A mutants were slightly hypersensitive to 2 μg/ml paromomycin, as were Y52A, Y52N and Y52H. In contrast, R51A, F57A and R61A were all paromomycin resistant (Figure 2B). Anisomycin competes with the 3' end of the aa-tRNA for binding to the A-site pocket of the ribosome (32,33). Both S1-4A and S4-7A showed anisomycin resistance at 40 μg/ml, as did several Y52* mutants, and R61A (Figure 2B). Sparsomycin binds to the P-site and interferes with peptidyl-tRNA binding and peptidyl transfer (33,34). S1-4A and S4-7A mutants were hypersensitive to 30 μg/ml sparsomycin, as were most of the Y52* mutants, with the exception of Y52F, which conferred slight resistance to this drug (Figure 2B).

The yeast ‘killer’ system is composed of the L-A helper and M1 satellite dsRNA viruses (35). The L-A dsRNA viral genome encodes a capsid protein (Gag), and an RNA-dependent RNA polymerase (Pol) that is synthesized as a Gag-pol fusion protein consequent to a near-cognate amino acid. In JD1313 cells expressing wild-type RPL11B, −1 PRF directed by the L-A dsRNA viral signal was 6.07% ± 0.16%. This compares favorably with other ‘wild-type’ strains in our laboratory (normal range from 4% to 8% (20,25). The S1-4A mutant promoted increased −1 PRF (1.33 ± 0.06-fold relative to wild type), while S4-7A trended in the opposite direction (0.84 ± 0.03-fold relative to wild type) (Figure 3, and Table 1). Both these values were statistically significant and correlate well with the Killer− phenotypes. Y52Δ, Y52N, Y52E and Y52H mutants also showed increased rates of −1 PRF, with statistically significant rates ranging from Y52H at 1.20-fold wild type to Y52E at 1.49-fold wild-type, Y52A, Y52S, Y52Q and Y52F all had wild-type rates of −1 PRF.

While both −1 and +1 PRF are kinetic parameters, the substrates for the slippage are distinct: −1 PRF requires that both the ribosomal A- and P-sites are occupied by tRNAs, while +1 PRF occurs while the A-site is empty (38). Rates of +1 PRF were monitored using a cis-acting signal derived from the Ty1 retrotransposable element using pYDL-Ty1. Baseline +1 PRF efficiencies in cells expressing wild-type RPL11B were 10.98% ± 0.30%. S1-4A had no effects on +1 PRF, while S4-7A promoted a small but statistically significant increase (1.18 ± 0.06-fold of wild type; Figure 3). Significant changes in +1 PRF were also observed in the Y52A, Y52S, Y52N, Y52E, Y52H and Y52F mutants. mRNA decoding occurs in the small subunit decoding center, and changes in termination codon recognition (nonsense suppression) is another indicator of altered translational fidelity. pYDL-UAA (39), which contains an in-frame termination codon immediately 5' of the firefly luciferase gene, was used to monitor this parameter. The baseline rate of nonsense suppression in cells expressing RPL11B was 0.137% ± 0.003%. The S1-4A mutant slightly improved this aspect of translational fidelity, with nonsense suppression levels decreasing to 0.88 ± 0.04-fold of wild-type levels. S4-7A did not affect UAA recognition (Figure 3). Y52Δ, Y52A, Y52S, Y52N, Y52E and Y52H all promoted increased rates of nonsense suppression ranging from 1.31- to 1.78-fold wild type. pYDL-AGC 218 tests missense suppression levels by monitoring rates of incorporation of an arginine (AGA) near-cognate amino acid instead of a cognate serine (AGC) at the catalytic codon 218 within the firefly luciferase gene as previously described (24). Thus, in this assay, mis-utilization of near-cognate tRNA<sup>AGA</sup> at the Ser AGC codon restores firefly luciferase activity. Wild-type missense levels were measured at 0.074% ± 0.002, comparable to previous studies (24). Mutant S1-4A had significantly higher levels of missense suppression (measured at 1.21 ± 0.03-fold wild-type), while S4-7A did not significantly affect this phenomenon (1.07 ± 0.04 fold wild type).
The mutant rpl11b alleles promote opposing effects on tRNA binding to the ribosomal A- and P-sites

Sucrose gradient analyses were employed to fractionate cycloheximide arrested elongating ribosomes on mRNAs in lysates generated from JD1313 cells expressing wild-type L11B, 51-4A, and 54-7A. In all strains the 60S peak was smaller than that of the 40S fraction which can be attributed to the presence of only a single copy of RPL11B, which has previously been shown to effectively reduce the number of 60S subunits produced by the cell to 60–66% of true wild-type levels while having no visible phenotypic effect on growth (14). No significant differences were observed among the samples (data not shown). Phenotypic variation in PRF and in the presence of anisomycin and sparsomycin are indicative of altered interactions between the ribosome and tRNAs. P-site tRNA $K_d$ values were determined in vitro by binding 2-fold serial dilutions of N-acetylated-[14C]Phe-tRNA to ribosomes until saturation was achieved (Figure 4A), and the resulting data were used to determine steady-state single site binding $K_d$ values (Figure 4B). Wild-type ribosomes bound this P-site substrate with a $K_d$ of 72.3 ± 7.9 nM. The 51-4A mutants promoted a slight increase in affinity for P-site substrate ($K_d = 50.9 ± 11.2$ nM), while 54-7A had the opposite effect ($K_d = 89.3 ± 10.4$ nM). Given the physical interaction between the L11 P-site loop and peptidyl-tRNA, it was imperative to determine whether the observed small changes in P-site affinities promoted by the mutants were biochemically significant. To this end, multiple turnover puromycin reactions were performed. In these experiments, puromycin was added to ribosomes pre-incubated with excess P-site substrate, i.e. Ac-[14C]Phe-tRNA$^{Phe}$, and accumulation of the peptidylpuromycin product was monitored over time. In these reactions, the first round of peptidylpuromycin synthesis is very rapid. Next, in a slow step, the ribosome intrinsically translocates the deacylated tRNAPhe into the E-site (40), followed by the slow diffusion of Ac-[14C]Phe-tRNA$^{Phe}$ into the P-site where it can react with puromycin. Repetition of this cycle results in slow multiple rounds of product synthesis (Figure 4C). Assuming that the L11 mutants do not affect either rates of intrinsic translocation or of Ac-[14C]Phe-tRNA$^{Phe}$ diffusion into the P-site, changes in product accumulation, i.e. $K_{obs}$, should be due to differences in binding affinities for the P-site substrate. Consistent with this model 51-4A promoted 1.46 ± 0.14-fold increased $K_{obs}$ relative to wild-type ribosomes, while 54-7A decreased $K_{obs}$ to
0.51 ± 0.01-fold that of wild type (Figure 4D). To assay for changes in tRNA binding to the A-site, steady-state $K_d$ values were determined in vitro by binding 2-fold serial dilutions of [14C]Phe-tRNA to ribosomes pre-loaded with uncharged tRNA-Phe in the P-site. Wild-type $K_d$ values were 103 ± 11 nM. 51-4A promoted decreased affinity for the substrate with a value of 150 ± 19 nM, while 54-7A promoted a small increase in affinity (84 ± 7 nM) (Figure 4E and F).

The P-site loop is flexible depending on the occupancy status of the P-site

The highly basic nature of the P-site loop, its interaction with peptidyl-tRNA, and its proximity to 25S rRNA Helix 84 (H84) suggested that it might interact with either of these two RNA components depending on the occupancy status of the P-site. Changes in interactions between the P-site loop and local rRNA structures may in turn
propagate outward to more distant regions of the ribosome. To test this, SHAPE (41–43) was employed to probe for structural alterations in selected regions of the 25S, 18S and 5S rRNAs due to either the L11B mutants or in wild-type ribosomes with occupied or unoccupied P-sites. Due to the large size and complex three-dimensional structure of the ribosome, the entire rRNA content was not examined. Rather, approximately one-third of the rRNA bases were interrogated, focusing on those bases closest to L11, the A- and P-sites, and the decoding center.

In the first series of experiments, salt-washed wild-type and 51-4A, 54-7A, Y52Q and Y52F mutant ribosomes (chosen for structural analyses because they had the most pronounced genetic phenotypes) were treated with 1M7, an electrophile that adds an adduct onto the 2'OH groups of solvent exposed base sugars. Modifications were performed on salt-washed ribosomes because they represent the thermodynamic ‘ground state’ of the ribosome. Thus, the structural changes observed are indicative of changes in the full ‘dynamic potential’ of the ribosome as opposed to conformations locked in by e.g. occupation of binding sites by tRNAs or ribosome-associated factors. rRNAs were extracted, hybridized with 5'[^32P]-labeled oligonucleotide primers and reverse transcriptase primer extension reactions were performed. The products were separated through urea-acylamide denaturing gels, and visualized using a phosphorimager. 2'-OH ribose modification results in a strong stop 1-nt 5' of modified bases, and the intensity of the stops are proportional to the solvent accessibility and flexibility of riboses. Comparison of the protection patterns between wild-type and mutant ribosomes enables identification of specific bases which became protected or deprotected relative to WT.

In all areas examined, rpl11b ribosomes Y52Q and Y52F matched the wild-type rRNA base modification profile (data not shown), while 51-4A and 54-7A ribosomes revealed consistently reproducible differences. The most significant changes in rRNA structure were observed in bases C2675-A2679 (E. coli numbering: C2306-2310) located in the terminal loop of 25S rRNA H84 (Figure 5A and E). The two mutants promoted opposing patterns of base protection/deprotection in this structure. Specifically, as compared to wild-type ribosomes, 51-4A promoted enhanced protection of this loop, while the loop was deprotected in the 54-7A mutants. Analysis of the recent cryo-EM yeast ribosome structure (4) revealed that these H84 loop bases are located within 3Å of the stretches of amino acids changed to alanines in both the 51-4A and 54-7A mutants (Figure 5B). These findings suggested that the two mutants had the effects of displacing the P-site loop into two opposing conformational states: extended toward the P-site (54-57A), or retracted into H84 (51-54A). To test whether these two states are naturally dependent on P-site occupancy, the experiments were repeated with wild-type and mutant ribosomes with or without tRNA<sup>the</sup> in their P-sites. Consistent with this model, addition of tRNA to the P-site of wild-type ribosomes resulted in slightly enhanced protection of the H84 terminal loop bases closest to the P-site loop (A2676-A2679). Interestingly, C2675 showed significant deprotection when the P-site was occupied by tRNA. This base is on the far side of the terminal end of H84 from the P-site loop, suggesting that H84 itself alters its conformation upon tRNA occupancy of the P-site (Figure 5C). 51-4A's H84 bases were unchanged between P-site bound and unbound ribosomes, consistent with the P-site loop positioned in the ‘retracted’ state in this mutant, although small differences in the protection patterns suggest that the P-site loop is in a slightly different orientation in this mutant. In contrast, while 54-7A ribosomes, i.e. the P-site loop ‘extended’ state, showed deprotection at all bases (C2675-A2679) for both P-site bound and salt washed ribosomes, bases A2676-A2679 were less deprotected when tRNA was in the P-site and C2675 was even more reactive, consistent with the notion that the P-site loop interacts with H84 when peptidyl-t-tRNA is in the P-site.

Although no other SHAPE-specific changes were observed, several other phosphodiester bonds 3' of specific 25S rRNA bases were reproducibly more, or less, intrinsically labile as compared to wild type (Figure 5D). In both mutants, G2531 and G2534 located in expansion segment 31 (ES31) were more stable than in wild-type ribosomes as evidenced by reduced intensity of strong reverse transcriptase stops 1-nt 5' of these bases. Additionally, bases A2779-A2780 (E. coli A2407-U2408) located in the terminal loop of Helix 88 were hyper-labile in 51-4A mutant ribosomes as compared to WT, as shown by the presence of strong stops with increased intensity 1-nt 5' of these bases. These are mapped onto the two-dimensional structure of yeast 25S rRNA (Figure 5E).

**DISCUSSION**

The L11 P-site loop is largely comprised of polar amino acids and carries a net positive charge, making it ideal for interactions with the phosphate backbones of nucleic acids, e.g. rRNA and tRNA. Positioned between H84 and the peptidyl-tRNA T-loop, several of its amino acids are within H-bonding distance of H84 (~3.3 Å), while C56 of the peptidyl tRNA T-loop comes within 2.1 Å of G58 in the L11 P-site loop (4.5), suggesting that the L11 P-site loop can directly interact with both of the RNA-based structures. While currently available X-ray crystal structures are unavailable for ratchet-state ribosomes, a recently published examination of tRNA movement through the E. coli ribosome using large-scale analysis of cryo-EM images implicates the P-site loop as a dynamic arm interacting with and moving in relation to tRNAs passing across the P-site (44). Although these studies were performed at resolutions of 9–20 Å, leaving considerable ambiguity regarding the precise residues involved, they clearly reveal highly dynamic interactions between the P-site loop and both P-site, and E-site tRNAs.

Although death is not a phenotype per se, the inviable mutants are informative nonetheless in so far as they demonstrate that the amino acids F<sub>57</sub> and G<sub>58</sub> are absolutely required for viability. While F<sub>57</sub> is universally conserved,
it does not appear to be essential on its own for viability, as witnessed in the mild phenotypes of the F57A mutant. Similarly, all single amino acid changes explored here resulted in viable cells, suggesting a certain degree of biochemical/biophysical redundancy within this essential loop. In support of this notion, the strongest growth phenotypes observed across a range of temperatures and small molecule translational inhibitors were concentrated in the multiple alanine substitutions, i.e. 51-4A and 54-7A, thus directing the bulk of the biochemical and structural analyses to these two mutants.

Analysis of the results of the assays performed on the viable multiple alanine substitution mutants (summarized in Table 1) provoke the hypothesis that the L11 P-site loop may dynamically function to help the ribosome sense the occupancy status of the large ribosomal subunit P-site. This is modeled in Figure 6. When the P-site is unoccupied, the P-site loop can extend into this space, moving away from the terminal loop of H84. Upon occupation of the P-site, the peptidyl-tRNA T-loop displaces the L11 P-site loop, causing its retraction into H84. By this model, the rRNA SHAPE analyses depicting increased
where it promotes misreading of near-cognate codons in the A-site by stabilizing codon–anticodon interactions (45). This sensitivity may be attributable to an observed increase in missense incorporation of a near cognate arginine (AGA) over that of the sense serine codon (AGC) in mutant 51-4A. Intriguingly, 54-7A had wild-type levels of missense incorporation suggesting that its sensitivity to paromomycin was indirect. The reciprocal anisomycin/paromomycin phenotypes of the L11 mutants demonstrate the effects of this protein on A-site ligand based ribosomal functions over very long distances. Similar phenotypic patterns were previously observed with mutants of other large subunit components (46,47).

The observed effects on -1 PRF are consistent with a recent kinetic analysis demonstrating that aa–tRNA slippage is the most highly weighted parameter in determining the rate at which this process occurs (Liito,P.Y. et al., submitted for publication). Here, increased affinity for aa–tRNA by the large subunit suggests that the 51-4A ribosomes stabilize the frame-shifted (i.e. near-cognate) tRNAs, reducing their ability to be proofread, thus promoting increased rates of −1 PRF. This is consistent with the observed increased rates of missense decoding in this mutant. Conversely, post-slippage A-site tRNAs are even less stable in the 54-7A mutants, leading these to be more efficiently proofread, and thus promoting decreased −1 PRF efficiency. In both cases, altering −1 PRF from the optimum ‘golden mean’ precludes these cells from maintaining the yeast killer virus (19,48). Programmed +1 frameshifting is completely dependent on peptidyl-tRNA slippage. Increased +1 PRF in the 54-7A mutant is consistent with decreased affinity for this substrate. The failure to observe decreased +1 PRF in the 51-54A mutant, despite its increased affinity for peptidyl-tRNA, is not entirely clear, although this may be due to the inability of these ribosomes to achieve a threshold beyond which +1 PRF effects can be observed.

The changes in rRNA stability observed in the terminal loop of Helix 88 and in ES31 are intriguing. Chemical protection experiments revealed the terminal loop of Helix 88 is involved in a kissing loop interaction with the terminal loop of Helix 22, and this interaction is apparent in the X-ray crystal and cryo-EM structures (4,49). Increased lability at A2779 and A2780 was
previously observed in the Y11C mutant of ribosomal protein L10 (homolog of E. coli L16) located at the base of the aa-tRNA accommodation corridor, and in the \( \Psi2922C \) (E. coli U2554) 25S rRNA mutant located in the peptidyltransferase center (50,51). The observation that mutations located in three very different and topologically distinct regions of the large subunit conferred similar structural effects suggest that this kissing loop interaction plays an important role in ribosome function. Its location on the cytoplasmic face of the ribosome where deacylated tRNA leaves the molecule implies that the interaction between the terminal loops of Helices 88 and 22 may be involved in gating this deacylated tRNA exit corridor open and closed. This is consistent with the model of allosteric coordination between the A- and E-sites (52,53), which would indicate that the defects conferred by all of these mutants on aa-tRNA binding might impair this E-site gating function. The decreased lability of C2531 and G2534 in ES31 is similarly intriguing, raising more questions than answers. No function is currently associated with this expansion of Helices 88 and 22 may be involved in A-site/E-site gating function. Its location on the cytoplasmic face of the large subunit (4). Perhaps this site is also involved in A-site/E-site gating function. Alternatively, it may be a site for recognition of defective ribosomes by the nonfunctional ribosome decay apparatus.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

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