Analysis of mutations at residues A2451 and G2447 of 23S rRNA in the peptidyltransferase active site of the 50S ribosomal subunit

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On the basis of the recent atomic-resolution x-ray structure of the 50S ribosomal subunit, residues A2451 and G2447 of 23S rRNA were proposed to participate directly in ribosome-catalyzed peptide bond formation. We have examined the peptidyltransferase and protein synthesis activities of ribosomes carrying mutations at these nucleotides. In *Escherichia coli*, pure mutant ribosome populations carrying either the G2447A or G2447C mutations maintained cell viability. *In vitro*, the G2447A ribosomes supported protein synthesis at a rate comparable to that of wild-type ribosomes. In single-turnover peptidyltransferase assays, G2447A ribosomes were shown to have essentially unimpaired peptidyltransferase activity at saturating substrate concentrations. All three base changes at the universally conserved A2451 conferred a dominant lethal phenotype when expressed in *E. coli*. Nonetheless, significant amounts of 2451 mutant ribosomes accumulated in polysomes, and all three 2451 mutations stimulated frameshifting and readthrough of stop codons *in vivo*. Furthermore, ribosomes carrying the A2451U transversion synthesized full-length β-lactamase chains *in vitro*. Pure mutant ribosome populations with changes at A2451 were generated by reconstituting *Bacillus stearothermophilus* 50S subunits from *in vitro* transcribed 23S rRNA. In single-turnover peptidyltransferase assays, the rate of peptide bond formation was diminished 3- to 14-fold by these mutations. Peptidyltransferase activity and *in vitro* β-lactamase synthesis by ribosomes with mutations at A2451 or G2447 were highly resistant to chloramphenicol. The significant levels of peptidyltransferase activity of ribosomes with mutations at A2451 and G2447 need to be reconciled with the roles proposed for these residues in catalysis.

Peptidyl transferase, the catalytic activity of the large ribosomal subunit that covalently links amino acids during protein synthesis, has been the subject of intense interest for over 30 years. An abundance of compelling biochemical and genetic evidence has supported the view that this biologically important reaction is catalyzed by 23S ribosomal RNA (reviewed in refs. 1 and 2). Recently, the x-ray crystal structure of the 50S subunit from *Haloarcula marismortui* was solved at atomic resolution (3, 4). Structures were solved of the vacant 50S subunit, and of complexes of the 50S subunit bound with a minimal A-site substrate or with C-C-dA-phosphoramide-puromycin, a peptidyltransferase inhibitor designed to mimic the tetrahedral intermediate in the proposed reaction pathway for peptide bond formation (4, 5). The high-resolution structures revealed that the site of peptide bond formation is more than 18 Å from the nearest protein residue, and that the catalytic center is composed solely of 23S rRNA.

In the complex of the 50S subunit with C-C-dA-phosphoramide-puromycin, the N-3 position of the adenosine at residue 2451 (*Escherichia coli* numbering used throughout), in domain V of 23S rRNA, was located ≈3 Å from one of the two nonbridging phosphoramide oxygen atoms, and ≈4 Å from the nitrogen corresponding to the attacking amine, in the tetrahedral center of the analog. This observation led to the proposal that A2451 functions as a general base, abstracting a proton from the amino group of aminoacyl-tRNA, and subsequently serves as a general acid, donating the proton to the 2′ hydroxyl leaving group of the P-site tRNA. An additional, or alternative, role for the protonated form of A2451 in transition-state oxyanion stabilization was also proposed (4). Based on the structure of the complex, it was further proposed that the catalytic role of A2451 is augmented by a charge-relay network, mediated through the nucleotide base of G2447 to the phosphate of A2450, that raises the pK_a of the N-3 position of A2451 toward the physiological range. A study of the pH dependence of the dimethyl sulfate (DMS) modification of A2451 also suggested an elevated pK_a of ≈7.6 for this residue (6).

Biochemical and genetic evidence had previously suggested the importance of A2451. This residue was a major site of high-yield photocrosslinking of 3-(4′-benzoylphenyl)propionyl-Phe-$tRNA^{Phe}$ when it was bound to the P site (7). Remarkably, the crosslinked BP-Phe-$tRNA^{Phe}$ was reactive in peptidyl transfer when ribosomes were supplied with aminoacyl-tRNA as an A-site substrate. A2451 was protected from DMS modification by the aminoacyl moiety of P-site bound tRNA (8, 9), by aminoacyl tRNA bound in the A/P hybrid state (10), and by chloramphenicol and carbomycin, antibiotics that inhibit peptide bond formation (11). Thus, interestingly, the DMS reactivity of A2451 is diminished even in the absence of an A-site substrate. Finally, in modification-interference experiments, A2451 was one of four 23S rRNA bases critical for P-site binding (12).

Bases essential for the mechanism of peptidyltransferase would be expected to be phylogenetically invariant. Whereas A2451 is completely conserved in all known sequences (ref. 13; and http://www.rna.icmb.utexas.edu), an A to U mutation of the mouse mitochondrial equivalent of nucleotide A2451 rendered cells resistant to chloramphenicol (14). G2447, while present as a guanine in over 95% of known sequences, is not absolutely conserved (ref. 13; and http://www.rna.icmb.utexas.edu); in yeast mitochondria, a G to A mutation at this position rendered cells resistant to chloramphenicol (15), whereas a G to C mutation in *Halobacterium cutirubrum* resulted in moderate resistance to anisomycin, another antibiotic inhibitor of peptidyl transfer (16). In this study, we constructed mutations at A2451 and G2447 in *E. coli* and *Bacillus stearothermophilus* 23S rRNA. We determined the effect of the mutations on cell viability, on

Abbreviations: DMS, dimethyl sulfate; Pm, puromycin.

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the ability of mutant ribosomes to participate in protein synthesis *in vivo*, and on the protein synthesis and peptidyltransferase activities of the mutant ribosomes *in vitro*. Ribosomes carrying mutations at either A2451 or G2447 synthesized full-length protein chains *in vivo* and *in vitro*, and catalyzed peptide bond formation in single-turnover peptidyltransferase assays. The peptidyltransferase and *in vitro* protein synthesis activities of the 2451 and 2447 mutant ribosomes were resistant to chloramphenicol at concentrations that abolished the activity of wild-type ribosomes.

Materials and Methods

Bacterial Strains and Plasmids. Site-directed mutagenesis of G2447 and A2451 of *E. coli* 23S rRNA was done as described (17). Mutations at A2451 were expressed from plasmid pGQ7, and mutations at G2447 were expressed in pLK35; both place the *E. coli rrnB* operon under control of the λ P_L promoter. DH1 pCI857 (encoding the *cI857* allele of the λ repressor) was transformed with the mutant plasmids. Synthesis of plasmid-encoded rRNA was induced by growth at 42°C for 2.5 h.

The Δ7 prrn strains AVS69009 (Δ*rrnE* Δ*rrnB* Δ*rrnA* Δ*rrnH* Δ*rrnG*::*lacZ* Δ*rrnC*::*cat* Δ*rrnD*::*cat* Δ*recA*/pTRNA66, pHKrrnC) and MC250 (Δ*rrnE* Δ*rrnB* Δ*rrnH* Δ*rrnG*::*cat* Δ*rrnA* Δ*rrnD*::*cat* Δ*rrnC*::*cat rpsL121 recA56*/prrnS12 p70) carry deletions of all seven *rrn* operons. MC250 also carries the *rpsL121* mutation. This strain is streptomycin sensitive because the resident neomycin-resistant *rrn* plasmid, prrnS12, carries a wild-type *rpsL* gene and streptomycin sensitivity is dominant. Displacement of prrnS12 by other *rrn* plasmids eliminates the wild-type *rpsL* gene and permits expression of the *rpsL121*-associated streptomycin resistance (18). The ability of pLK35 or its mutant derivatives to displace prrnS12 was tested by growing MC250 transformants on medium containing streptomycin, which permits growth only of those cells that have lost prrnS12 and express rRNA exclusively from the pLK35 plasmid. Exclusive expression of mutant rRNA in the resulting strains was confirmed by primer extension analysis of total RNA (19).

Fractionation of Cell Lysates and Quantification of Mutant rRNA. DH1 pCI857 pGQ7 (wild-type) and derivatives encoding the three 2451 mutations were grown to saturation at 30°, diluted into fresh medium and grown at 42°C for 2.5 h. Cell lysates were fractionated through sucrose gradients as described (20, 21). Gradient fractions containing 50S subunits, 70S ribosomes, or polysomes were collected, and rRNA was extracted and analyzed by primer extension with a primer complementary to residues 2450–2469 of 23S rRNA (19). Relative intensity of bands corresponding to extension products from the mutant and wild-type templates was determined with a PhosphorImager (Molecular Dynamics) and IMAGEQUANT software.

Miscoding Assays. The pSG series of *lacZ* plasmids contain nonsense or frameshift mutations in the 5′ end of the *lacZ* coding region (22, 23). Synthesis of β-galactosidase requires that ribosomes read through stop codons or shift reading frame. The β-galactosidase activities of cells harboring plasmid pLG857 (encoding the λ *cI857* repressor), pLK35- or pGQ7-derived *rrn* plasmids, and the pSG series of plasmids were measured after mutant rRNA synthesis was induced by growth at 42°C for 2.5 h, as described (22).

***In Vitro* Coupled Transcription-Translation.** DH1 pCI857 pGQ7 (wild-type) and the A2451U derivative were grown as described above, MC250 pLK35 and the G2447A mutant derivative were grown to mid-log at 37°C, and ribosomes were prepared as described (24), except that cells were lysed by grinding with alumina. Extracts from *E. coli* strain MRE600 were prepared as described (25, 26) and depleted of DNA with staphylococcal nuclease. Protein synthesis was completely dependent on added plasmid (pUC19) and ribosomes. Assays for the incorporation of [^{35}S]methionine (>1000 Ci/mmol, 15 mCi/ml, Amersham Pharmacia; diluted with unlabeled methionine to 1000 cpm/pmol) contained fractionated MRE600 extract, 3–7 pmol ribosomes, 0.57 pmol pUC19, purified IF1, IF2, and IF3 (10 pmol each), and synthesis mix (25, 26) in 15 μl. Where indicated, chloramphenicol (250 μM) was added to ribosomes for a 10-min preincubation at 25°C before the addition of the remaining reaction components. Synthesis was followed at 37°C; 3-μl samples were removed at times indicated into 10% (wt/vol) trichloroacetic acid, boiled, and filtered through Whatman GF/A discs. Radioactivity was detected by liquid scintillation counting. Analysis of reaction products by 10% polyacrylamide-SDS electrophoresis was done as described (25).

***In Vitro* Reconstitution of *B. stearothermophilus* 50S Subunits Carrying Mutations at A2451.** Nucleotide substitutions at A2451 were incorporated into the plasmid pBST7-23S by using the QuikChange protocol (Stratagene) and confirmed by sequencing. *B. stearothermophilus* 50S subunits were reconstituted from *in vitro* transcripts of 23S rRNA as described (27).

Peptidyltransferase Assays. Assay A was performed as described (28) with *B. stearothermophilus* 50S subunits reconstituted with wild-type or 2451 mutant 23S rRNA. Reaction products were resolved by denaturing 24% PAGE and quantified on a PhosphorImager. In assay B, reconstituted *B. stearothermophilus* 50S subunits (68 nM) were incubated with highly purified *E. coli* 30S subunits (100 nM), 4 μM gene 32 mRNA, and 180 nM *N*-Ac-[^{35}S]Met-tRNA^{Met} at 45°C for 2 min. Puromycin (1 mM) was added to start the reaction, and the incubation was continued at 45°C. Samples were removed at indicated times, and the reaction was stopped with 0.2 M KOH. Samples were heated at 37°C for 30 min and loaded on Whatman 3MM paper for electrophoresis in pyridine acetate buffer at pH 3.5 (29). Radioactive species were quantified on a PhosphorImager. Both assay A and assay B were performed as described above with pure *E. coli* G2447A mutant ribosomes, except that reactions were incubated at 37°C and shorter time courses were followed.

Results

Effects of Mutation of Residues G2447 and A2451 on Growth and Viability of *E. coli*. We constructed all three base changes at positions G2447 and A2451 of *E. coli* 23S rRNA by site-directed mutagenesis, and the mutations were expressed in a background of wild-type ribosomes, from a plasmid-borne *rrnB* operon under inducible control of the λ P_L promoter. Expression of each of the three 2451 mutations conferred a dominant lethal phenotype. In contrast, expression of the 2447 mutant rRNAs resulted in only moderately retarded growth rates.

A stringent test of the effect of an rRNA mutation on ribosome function is to determine whether a mutant plasmid can displace the plasmid encoding wild-type rRNA from Δ7 prrn strains of *E. coli*, in which all chromosomal *rrn* operons have been deleted (18, 30, 31). If a plasmid encoding mutant rRNA displaces the wild-type plasmid, the resulting strain will contain ribosomes assembled solely with mutant rRNA, which must thus be capable of supporting cell viability. The first requirement is transformation of the Δ7 prrn strain with the mutant plasmid. Because the Δ7 prrn strains do not contain the λ repressor, transformation with *rrn* plasmids under control of the P_L promoter results in constitutive expression of mutant rRNA, yielding cells that express both mutant and wild-type rRNA (from the resident *rrn* plasmid). Whereas pGQ7 (wild-type control) readily yielded colonies in the Δ7 prrn strain AVS69009 (31), no transformants were obtained with the 2451 mutant plasmids,

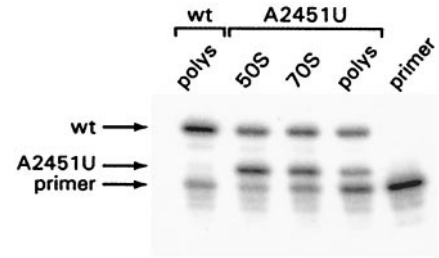

Fig. 1. Presence of A2451U mutant ribosomes in polysomes. Primer extension of rRNA extracted from sucrose gradient fractions containing wild-type (wt) polysomes (polys), and from fractions containing 50S subunits, 70S ribosomes, or polysomes from cells induced to express A2451U 23S rRNA. The last lane shows the position of the oligonucleotide primer alone.

consistent with the dominant lethal phenotypes of the 2451 mutations.

In contrast to the results with the 2451 mutants, the Δ7 prrn *rpsL121* strain MC250 was readily transformed with all three 2447 mutant plasmids. The G2447A and G2447C mutants grew on streptomycin-containing medium, indicating that they had lost the resident *rrn* plasmid, prrnS12 (18). Primer extension of total RNA from these strains confirmed the exclusive expression of mutant 2447 rRNAs. Thus, the G2447A and G2447C mutant ribosomes supported the total protein synthetic needs of the cell. However, growth of the G2447A mutant was detectably slower than wild type, and growth of the G2447C mutant was even further retarded. MC250 transformed with the G2447U mutant plasmid was unable to grow on streptomycin-containing medium, indicating that these mutant ribosomes could not maintain cell viability in the absence of wild-type ribosomes.

Incorporation of 2451 Mutant Ribosomes into Polysomes. To determine whether 50S subunits with mutations at position 2451 participate in protein synthesis *in vivo*, we measured the relative amounts of mutant and wild-type rRNA present in 50S subunits, 70S ribosomes, and polysomes in cells induced to express mutant rRNA. DH1 pCI857 pGQ7 (encoding wild-type rRNA), and derivatives encoding each of the three mutations at position 2451, were grown at 42°C for 2.5 h to induce synthesis of plasmid-encoded rRNA. Cell lysates were separated through sucrose gradients, and fractions were collected containing 50S subunits, 70S ribosomes, or polysomes. Polysomes were collected beginning with the tri-ribosome peak to exclude potential contamination from the 70S ribosome peak. The relative amounts of mutant and wild-type rRNA in each fraction were quantified by primer extension (19).

When expression of A2451U mutant rRNA was induced, mutant rRNA represented 57% of the 23S rRNA in 50S subunits, 50% in 70S ribosomes, and 34% in the polysome fraction (Fig. 1). Similar results were obtained for the A2451C and A2451G mutant ribosomes, where the mutant rRNA constituted 32% and 34%, respectively, of the 23S rRNA in polysome fractions (data not shown). No aberrant peaks, indicative of stalled or incorrectly assembled intermediates, were observed in the profiles from the mutant lysates. Thus, 23S rRNA with mutations at 2451 is assembled into 50S subunits that associate with 30S subunits, and the mutant subunits accumulate in polysomes, albeit at decreased levels.

Translational Fidelity Phenotypes of 2451 and 2447 Mutant Ribosomes. Several mutations in regions of 23S rRNA implicated in peptidyltransferase function have been shown to perturb translational fidelity. These include mutations in the A loop (22) and P loop (23) at, or adjacent to, nucleotides that base pair with the CCA end of A-site or P-site tRNAs, respectively, and mutations

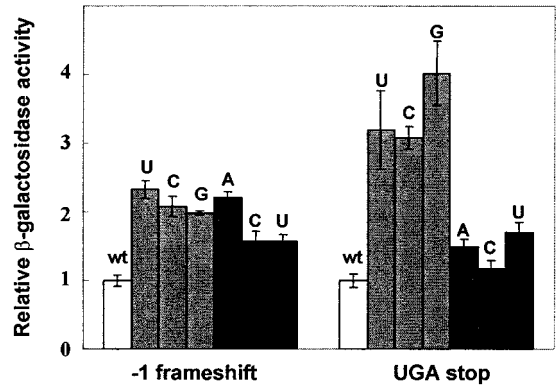

Fig. 2. Effects of 2447 and 2451 mutations on −1 frameshifting (*Left*) and UGA readthrough (*Right*). Cells carrying pLG857 and the *lacZ* −1 frameshift reporter plasmid pSG12DP or the *lacZ* UGA stop codon plasmid pSG34-11 were cotransformed with each of the mutant or wild-type rRNA plasmids, and β-galactosidase activity was measured after induction of plasmid-encoded rRNA expression for 2.5 h. β-Galactosidase levels obtained with wild-type rRNA plasmids (pLK35 or pGQ7) were set at 1.0, and values obtained with each of the 2447 (filled bars) and 2451 (gray bars) mutants were expressed relative to that obtained with the respective wild-type rRNA plasmid (open bars). Qualitatively similar results were obtained with the +1 frameshift construct pSGlac7 and the UGA stop codon construct pSG12-6 (data not shown).

at domain V central loop residues D2449 (18), U2504 (ref. 32; and M.O'C. and A.E.D., unpublished results), and in helix 89 (33), which extends from the central loop of domain V. We tested whether ribosomes with mutations at 2451 or 2447 perturbed the accuracy of mRNA decoding, by determining whether the mutations affected the ability of ribosomes to read through engineered frameshift mutations or stop codons in β-galactosidase mRNA (22, 23). All of the base changes at 2451 and 2447 increased both −1 and +1 frameshifting, and all three 2451 mutants stimulated readthrough of UGA and UAG stop codons (Fig. 2). Consistent with the presence of the 2451 mutant ribosomes in polysomes, and with the ability of two of the 2447 mutants to support cell growth with 100% mutant ribosomes, these results indicate that 50S subunits with mutations at 2451 or 2447 are engaged in protein synthesis and produce active β-galactosidase *in vivo*. Whereas the mechanisms by which mutations in regions of 23S rRNA associated with peptidyltransferase activity promote miscoding are not yet understood, mutations expected to perturb the geometry of the tRNAs in elongating ribosomes, in both the CCA end of tRNA and in residues of 23S rRNA that interact with the CCA end of tRNA, have been shown both to decrease the rate of peptidyl transfer (28, 29) and to promote errors in mRNA decoding (22, 23, 34).

Analysis of Protein Synthesis by A2451U and G2447A Mutant Ribosomes *in Vitro*. We examined the activity of ribosomes with mutations at 2451 or 2447 in *in vitro* protein synthesis, by using a coupled transcription-translation system. A2451U mutant ribosomes were prepared after induction of mutant rRNA synthesis for 2.5 h. Mutant rRNA represented 40–50% of the 23S rRNA in the total ribosome population, and was measured for each ribosome preparation. Because an A to U transversion at residue 2451 had been shown to give chloramphenicol resistance in mouse mitochondria (14), we tested whether the mixed population of *E. coli* ribosomes (A2451U and wild type) might also be resistant to the drug. In the absence of chloramphenicol, the rate of [^{35}S]methionine incorporation into trichloroacetic acid-precipitable material by the mixed ribosome population was lower than the rate in reactions with pure wild-type ribosomes (Fig. 3 *A* and *B*). In the presence of 250 μM chloramphenicol,

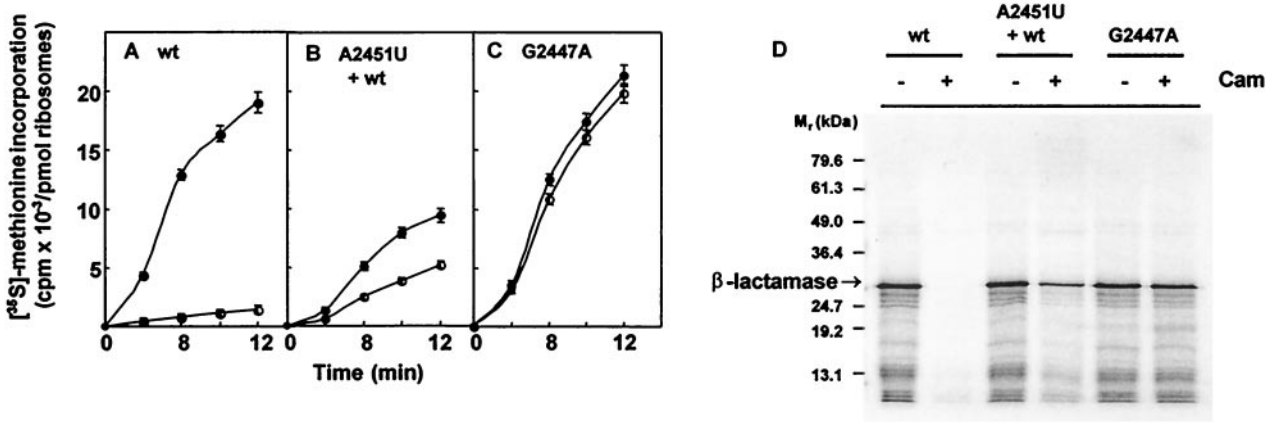

Fig. 3. *In vitro* protein synthesis by wild-type and mutant ribosomes. Ribosomes (3–7 pmol) from (*A*) DH1 pCI857 pGQ7 or MC150 pLK35 (wild-type controls that gave similar protein synthesis levels in multiple assays), (*B*) DH1 pCI857 pGQ2451U, or (*C*) MC250 pLK2447A were assayed as described in *Materials and Methods*. [^{35}S]methionine incorporation into hot trichloroacetic acid-precipitable material was measured at time points indicated (●). Chloramphenicol (250 μM) was added to ribosomes for a 10-min preincubation at room temperature in a parallel series of assays (○). Data are from four assays, with two preparations of each set of ribosomes. The percentage of A2451U ribosomes in the two preparations was 46% and 48%, as determined by primer extension (19). (*D*) Autoradiograph of a 10% polyacrylamide-SDS gel of the *in vitro* protein products of the coupled transcription-translation system. Synthesis was carried out for 20 min in the presence of [^{35}S]methionine (15 mCi/mmol), followed by a 10-min chase with cold methionine. Lanes show reactions with (+), or without (−), 250 μM chloramphenicol, by 100% wild-type ribosomes (wt), 46% A2451U ribosomes in a wild-type background (A2451U + wt), and 100% G2447A ribosomes (G2447A).

a concentration well in excess of that required to inhibit fully the wild-type ribosomes (Fig. 3*A*), significant levels of peptide synthesis were catalyzed by the mixed A2451U and wild-type ribosomes (Fig. 3*B*). Thus A2451U mutant ribosomes were resistant to chloramphenicol, and engaged in *in vitro* protein synthesis, although at an apparently decreased rate relative to wild-type ribosomes.

Analysis of the products of the coupled transcription-translation reactions on denaturing gels (Fig. 3*D*) showed that the A2451U ribosomes synthesized full-length protein. β-Lactamase (encoded by pUC19; molecular mass 34 kDa) was synthesized by pure wild-type ribosomes in the absence, but not in the presence, of chloramphenicol. Significantly, this protein product was synthesized by the A2451U mixed ribosome population both in the absence, and in the presence, of the drug.

A similar analysis was carried out with pure mutant G2447A ribosomes isolated from strain MC250. Consistent with their ability to support cell growth at near wild-type rates, the G2447A ribosomes were as active as wild-type ribosomes in their rate of protein synthesis (Fig. 3*C*); translation of full-length β-lactamase was observed, which was resistant to high levels of chloramphenicol (Fig. 3*D*). This finding is consistent with the report of a chloramphenicol-resistance mutation at this residue in yeast mitochondria (15).

Peptidyltransferase Activity of Mutant 50S Subunits. Peptidyl transfer may not be rate limiting in the processive reactions of protein synthesis, and thus the magnitude of a catalytic rate decrease for the mutant ribosomes could be masked in *in vitro* translation reactions. To determine the effects of altering A2451 or G2447 on catalysis more directly, we measured the activity of mutant ribosomes in assays that measure single-turnover peptidyl transfer events. Pure populations of 2451 mutant 50S ribosomal subunits were reconstituted from *in vitro* transcribed *B. stearothermophilus* 23S rRNA (27), and their peptidyltransferase activity was measured in two different single-turnover assays.

In assay A, *E. coli* 30S subunits and poly(U)$^{+}$ mRNA were added to the *B. stearothermophilus* 50S reconstitution reaction to form mRNA-programmed 70S ribosomes, followed by addition of the P-site substrate, *N*-Ac-Phe-tRNA^{Phe}. The reaction was initiated by addition of the minimal A-site substrate, [^{32}P]CpPm (Pm = puromycin), at a limiting concentration. In this assay, reaction rate is proportional to ribosome concentration, the P site of the mutant and wild-type ribosomes is saturated, and the rate of conversion to product ([^{32}P]CpPm-Phe-*N*-Ac) is sensitive to defects in A-site binding, or in steps that occur following initial A-site binding, including the chemical step(s) of peptidyl transfer (data not shown). Control experiments, performed in the presence of a "chase" of competitor tRNA, or with peptidyl-tRNA hydrolase (to deacylate unbound *N*-Ac-Phe-tRNA^{Phe} that might contribute to multiple-turnover catalysis), confirmed that the reaction rates measured reflect single-turnover events (data not shown). This result is consistent with the expectation that, at the high magnesium concentration (20 mM) used in the assays, the deacylated tRNA^{Phe} reaction product remains stably bound to 70S ribosomes (35). In this single-turnover assay, all three mutations at 2451 decreased the rate of the reaction, ranging from ≈3-fold for A2451C and A2451U, to 11-fold for A2451G (Fig. 4; Table 1). The same ratios of rate constants between the wild-type and mutant reconstituted ribosomes were consistently observed when A-site substrate concentration was varied over a 25-fold range (from 40 nM to 1.6 nM; data not shown).

An alternative single-turnover assay (assay B) was performed with the mutant ribosomes, where *E. coli* 30S subunits and gene 32 mRNA were added to the reconstitution reactions. *N*-Ac-[^{35}S]Met-tRNA^{Met} was then added, at a concentration that saturated the P site of the mutant and wild-type ribosomes; next, puromycin was supplied as the A-site substrate, at a concentration that was saturating for both mutant and wild-type ribosomes (data not shown). In this assay, A-site binding deficiencies of the mutant ribosomes may be overcome by the high concentration of puromycin, allowing the effects of the mutations on subsequent steps to be monitored. Again, control experiments were performed in the presence of a chase of competitor tRNA or peptidyl-tRNA hydrolase to confirm that the observed reaction rates represent single-turnover events (data not shown). Similar to the effects observed in assay A, all three mutations at 2451 reduced the peptidyltransferase reaction rate, by ≈4-fold for A2451C, 5-fold for A2451U, and up to 14-fold for A2451G (Table 1).

G2447A ribosomes, isolated from strain MC250, were tested in the two peptidyltransferase assays. The temperature was

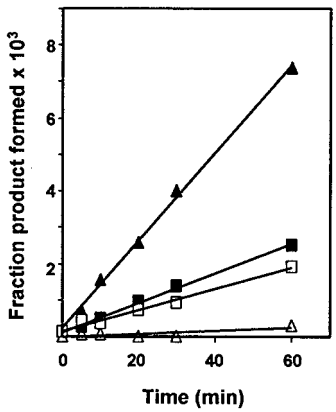

Fig. 4. Effect of mutations at 2451 of 23S rRNA on peptidyltransferase activity with limiting A-site substrate (assay A). Quantification of data from denaturing PAGE of peptidyl transfer reactions catalyzed by *B. stearothermophilus* 50S subunits reconstituted from either wild-type or A2451C *in vitro* transcribed 23S rRNA. The fraction of ^{32}P-labeled CpPm converted to product ([^{32}P]CpPm-Phe-*N*-Ac) is plotted as a function of time (min). Closed symbols represent data from samples not treated with chloramphenicol whereas open symbols represent data from samples treated with 62.5 μM chloramphenicol; triangles indicate wild-type ribosomes (A2451), and squares indicate A2451C ribosomes.

lowered to 37°C, and earlier time points were taken to accommodate the higher rates of reaction with the pure native *E. coli* ribosomes (as opposed to ribosomes containing 50S subunits reconstituted from *in vitro* transcripts). In the A-site limiting assay, the G2447A mutant ribosomes were 14-fold less active than wild-type ribosomes (Table 1). However, in the A-site saturating assay (assay B), peptide bond formation was catalyzed by the G2447A mutant ribosomes at a rate indistinguishable from that of wild-type (Table 1).

Finally, we tested whether the peptidyltransferase activity of the 2451 or 2447 mutant ribosomes was resistant to chloramphenicol. Peptide bond formation by wild-type ribosomes isolated from strain MC250, or by ribosomes containing reconstituted wild-type *B. stearothermophilus* 50S subunits, was inhibited 100-fold by 62.5 μM chloramphenicol in assay A (Fig. 4; Table 1). However, at this antibiotic concentration, the three 2451 mutants retained 33–68% of their catalytic activity (Fig. 4; Table 1), whereas the G2447A ribosomes were essentially unimpaired by the drug (Table 1).

Table 1. Relative peptidyl transferase rates of mutant ribosomes

	Assay A		Assay B
	− Cam	+ Cam	− Cam
A2451 (wt)	**1.0**	0.01	**1.0**
A2451C	0.32	0.19	0.25
A2451G	0.09	0.03	0.07
A2451U	0.31	0.21	0.20
G2447 (wt)	**1.0**	0.01	**1.0**
G2447A	0.07	0.06	1.1

Initial rates were determined from time points in the linear range of the reactions and normalized to the rate of the wild-type (wt) for each set and assay. The normalized rates (in bold type) are shown for assays performed with (+), or without (−), 62.5 μM chloramphenicol (Cam). The 2451 mutants were assayed using 70S ribosomes containing *B. stearothermophilus* 50S subunits reconstituted *in vitro*; G2447A ribosomes were prepared from *E. coli* strain MC250 carrying solely mutant ribosomes. Data represent the average of multiple experiments. Standard deviations were less than 50% of the average values derived from multiple experiments. Representative data for assay A are shown in Fig. 4.

Discussion

We have constructed mutations at A2451 and G2447 of 23S rRNA, proposed to be directly involved in the catalytic mechanism of ribosomal peptidyltransferase. Based on its 3- to 4-Å proximity to the tetrahedral center of C-C-dA-phosphoramidepuromycin in the crystal structure of the *H. marismortui* 50S subunit, A2451 of 23S rRNA was proposed to serve as a general base, abstracting a proton from the amine of the A-site substrate, and subsequently as a general acid, donating the proton to the 3′ hydroxyl of the leaving group (4). It was later suggested that a more likely substrate for general base catalysis by A2451 is the ammonium form of the A-site substrate (36). An additional or alternative proposal was that A2451 (in its protonated form) promotes catalysis by stabilizing the negative charge on the oxyanion (4). Based on the 23S rRNA structure in the vicinity of A2451, it was further proposed that an altered pK_a for A2451 (6) that allows it to perform these catalytic roles was generated through a charge-relay network, mediated through the nucleotide base of G2447 to the phosphate of A2450 (4).

All three base changes at the universally conserved residue A2451 conferred dominant lethality in *E. coli*, consistent with its phylogenetic conservation. However, ribosomes with mutations at 2451 were represented at significant levels in polysomes in these cells, and all three mutations at 2451 stimulated frameshifting and stop codon readthrough *in vivo*, indicating that the 2451 mutant ribosomes synthesized full-length β-galactosidase *in vivo*. The rate of protein synthesis by A2451U mutant ribosomes *in vitro* was apparently slower than that of wild-type ribosomes, suggesting that the deleterious effect of the mutations is manifested during protein synthesis. In contrast, none of the mutations at the less well conserved G2447 exhibited such extreme lethality. Indeed, ribosomes containing two of the three base changes (to A or C) supported cell growth in the absence of wild-type ribosomes, with the G2447A mutant demonstrating *in vitro* protein synthesis at close to wild-type levels.

The chloramphenicol resistance of the 2451 and 2447 mutant ribosomes in protein synthesis and peptidyltransferase assays *in vitro* is in agreement with earlier findings that mutations at these positions in mitochondrial rRNAs conferred resistance to this antibiotic in yeast (15) and mouse (14) cells. The high levels of chloramphenicol resistance afforded by the 2451 and 2447 mutations, together with protection of A2451 from DMS modification by this antibiotic (11), suggest that these residues form part of the chloramphenicol binding site.

If the peptidyltransferase reaction proceeds through the most plausible reaction pathway of direct nucleophilic attack of aminoacyl-tRNA on the acyl ester of peptidyl-tRNA, then 23S rRNA might make a variety of contributions to catalysis, including the use of intrinsic substrate binding energy and precise substrate positioning, which have been shown to provide substantial rate increases (37). These binding interactions might extend to specific transition-state contacts, including oxyanion stabilization. Finally, the reaction could be accelerated by specific chemical contributions from the ribosome, such as metal ion positioning or general acid and/or general base catalysis.

The decreases in peptidyltransferase activity of the 2451 and 2447 mutant ribosomes were significant, yet surprisingly modest given the roles proposed for these residues based on the 50S subunit x-ray structure (4). In particular, it might be expected that mutations at 2451, poised within 3–4 Å of the tetrahedral intermediate analog (4), would be severely detrimental to catalysis. Because we have not determined rate constants for individual steps in the peptidyltransferase reaction, we cannot be certain that the decreased rate of peptidyl transfer measured for the 2451 or 2447 mutants fully represents the effect of these mutations on the chemical step(s) of catalysis. In assay A (A-site substrate limiting conditions), diminished rates measured for the

mutant ribosomes could reflect an altered dependence of reaction rate on A-site substrate concentration, or an alteration in the rate of any steps that occur after initial A-site binding, including the chemical step(s) of the reaction (or both). Assay B, with puromycin at a saturating concentration, is less likely to be sensitive to differences in A-site substrate affinity. The similar results for the 2451 mutants in the two assays suggest that A-site substrate binding is not severely compromised in these mutated ribosomes, and that the rate decreases are most easily explained by effects on the chemical step(s). In contrast, the 14-fold rate reduction observed for the G2447A ribosomes in assay A was overcome by saturating concentrations of A-site substrate, suggesting that A-site substrate interaction, and not subsequent steps in the reaction pathway, is perturbed by mutations at this position. The absence of effect on peptidyltransferase reaction rate observed for the G2447A mutant ribosomes at high substrate concentrations is consistent with the modest effects of this mutation on cell growth, and its lack of effect on the rate of *in vitro* protein synthesis. These results do not support a central role for G2447 in the proposed charge-relay system (4).

The failure of mutations in nucleotides that are crucial to the proposed mechanism (4) to abolish peptide bond formation can be rationalized in various ways. If the peptidyltransferase assays in this study reflect the chemical step(s) of catalysis, it is possible that A2451 and G2447 are not critical to the catalytic mechanism. This would indeed appear to be the case for G2447. However, whereas the 3- to 14-fold rate decreases observed for the 2451 mutant ribosomes are inconsistent with the large (10^6-fold) decreases in catalytic power caused by mutation of the general base histidine of serine proteases (38), they are consistent with a role for A2451 in a binding contact important for catalysis, possibly even a contribution to transition-state oxyanion stabilization. For example, some mutations in proteases and lipases that result in the loss of a hydrogen bond to the transition-state oxyanion caused rate decreases ranging from 20- to 500-fold, with the mutant enzymes retaining considerable catalytic activity (ref. 39, and references therein). Alternatively, moderate rate impairments of 5- to 100-fold were also predicted for 2451 mutant ribosomes, on chemical grounds, if this nucleotide serves as a general-base catalyst with the ammonium form, rather than the amine form, of the A-site nucleophile (36).

It is also possible that the chemical step(s) of peptidyl transfer are not rate limiting, either *in vivo* or *in vitro*. A conformational rearrangement in the catalytic center (subsequent to initial A-site substrate recognition but preceding the chemical step), proceeding at a slower rate than the chemical step, might be required. In this case, a peptidyltransferase reaction greatly compromised by mutation would need only to be able to keep up with the rate of the slow step, or nearly so. Indeed, the decreased peptidyltransferase rates of the 2451 mutant ribosomes could derive from impairment of such a requisite structural rearrangement. However, if a conformational change does occur, it raises the further question as to whether the crystallographically observed structure describes the active, catalytic state, or an inactive ground state, which must undergo the postulated rate-limiting conformational change, before the chemical step.

The participation of ribosomes with mutations at 2451 or 2447 in peptidyl transfer and protein synthesis, both *in vivo* and *in vitro*, indicates that the identities of these conserved residues are not strictly indispensable for ribosome-catalyzed peptidyl transfer. Given the modest impairments of the 2451 and 2447 mutant ribosomes in peptidyl transfer, and the absence of 50S ribosomal protein residues in the vicinity of the peptidyltransferase active site, it seems likely that additional 23S rRNA elements contribute to substrate positioning, specific chemical steps, or conformational rearrangements in the peptidyltransferase catalytic mechanism.

BIOCHEMISTRY

Note Added in Proof. After submission of this paper, Polacek *et al*. (40) reported that ribosomes containing *Thermus aquaticus* 50S ribosomal subunits, reconstituted *in vitro* using *in vitro*-transcribed 23S rRNA bearing mutations at position 2451 or 2447, retain levels of peptidyltransferase activity similar to those found in this study.

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