

## Conserved Nucleotides of 23 S rRNA Located at the Ribosomal Peptidyltransferase Center\*

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Two nucleotides of the 23 S rRNA gene were mutated; the nucleotides correspond to the first two positions of the universally conserved sequence  $\Psi$ GG2582 at the peptidyltransferase ring of 23 S rRNA. The ribosomes containing the altered 23 S rRNA were analyzed. Previously, it was shown that ribosomal assembly was indistinguishable from that in wild-type cells, that the flow of the corresponding 50 S subunit into the polysome fraction was not restricted, but that the ribosomes were strongly impaired in poly(Phe) synthesis (C. M. T. Spahn, J. Remme, M. A. Schäfer, and K. H. Nierhaus (1996) *J. Biol. Chem.* 271, 32849–32856). Here we apply assay systems exclusively testing the puromycin reaction of ribosomes carrying plasmid-born rRNA, a dipeptide assay using the minimal P site donor pA(fMet) and a translocation system not depending on the puromycin reaction. The mutations in helix 90 exclusively abolish or severely impair the ribosome capability to catalyze AcPhe-puromycin formation. A possible explanation of these observations is that G2581 and  $\Psi$ 2580 (and possibly also G2582) are part of the binding site of C75 of peptidyl-tRNA in the P site. The results suggest that in this case, however, such an interaction would disobey canonical base pairing.

Two out of the 55 UGG sequences in 23 S rRNA from *Escherichia coli* are universally conserved in the non-mitochondrial rRNAs of the large ribosomal subunit, UGG2251 and  $\Psi$ GG2582. The first two positions of both sequences were mutated, and the effects on ribosomal assembly and functions were analyzed (1). Mutants of the first sequence did not influence ribosomal assembly and bacterial growth but caused some defects in *in vitro* poly(U) translation. Those of the second sequence are characterized by a seemingly normal assembly but with severe defects in peptide synthesis (1). The  $\Psi$ GG2582 sequence is located at the “peptidyltransferase ring” (see Fig. 1). Here we analyze the peptidyltransferase (PTF)<sup>†</sup> activity in various systems and the translocation reaction. All mutant ribosomes were able to translocate. The helix 80 mutants show a normal puromycin reaction. In contrast, the helix 90 mutations severely interfere with the PTF activity.

### MATERIALS AND METHODS

Plasmids and strains, the preparation of ribosomes and polysomes, and the quantification of plasmid-born 23 S rRNA have been described

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<sup>†</sup> The abbreviation used is: PTF, peptidyltransferase.

in the preceding paper (1).

**Puromycin Kinetics with AcPhe-tRNA Bound to the P-sites of Poly(U)-programmed 70 S—tRNA binding and puromycin reaction was essentially as described (2) except that the buffer conditions (20 mM Hepes-KOH (pH 7.6), 6 mM MgCl<sub>2</sub>, 150 mM NH<sub>4</sub>Cl, 4 mM  $\beta$ -mercaptoethanol, 2 mM spermidine, and 0.05 mM spermine) were kept constant in all steps (3). 70 S ribosomes were programmed with Ac[<sup>14</sup>C]Phe-tRNA<sup>Phe</sup> (1,030 dpm/pmol, 1.5-fold excess over 70 S) and poly(U). After an incubation at 37 °C for 30 min, two aliquots were filtered over nitrocellulose to determine the binding. To the remaining aliquots, puromycin was added, and the kinetics of AcPhe-puromycin formation were followed at 0 °C.**

**Measuring AcPhe-Puromycin Formation of Exclusively 70 S Ribosomes Containing Plasmid-born 23 S rRNA—**The buffer conditions were as described in the preceding section. 70 S ribosomes were programmed with the heteropolymeric MF-mRNA coding for Met and Phe (4). P-sites were blocked with a 1.5-fold excess of tRNA<sup>Met</sup> over 70 S ribosomes (20 min, 37 °C). Ac[<sup>14</sup>C]Phe-tRNA<sup>Phe</sup> (1030 dpm/pmol, 1:1 molar ratio to 70 S) was bound to the A sites at 37 °C for 30 min. Thiostrepton was added (1  $\mu$ M final concentration), and the incubation proceeded for 10 min. An EF-G-dependent translocation followed (10 min, 37 °C), and the kinetics of AcPhe-puromycin formation were measured at 0 °C. Controls were done with and without thiostrepton and EF-G.

**Translocation Measurement via Assessment of the Movement of the mRNA—**The technique followed the procedure described in Beyer *et al.* (5). A chemically synthesized mRNA with the sequence A AUG UUC AAA C<sub>30</sub> was used. This MFpC-mRNA was labeled with <sup>32</sup>P at its 5'-end. The length of the mRNA protected by the ribosome can be determined precisely by trimming the overhanging oligo(C) sequence by the cytidyl-specific RNase CL3 (5). The pretranslocational state was established by binding first deacyl-tRNA<sup>Met</sup> to the P site of ribosomes programmed with the MFpC-mRNA, then AcPhe-tRNA was bound to the A site. The complex was purified via a spun-column centrifugation. The probe was divided in three aliquots according to the strategy shown in Fig. 3. With two aliquots, EF-G-dependent translocations were performed in the presence and the absence of 1  $\mu$ M thiostrepton, respectively. The third aliquot was used as a control of the pretranslocational state. The puromycin reaction and the movement of the mRNA in the course of translocation were determined in parallel (for experimental details, see Ref. 5).

**Fragment Reaction with pA-fMet—**The conditions were essentially as described (6) except that the amount of the 70 S ribosomes was reduced to achieve a linear dependence of the reaction on the ribosome concentration. The 25  $\mu$ l of the reaction mixture contained 50 mM Tris-HCl, pH 7.4, 400 mM KCl, 20 mM MgAc<sub>2</sub>, 1 pmol of 70 S, 1.5–2 pmol of [<sup>3</sup>H]Phe-tRNA<sup>Phe</sup> (~80,000 dpm/pmol), 1 mM pA-fMet, and, when indicated, 1 mM AMP, CMP, GMP, or UMP. The reaction was initiated by addition of 25  $\mu$ l of methanol; incubation was for 60 min at 0 °C. The reaction was terminated by addition of 25  $\mu$ l of 3 M NaOH, and the mixture was incubated for 30 min at 37 °C to hydrolyze the ester bonds. 100  $\mu$ l of 10 M HCl were added, and fMet-Phe was extracted with ethyl acetate. To decrease the background, the ethyl acetate phase was washed successively with 100  $\mu$ l of 1 M HCl and 100  $\mu$ l of water and then counted. Blank values were obtained by omitting pA-fMet, ribosomes, or methanol.

### RESULTS

The mutants carried the altered 23 S rRNA gene on a plasmid in addition to the seven wild-type rRNA genes on the chromosome. In addition to the mutation in the peptidyl trans-

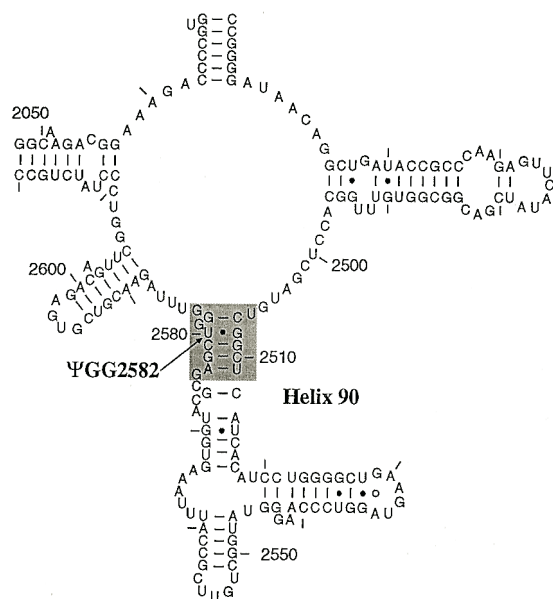


FIG. 1. The secondary structure of the "peptidyltransferase ring" in domain V of *E. coli* 23 S rRNA according to Gutell, *et al.* (20). Helix 90 and the highly conserved ΨGG2582 are indicated.

ferase region, all plasmid genes bore the A1067U mutation that confers thiostrepton resistance (7). About 50% of the various ribosomal fractions (50 S subunits, 70 S ribosomes, and polyosomes) of the mutants contained an altered 23 S rRNA. The binding of AcPhe-tRNA to the P site of poly(U)-programmed 70 S ribosomes was not affected by the plasmid-born 23 S rRNA, but poly(Phe) synthesis was severely impaired, especially by the helix 90 mutations (1).

If the severely reduced activity of some of the mutants in poly(Phe) synthesis is not caused by an impaired tRNA binding, then the PTF center could be affected. To test this possibility, AcPhe-tRNA<sup>Phe</sup> was bound to the P site of poly(U)-programmed ribosomes, and kinetics of the puromycin reaction were performed. The results with the mutations in the helix 90 are compiled in Fig. 2A. These mutations show about half the activity of wild-type ribosomes. Since half of the ribosomes carry the mutation (Table I in Ref. 1), this result indicates that the mutations are not active in peptide-bond formation in this assay. The only exception is the C2507U mutation that takes up an intermediate position and is thus significantly active at a reduced level similar to the results in the poly(Phe) system (1). In contrast, all mutations at the helix-loop 80 are only slightly reduced in the kinetics of peptide-bond formation (Fig. 2B).

An unsatisfying feature of this kind of puromycin reaction is the fact that the activities of the ribosomes carrying mutated rRNA are detected only on top of the activities of the 50% ribosomes with chromosomal derived 23 S rRNA. We therefore developed a technique which is able to test exclusively the PTF activity of the ribosomes with plasmid-born 23 S rRNA without interference of ribosomes with chromosomal born rRNA. The strategy is outlined in Fig. 3. As already mentioned a preparation of 70 S ribosomes contains roughly equal amounts of 23 S rRNA derived from the chromosome or from the plasmid. The chromosomal derived ribosomes are thiostrepton sensitive (A1067 in 23 S rRNA), those derived from the plasmid are thiostrepton resistant (A1067U). AcPhe-tRNA is bound to the A site in the presence of the heteropolymeric MF-mRNA, which is 46 nucleotides long and contains the codons AUG-UUC only in the middle (4); the P site has to be pre-filled with deacylated-tRNA<sup>Met</sup>. Then thiostrepton is added which is the strongest

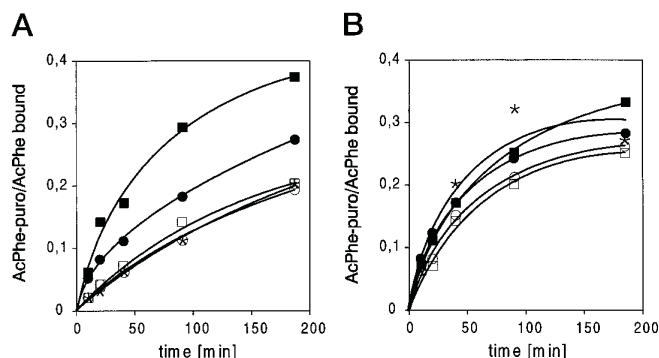


FIG. 2. Kinetics of the puromycin reaction. AcPhe-tRNA<sup>Phe</sup> was bound to the P site of poly(U)-programmed 70 S ribosomes. Puromycin was added and the time course of AcPhe-puromycin formation was followed. The results are normalized to the bound AcPhe-tRNA<sup>Phe</sup>. In A, the results of the mutations within helix 90 of 23 S rRNA are given (■, wild type; ●, C2507U; ★, Ψ2580C; □, G2581A; ○, C2507U/G2581A), in B, those of the mutations within helix 80 (■, A1067U; ●, G2250A; ★, C2254U; □, U2249C; ○, G2250A/C2254U).

inhibitor of translocation known (9). The ribosomes with chromosome derived 23 S rRNA will be locked in the pre-translocational state and hold their AcPhe-tRNA in the puromycin insensitive A site, whereas those with 23 S rRNA from the plasmid are thiostrepton resistant and can be translocated to the puromycin sensitive P site in an EF-G dependent reaction. A puromycin reaction can therefore be observed exclusively with ribosomes carrying plasmid born 23 S rRNA.

Figs. 4A and B demonstrate the described effects. Thiostrepton sensitive ribosomes (pNO(wt); wt, wild type) show a puromycin reaction only upon EF-G dependent translocation in the absence of thiostrepton. In the presence of thiostrepton EF-G cannot promote a translocation and the puromycin reaction does not occur since AcPhe-tRNA remains at the A site. However, when the plasmid carries the A1067U mutation in the 23 S rRNA gene a significant puromycin reaction is observed upon translocation in the presence of thiostrepton. The activity amounts to about half the activity seen in the absence of thiostrepton in accordance with the fraction of plasmid born 23 S rRNA. The puromycin reaction was hardly reduced in the presence of the drug when the mutations of the helix-domain 80 were tested (see for example G2250A; Fig. 4C). These nucleotides play therefore no essential role for the translocation reaction as well as for the peptidyltransferase (PTF). The PTF activity, however, was abolished in the presence of the mutations G2581A (Fig. 4D), C2507U/G2581A and C2507A/G2581A (not shown). Little activity was observed with the mutation Ψ2580C (Fig. 4E). The mutation C2507U (Fig. 4F) had the relatively highest activity of the helix 90 mutations similar to the puromycin reaction of the P-site bound AcPhe-tRNA (Fig. 2A). Inhibition with this mutation was around 50%. The results unequivocally show that all the constructed mutations in helix 90 have a disastrous effect on the puromycin reaction.

The severe inhibition of the puromycin reaction in Fig. 4D observed with, for example, G2581A, however, could be an indirect effect, in that the mutation blocks the translocation reaction in addition to the PTF activity. Since the success of the translocation is tested with the puromycin reaction, a block of the translocation would freeze the AcPhe-tRNA in the puromycin insensitive A site and therefore would also result in a block of the puromycin reaction. In order to test this possibility we tested the translocation reaction directly *via* the movement of the mRNA (5) without the participation of a puromycin reaction. An mRNA with a codon for Met and Phe at the 5'-end and a 30 nucleotides long oligo(C) sequence at the 3'-end (MFpC-mRNA) was 5'-labeled with [<sup>32</sup>P]. When the mRNA is

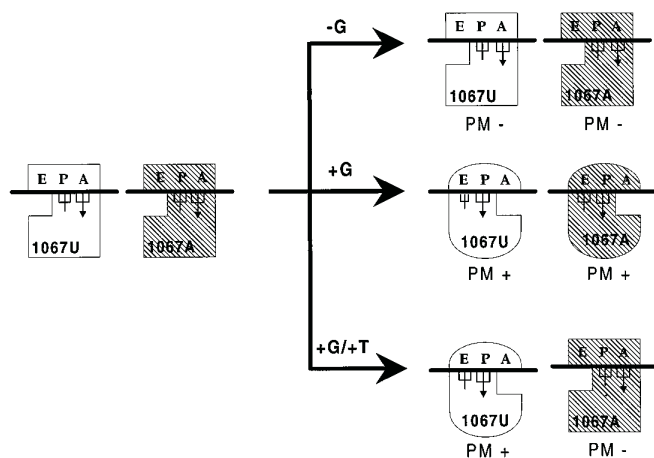


FIG. 3. Strategy to measure the puromycin reaction exclusively of ribosomes with plasmid born 23 S rRNA. The strategy makes use of the thioestrepton resistance mutation A1067U. Pretranslocational complexes were prepared in the presence of MF-mRNA and carried a tRNA<sup>Met</sup> in the P site and an AcPhe-tRNA in the A site. After an EF-G dependent translocation in the presence of thioestrepton (+G/+T) the puromycin reaction was measured. Since all ribosomes with 23 S rRNA derived from the pNot derivatives contain this mutation they should be able to translocate and subsequently to react with puromycin (PM+), whereas the wild-type ribosomes with chromosomally encoded 23 S rRNA (hatched ribosomes) should be blocked in the puromycin-insensitive pretranslocational state (PM-). One aliquot of the complexes treated without EF-G and thioestrepton (-G) controlled that the pretranslocational state was achieved, whereas the sample treated with EF-G but without drug (+G) measured the capability of the wild type fraction of the ribosomes to translocate and to perform a puromycin reaction. The fork with a triangle represents AcPhe-tRNA; the tRNA without the triangle represents deacylated tRNA<sup>Met</sup>. The rectangular ribosome indicates a pretranslocational state with occupied A and P sites, the round ribosome a posttranslocational state with occupied P and E sites (for review, see Ref. 8).

fixed with cognate tRNAs that part of the oligo(C) sequence protruding from the ribosome can be removed by a C-specific RNase (RNase CL3) and the length of the mRNA covered by the ribosome can be determined on a sequence gel. A translocation reaction will prolong the mRNA sequence covered by the ribosome for three nucleotides. The general strategy followed that of the experiment described in Fig. 3: A pre-translocational complex was formed with AcPhe-tRNA in the A site. Then thioestrepton was added and an EF-G dependent translocation performed, which could be executed in the presence of the drug only by the ribosomes carrying plasmid born 23 S rRNA. In Table I, the relative values of the resulting puromycin reactions are compared with the translocation efficiencies determined from the mRNA prolongation. Wild type ribosomes have no activity in either system since they are thioestrepton sensitive, whereas the control containing thioestrepton resistant ribosomes derived from the mutant A1067U are active in both systems. The mutants G2581A and C2507U/G2581A have no detectable activity in the puromycin reaction, whereas C2507U and  $\Psi$ 2580C showed a low but significant activity in agreement with the experiment of Fig. 4. However, all the mutant ribosomes could translocate as efficient as the control, only the translocation of the double mutant C2507U/G2581A was somewhat reduced. It is clear that the mutant ribosomes can translocate and therefore have a specific block in the PTF activity.

In order to characterize the lesion of the PTF center in more detail we made use of results concerning the minimal donor substrate of the ribosomal PTF. pA-fMet is the minimal donor (6), pCA-fMet is significantly better and a further improvement is seen with CCA-fMet (10, 11). The intriguing observation was that the low donor activity of pA-fMet could be strongly stimulated by adding CMP (12), the other nucleotides had no effect.

In contrast, CMP even impaired the substrate activity of pCA-fMet, which is already a good substrate *per se* (13). The interpretation was that C75 of the universally conserved CCA-3' end of the tRNAs plays a critical role for the binding of the peptidyl-residue at the PTF center (11, 14).

In Fig. 5A the fMet-Phe formation is shown, where pA-fMet is bound to the P-site region of the PTF center and Phe-tRNA to the A site in the presence of 50% methanol. The stimulation of the peptide-bond formation by CMP in contrast to all other nucleotides is clearly visible. Since all the ribosome preparations from the mutants contain about 50% wild type ribosomes, 50% of the activity found with control ribosomes (pNot) was subtracted from the data obtained with the mutant preparations (pNotG2581A). The resulting activities should mirror the activities seen with fraction of ribosomes containing mutant 23 S rRNA (Fig. 5B). Ribosomes of the G2581A mutant had a residual activity but completely lost the stimulatory effects of CMP; UMP also was not effective, as were AMP and GMP. The  $\Psi$ 2580C mutant also lost the stimulatory effect of CMP, whereas all other mutants tested (C2254U, C2507U) showed still some specific responds upon addition of CMP (Fig. 5C). The double mutants, however, were hardly active (not shown).

We further tested whether the mutation C75U at the CCA-end of AcPhe-tRNA could cure the severe impairment of the PTF activity seen with the G2581A mutant, in other words, how an AcPhe-tRNA with a CUA-3' end behaves in the puromycin reaction as compared with an AcPhe-tRNA containing the normal CCA-3' end. Wild type ribosomes and those derived from the G2581A mutant were analyzed. Both AcPhe-tRNA were made from *in vitro* transcribed tRNA<sup>Phe</sup>. The AcPhe-tRNA-CUA was totally inactive with ribosomes from both sources, whereas the AcPhe-tRNA-CCA gave the expected results as demonstrated in Fig. 6. Note that the transcribed AcPhe-tRNA shows a higher puromycin reaction than the native AcPhe-tRNA containing the modified bases in contrast to a recent report (15). Similarly, we reproducibly observed a higher translation efficiency with the transcribed tRNA<sup>Phe</sup> in a poly(Phe) synthesis system.<sup>2</sup> These observations have not yet been analyzed.

## DISCUSSION

A mutation of a nucleotide specifically involved in peptide-bond formation should fulfill all the following criteria: i) The mutation should cause a dominant lethal phenotype thus indicating a severe ribosomal defect. ii) The mutation should not affect the assembly of the large subunit indicated by the facts that no precursor particles accumulate and the mutant 23 S rRNA is found in equal amount in all three ribosomal fractions, *i. e.* 50S subunits, 70 S ribosomes and polysomes. iii) The PTF activity should be practically abolished, whereas other functions such as subunit assembly, tRNA binding to A and P sites and translocation should be not affected.

Mutations within the universally conserved UGG2251 in helix domain 80 only weakly hamper peptide-bond formation. However, the adjacent nucleotides G2252 and G2253 have been shown to be at or near the ribosomal PTF center. The G's in the loop of helix 80 are protected by tRNAs and CCA-containing oligonucleotide analogues of acetyl-aminoacyl-tRNAs at the P site (16, 17). Mutations of G2252 affected the binding of ligands to the PTF center, the effects could be cured by the complementary base replacing C74 of the mature ends of tRNAs arguing for a base pair between G2252 and C74 (18). However, the fact that ribosomes carrying the double mutation G2252C/G2253C showed at least a 50% activity in peptide-bond formation as

<sup>2</sup> M. A. Schäfer, M. Dabrowski, C. M. T. Spahn, and K. H. Nierhaus, unpublished result.



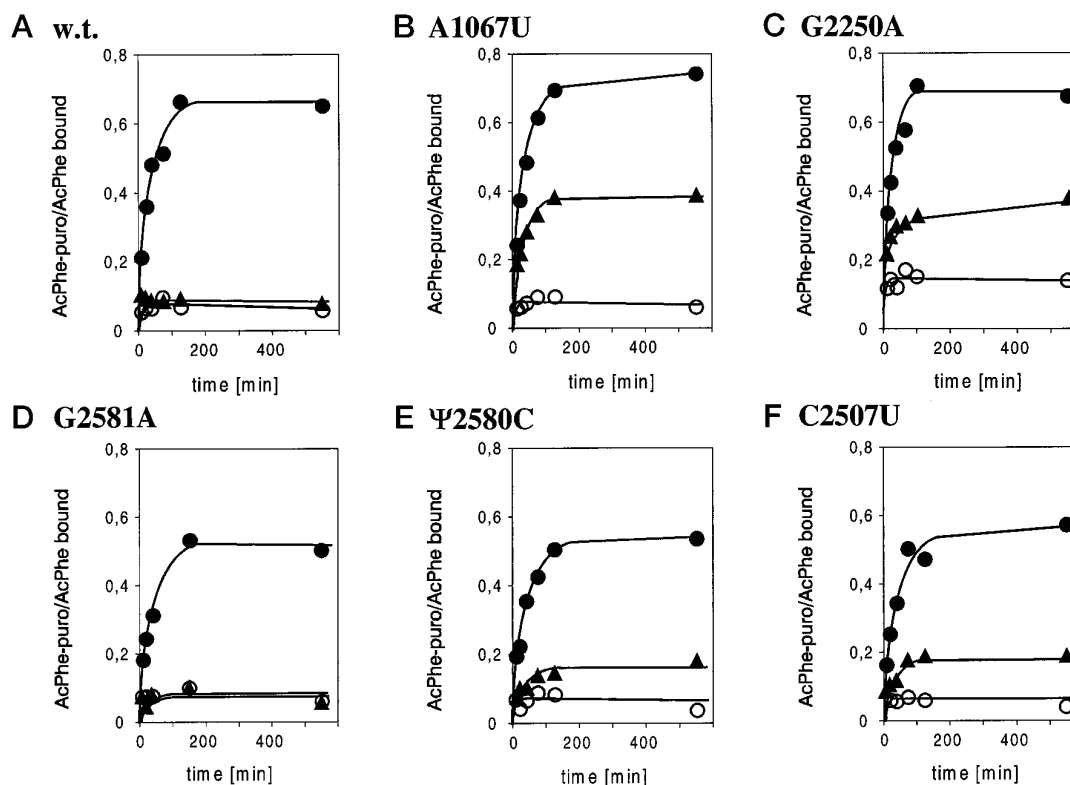


FIG. 4. **The strategy shown in Fig. 3** was applied to measure the puromycin reaction exclusively of ribosomes containing plasmid derived 23 S rRNA. Kinetics of the puromycin reaction of pretranslocational ribosomes ( $-G$ , open circles), those after translocation with EF-G ( $+G$ , closed circles), and those after translocation in the presence of EF-G and thiostrepton ( $+G/+T$ , filled triangles) are shown. The curve with EF-G and thiostrepton ( $+G/+T$ ) indicates the puromycin kinetics of ribosomes exclusively with plasmid-derived 23 S rRNA.

TABLE I

Analysis of the translocation reaction via both puromycin reaction and the movement of the mRNA

The value in the "puromycin" column is the relative amount of AcPhe-puromycin formed after a translocation reaction in the presence of thiostrepton ( $+G/+T$ ) in comparison to translocation in the absence ( $+G$ ). Background values ( $-G$ ) were subtracted. In the "mRNA protection" column, the ratio of translocation in the presence of thiostrepton is given in comparison with the translocation without thiostrepton determined by an mRNA protection assay according to Ref. 5 (under "Materials and Methods"). Before translocation, a strong band at position +16 (A in the AUG codon is +1) was observed. Upon translocation, the intensity of the bands corresponding to mRNA positions +17, +18, and +19 was increased. This increase was assessed by scanning the corresponding bands on a sequencing gel. Intensities were determined relative to the band at position +15, which served as an internal control. The background values ( $-G$ ) were subtracted, and the ratio ( $+G/+T$ ) versus ( $+G$ ) was calculated. The average values of these ratios of positions +17, +18, and +19 are given in the column "mRNA protection". Low values (e.g. 0.18) indicate that no translocation took place in the presence of thiostrepton, whereas values around 0.50 indicate translocation.

	Puromycin	mRNA protection
Wild type	$0.09 \pm 0.02$	$0.18 \pm 0.03$
A1067U	$0.61 \pm 0.14$	$0.48 \pm 0.12$
G2581A	$0.05 \pm 0.02$	$0.51 \pm 0.03$
C2507U	$0.16 \pm 0.03$	$0.45 \pm 0.11$
$\Psi 2580C$	$0.16 \pm 0.02$	$0.46 \pm 0.08$
C2507U/G2581A	$0.02 \pm 0.01$	$0.37 \pm 0.07$

compared to wild-type ribosomes does not easily explain the dominant lethal character of the mutant (15). Furthermore, tRNA mutations at position C74 were active in protein synthesis *in vivo* although these mutations induced frameshifts and read-through's (19). It is therefore possible that the base pair between G2252 of 23 S rRNA and C74 of tRNA is not essential for PTF and disruption of this base pair affects also other ribosomal functions, e. g. tRNA binding to one of the ribosomal

sites, translocation or the accuracy of translation.

Mutations within helix 90 ( $\Psi 2580C$ , G2581A, C2507U/G2581A and C2507 $\Delta$ /G2581A) fulfilled the above mentioned criteria. All mutants were exclusively and severely reduced in peptide-bond formation. Also C2507U showed a strong reduction of the puromycin reaction ( $\sim 50\%$ ). Other functions such as subunit assembly (Table I in Ref. 1), tRNA binding to A and P sites (Table II in Ref. 1) and translocation (Table I) are not affected. These results provide strong evidence that this region of helix 90 is highly important for peptide-bond formation.

The double mutation C2507U/G2581A had the strongest effects in the functional tests, *i. e.* the effects were stronger than those of the single mutations, and the portion of this mutated 23 S rRNA was reduced in the polysomal fraction (preceding paper, ref. 1). These observations are in line with the newer secondary structure map of 23 S rRNA (20) and indicate that G2581 is not base paired with C2507. In the latest map version C2507 is base paired with G2582 rather than with G2581 (as in the previous version) and, therefore, the effects of the single mutations should be exacerbated in the double mutant.

The  $\Psi 2580C$  mutation was also investigated by Porse and Garrett (21). They found a 90% inhibition of the fragment reaction which is in good agreement with our data. They also reported a severe reduction of growth when their construct was expressed from the  $\lambda_{PL}$  promoter. In contrast, we did not observe a growth defect of this mutant when expressed from the  $\lambda_{PL}$  promoter and only a small effect when expressed from the tac promoter (1). A possible reason for this discrepancy can be seen in the fact that Porse and Garrett (21) had an erythromycin resistance (A2058G) as a secondary genetic marker on their 23 S rRNA constructs which might effect the PTF center *per se*, since erythromycin binds adjacent to the PTF center probably blocking the entrance of the tunnel for the nascent peptide chain (22). We used thiostrepton resistance as a genetic marker

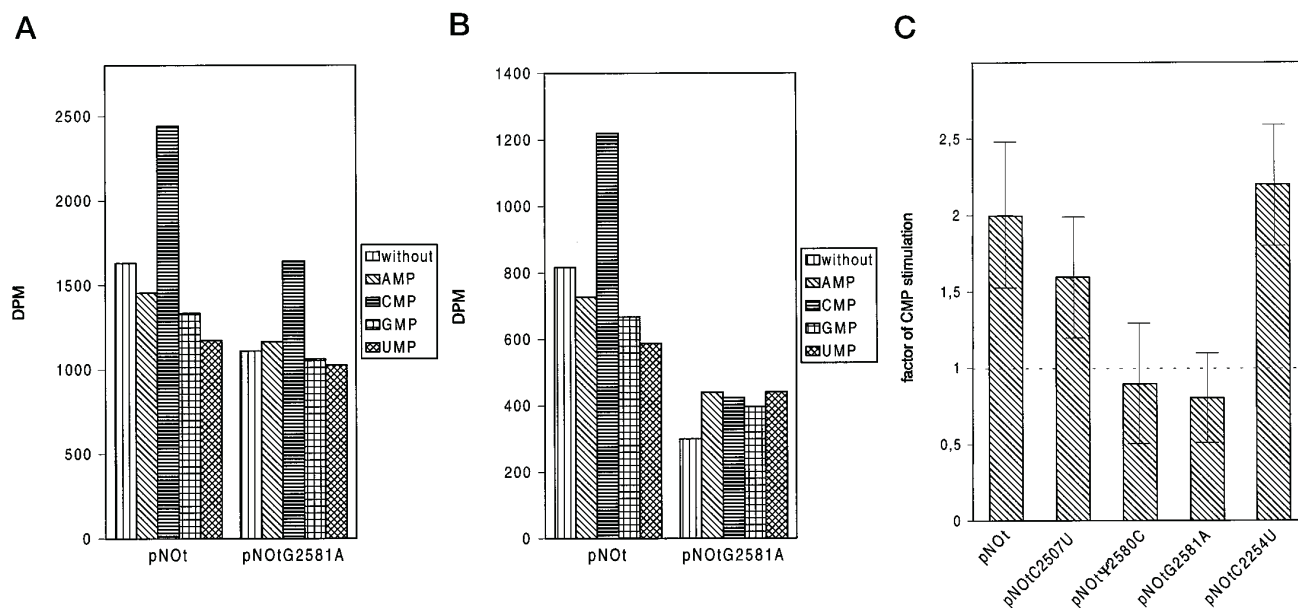


FIG. 5. **Fragment reaction with the minimal donor pA-fMet.** A, 70 S ribosomes were incubated in the presence of 50% methanol with pA-fMet and [ $^3\text{H}$ ]Phe-tRNA<sup>Phe</sup> (80,000 dpm/pmol) and the formation fMet-Phe was measured. When indicated AMP, CMP, GMP or UMP were present during the reaction. Results are shown for 70 S derived from pNOT or pNOTG2581A. B, 50% of the respective signals of the pNOT ribosomes shown in A were subtracted from the signals derived from both ribosomes in order to calculate the signal of the 70 S with plasmid derived 23 S rRNA. C, the CMP stimulation of the pA-fMet reaction derived from the data processed as described in B. The numbers presented are the average of two to six independent experiments, each done in duplicate.

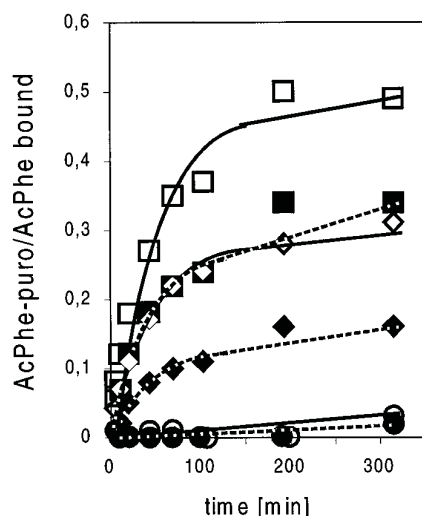


FIG. 6. **Puromycin kinetics with various kinds of AcPhe-tRNA.** AcPhe-tRNA<sup>Phe</sup> was made from natural tRNA<sup>Phe</sup> (wt-CCA), *in vitro* transcribed tRNA<sup>Phe</sup> (TR-CCA) or *in vitro* transcribed tRNA<sup>Phe</sup> containing a CUA-3' end (TR-CUA). These AcPhe-tRNA<sup>Phe</sup> were bound to the P sites of poly(U) programmed ribosomes. Puromycin was added, and the kinetics of AcPhe-Puromycin was followed at 0 °C. Ribosomes were derived from pNOTC2254U (open symbols) or pNOTG2581A (closed symbols), respectively. ◇, C2254U, wt-CCA; □, C2254U, TR-CCA; ○, C2254U, TR-CUA; ◆, G2581A, wt-CCA; ■, G2581A, TR-CCA; ●, G2581A, TR-CUA.

which does not significantly influence the elongation cycle under our conditions (1). An effect of the erythromycin resistance mutation A2058G alone is already seen in the distribution of the mutated 23 S rRNA. Only 30% of the 70 S population carry this mutation in contrast to 50% in the 50S population (21).

Specifically the modified base Ψ2580 was discussed as a possible candidate for being directly involved in the PTF activity due to its highly conserved nature with respect to the presence of this modification at this position (23), although yeast cytoplasmic ribosomes contain a Ψ residue not at the position 2580 but at 2575 (*E. coli* numbering).

70 S ribosomes with the Ψ2580C mutation were severely affected in formation of AcPhe-puromycin though a small activity was observed. In contrast to the G2581A mutation, this mutation hardly affected the cell growth of the bacteria, although both mutations were equally present in the 50S and 70 S fractions as well as in polysomes (1). Therefore, G2581 probably plays a more prominent role than Ψ2580 for peptide-bond formation. Our data indicate that Ψ2580 is important but not essential for the PTF activity.

AcPhe-tRNA with an U replacing C75 could not cure the PTF lesion of the mutant G2581A (Fig. 6). It is therefore unlikely that G2581 forms a canonical base pair with the universal C75. G2581 belongs to the only universally conserved UGG sequences which is highly important for PTF activity. However, if the universal CCA-3' end of tRNAs would base pair with a UGG sequence of the 23 S rRNA, this UGG sequence should be also universal. Consequently no continuous UGG sequence in 23 S rRNA base pairs with the CCA-3'-end of tRNA. Notwithstanding, the results of the pA-fMet assay in Fig. 5 provide evidence for an interaction of G2581 or Ψ2580 with C75. As mentioned already in the results section pA-fMet is the minimal ligand for the P-site region of the PTF center. The addition of CMP but not of UMP, GMP or AMP strongly stimulates the binding of pA-fMet and the following peptide-bond formation, which we measured with Phe-tRNA as the acceptor substrate. The specific stimulation seen with CMP (Fig. 5) was interpreted that the universal C75 is essential for the proper binding of the donor substrate at the PTF center (11, 14). The intriguing finding is that the residual activities observed with ribosomes with the mutants G2581A and Ψ2580C cannot be stimulated with CMP (Fig. 5), and the same was observed with G2582(C, A) mutations.<sup>3</sup> This functional pattern strikingly contrasted to ribosomes derived from the wild type, the A1067U mutant and all other tested constructs: In all cases a stimulation with CMP could be seen. A possible explanation of these conspicuous observations is that the whole sequence

<sup>3</sup> J. Remme, U. Saarma, C. M. T. Spahn, and K. H. Nierhaus, unpublished result.

$\Psi$ GG2582 is involved in binding the C75 of the peptidyl-tRNA at the P region of the PTF center. However, an indirect effect, *e.g.* a structural distortion of the PTF center, cannot be ruled out by the data. This possibility implies that such a putative distortion should be exclusively restricted to the PTF center since all other functions tested were not impaired, and that therefore in this case the sequence  $\Psi$ GG2582 should be at or adjacent to the PTF center.

Although mutations at all three positions of the  $\Psi$ GG2582 sequence severely affected the PTF activity and the ability of CMP to stimulate the pA-fMet reaction, it is tempting to speculate that G2581 plays a more prominent role in the putative interaction with C75: In addition to the strong effects of the G2581A mutation, this nucleotide exhibits a remarkable single-stranded state in the secondary structure map (20). If G2581 has this prominent role and a prominent position at least in the secondary map, it is astonishing that it cannot be modified with G specific modifying reagents such as kethoxal in the empty ribosome (16). Possibly, the 2581 region is shielded in the empty ribosome but interacts with the CCA end if a tRNA is bound to the P site. In fact, a structural change (melting) of this region upon association of the small and large subunits has been observed in eukaryotic ribosomes (24).

In conclusion, all our mutations within the helix 90 drastically impair growth of the bacteria with the exception of  $\Psi$ 2580C, and all severely reduce the PTF activities but not other ribosomal functions. This functional pattern together with the high degree of evolutionary conservation of this region and its topological relation to the PTF center suggests a direct involvement of this helical region in the PTF reaction.

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