Supplementary Data

The role of the universally conserved A2450-C2063 base pair in the ribosomal peptidyl transferase center

Anna Chirkova[1], Matthias D. Erlacher[1], Nina Clementi[1], Marek Zywicki[1], Michaela Aigner[2], and Norbert Polacek[1]*

[1] Innsbruck Biocenter, Medical University Innsbruck, Division of Genomics and RNomics, Fritz-Pregl-Strasse 3, 6020 Innsbruck, Austria

[2] CMBI, Leopold-Franzens-University Innsbruck, Institute of Organic Chemistry, Innrain 52a, 6020 Innsbruck, Austria

Correspondence to N.P. (norbert.polacek@i-med.ac.at)

Supplementary Figures

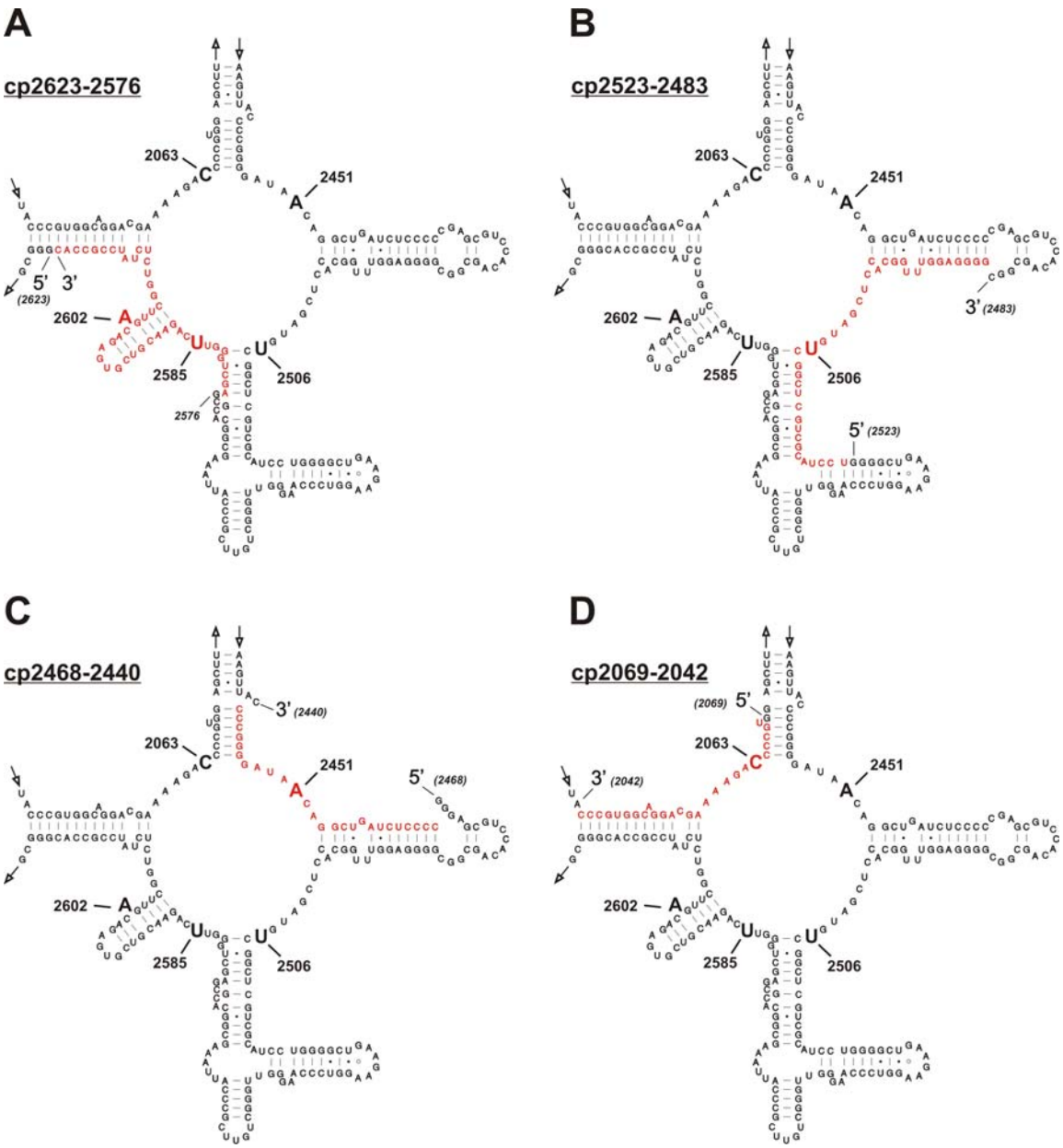

Figure S1. Secondary structures of the central loop of domain V of gapped-cp-23S rRNAs. To construct cp-23S rRNAs, the natural ends of 23S rRNA were covalently connected and new 5'- and 3'-ends have been introduced thus leaving a short sequence gap in the PTC (1). The chemically synthesized RNA fragments that were used to fill the gaps of the cp-23S rRNAs during 50S *in vitro* reconstitution are shown in red. Cp2623-2576 (**A**), cp2523-2483 (**B**), cp2468-2440 (**C**), and cp2069-2042 (**D**) were constructed to study the roles of residues A2602, U2585, U2506, A2453, A2451, A2450, A2062 and C2063, respectively, whereas the first nucleotide of every cp-23S rRNA construct always indicates the new 5'-end and the second residue the 3'-end of the transcript.

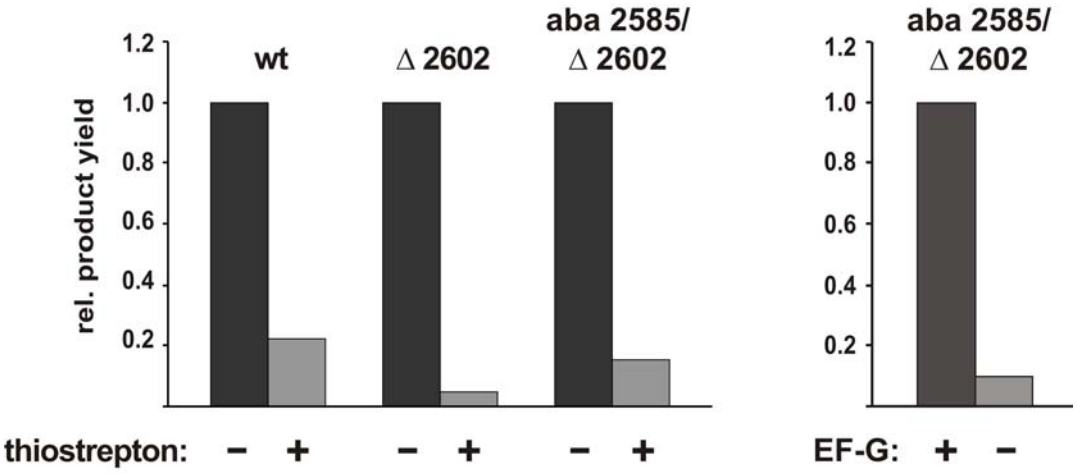

Figure S2. Poly(Phe) production on chemically engineered ribosomes is depending on an authentic tRNA translocation. The poly(U)-directed poly(Phe) synthesis employing reconstituted ribosomes carrying the full-length wt 23S rRNA transcript (wt), the deletion of A2602 (Δ 2602), or the double modification (aba 2585/Δ 2602) was performed in the absence (-) or presence (+) of 5 µM thiostrepton (left). This antibiotic binds to the GTPase-associated center of the 50S subunit and is known to inhibit EF-G turnover (2,3). Omitting EF-G during the *in vitro* translation reaction showed similar results (right). These controls demonstrate that product formation on chemically engineered ribosomes is EF-G-dependent and not the result of a potentially repetitive peptidyl-tRNA drop-off, rebinding to the P-site with subsequent peptidyl transfer to another A-site bound Phe-tRNA. The product yields in the absence of thiostrepton (left) or in the presence of EF-G (right) after 120 minutes of incubation were taken as 1.0.

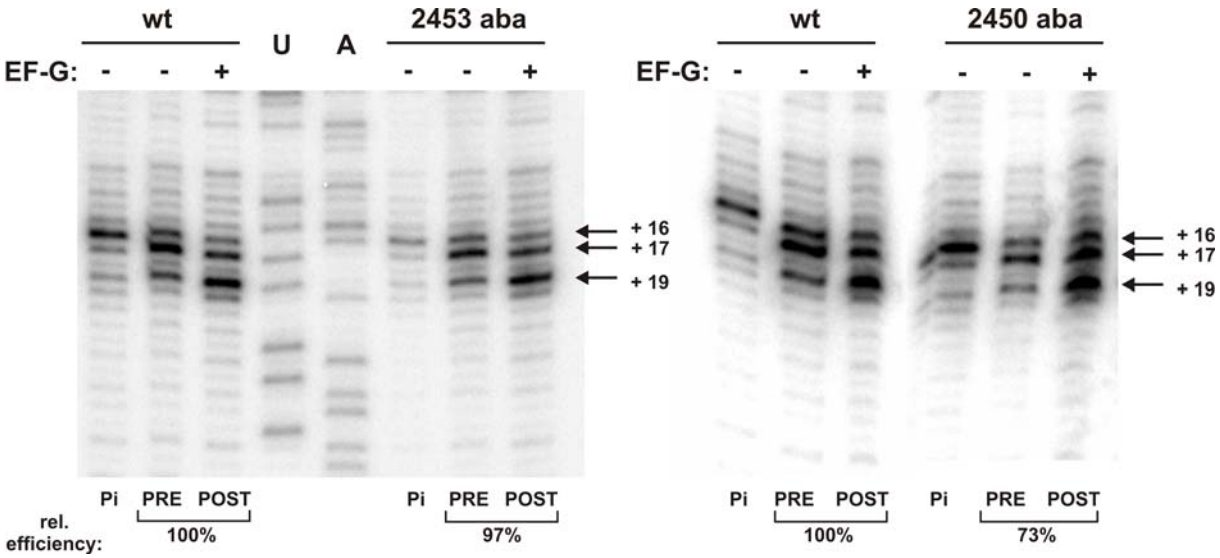

Figure S3. Toeprinting analysis of chemically engineered ribosomes. To assess EF-G-dependent tRNA translocation efficiencies, ribosomes carrying the wt RNA oligo in the PTC or harboring an abasic site (aba) at A2453 (left) or A2450 (right) were programmed with an mRNA analog and used to construct various tRNA/70S/mRNA complexes for toe-printing analysis. Pre-translocational complexes (PRE) were established by binding of deacyltated $tRNA^{fMet}$ to the P-site, followed by binding of AcPhe-$tRNA^{Phe}$ to the A-site. To construct the post-translocational complex (POST) EF-G•GTP (+) was added. Lanes designated Pi represent control complexes carrying only one deacylated $tRNA^{fMet}$ in the P-site. These control complexes produced a primer extension stop at position +16, if the first nucleotide auf the AUG codon is assigned as +1. The additional stop at +17 in PRE complexes has been amply observed also in other toe-printing assays using native 70S particles (e.g. (4)). The mean of the relative translocation efficiencies of at least three independent toeprinting experiments are given underneath the respective lanes, whereas the total fraction of ribosomes that participated in translocation from the PRE to the POST state in the wt control (typically ~ 15 - 25% of reconstituted ribosomal complexes) was taken as 100%. U, A denote dideoxy sequencing lanes.

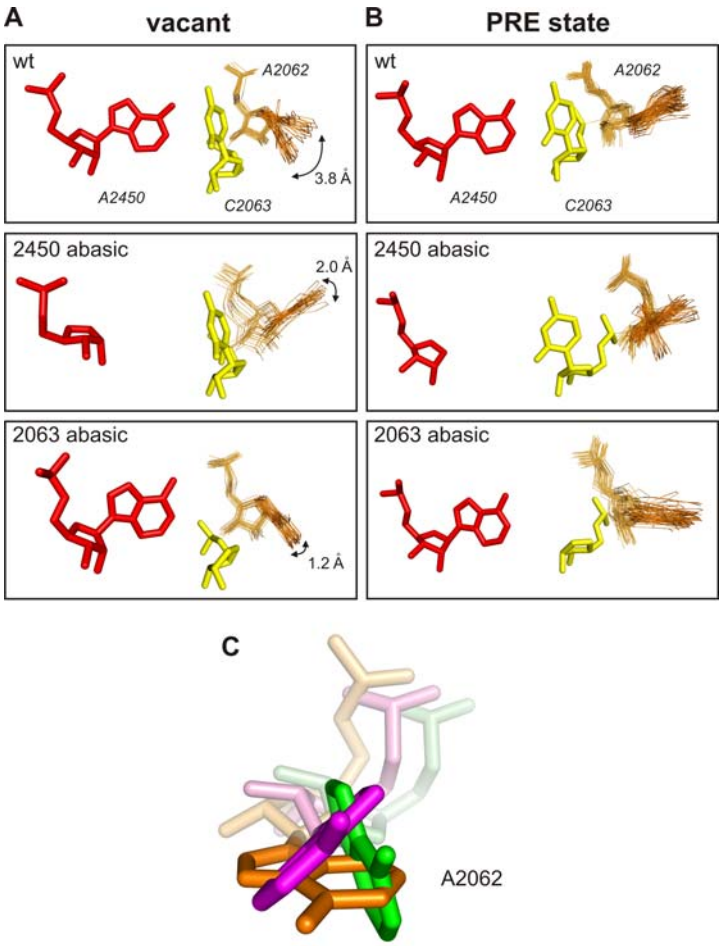

Figure S4. Molecular dynamics (MD) simulations of the PTC. MD simulations of active site residues were performed for 2 nsec in a radius of 20 Å from residues A2450, A2062 and C2063 in the context of (**A**) vacant ribosomes (5) as well as (**B**) in the presence of two tRNA substrates in the P- (deacylated tRNA) and A-sites (aminoacyl-tRNA analog) (6), representing the situation after peptidyl transfer but before translocation, thus mimicking the pre-translocational (PRE) state. The effects of manipulations of the A2450-C2063 base pair (by introducing abasic sites at either of the two residues) on the conformational dynamics of A2062 are shown (arrows an (A)). In the PRE state wt ribosome (B) A2062 reaches the amino acid of A-site tRNA and is thus stabilized, while in the PTC with disrupted A2450-C2063 pairs the nucleobase at A2062 does not interact with the amino acid side chain of A-site tRNA and is confined in an unusual conformation. (**C**) The conformation of the nucleobase at A2062 is highlighted as seen in simulations of the PRE state active site with the wt sequence (orange) or with a disrupted A2450-C2063 base pair in the presence of the 2450 purine (green) or 2450 abasic (magenta) nucleotide analogs. Even though the adenine positions are markedly different in the presence of the 2450 purine or 2450 abasic modifications, the location of the N7 of the Hoogsteen edge of A2062 is similar, but clearly distinct from the A2062 N7 position in the wt situation (orange).

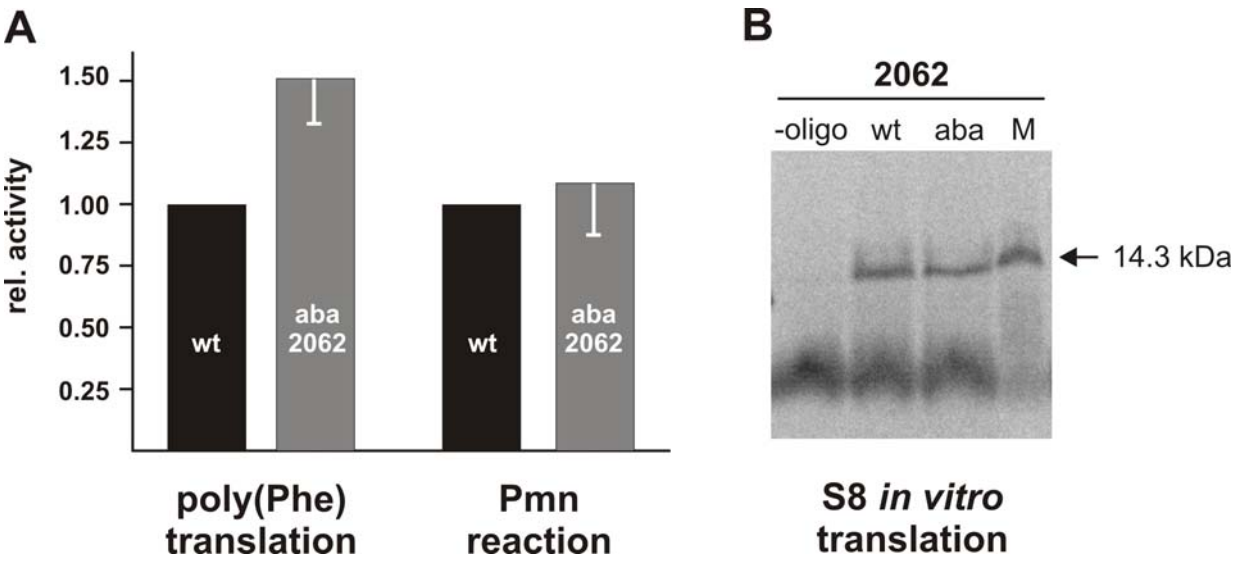

Figure S5. Activities of ribosomes carrying an abasic site at position A2602. **(A)** The product yield in poly(U)-directed poly(Phe) synthesis after 120 minutes of incubation and the initial rates in the puromycin (Pmn) reaction of reconstituted wt ribosomes were compared to particles harboring the abasic site analog (aba) at position 2062. The values given represent the mean and standard errors of at least two independent experiments, whereas the activities observed with wt control ribosomes were taken as 1.00. (**B**) Activities of A2062 modified ribosomes were also tested in the *in vitro* translation assay using an mRNA coding for r-protein S8. A representative SDS-PAGE shows the translation products of ribosomes reconstituted with the wt oligo (wt), carrying an abasic site at 2062 (aba), or ribosomal particles assembled without the compensating synthetic RNA oligo (-oligo). The marker lane (M) shows an aliquot of [^{35}S]-labeled S8 protein produced by native *E. coli* 70S ribosomes.

Supplementary Methods

Toeprinting assay

The toeprinting assay was adapted from protocols published by the Nierhaus and Noller labs (7,8). Annealing of 4.5 pmol [^{32}P]-labeled primer 5'-CGTTAATCTGTGATG-3' to 36 pmol of an *in vitro* transcribed and purified mRNA analog coding for MFKSIRYV (8) was performed in 8.5 µl buffer containing 20 mM Hepes/KOH pH 7.6, 150 mM NH_4Cl, 4 mM β-mercaptoethanol, 2 mM spermidine, 0.05 mM spermine by heating at 66°C for 3 min and direct transfer on ice. 10.45 µl of 70S ribosomes (containing 50S subunits reconstituted from 7.5 pmol cp-23S rRNA and 3 pmol of native *E. coli* 30S subunits) were activated by incubation at 37 °C for 15 minutes. Subsequently, 7.5 pmol of mRNA/primer and 7.5 pmol deacylated $tRNA^{fMet}$ were added and the reaction (13.15 µl) incubated for 10 minutes at 37 °C (Pi complex formation). 4 µl of the reaction were removed and placed on ice for later reverse transcription (RT). The PRE complex was formed by the addition of 7.5 pmol AcPhe-$tRNA^{Phe}$ and incubation at 25°C for 10 minutes (reaction volume 10.15 µl). Two 4 µl aliquots were incubated with either 1 µl 1.5 mM GTP (PRE complex) or with 1 µl 1.5 mM GTP and 5 pmol *E. coli* or *T. thermophilus* EF-G in the buffer containing 49 mM Tris/HCl pH 7.4, 264 mM NH_4Cl, 17 mM $MgCl_2$, 0.1 mM EDTA, 4.6 mM β-mercaptoethanol, 0.18 mM spermidine, 0.004 mM spermine for 10 minutes at 37 °C (POST state). In all samples (Pi, PRE, POST) RT was initiated by the addition of 1 µl mix containing 1 unit AMV reverse transcriptase (*Promega*), 20 mM Tris/HCl pH 7.4, 100 mM NH_4Cl, 20 mM $MgCl_2$, and 1.45 mM dNTPs. The RT reactions were performed at 42 °C for 15 minutes and stopped by the addition of 20 µl water and subsequent precipitation with 2.5 volumes EtOH/NaOAc (0.3 M) for 40 minutes at -20 °C. Samples were centrifuged, pellets dissolved in 5 µl formamide loading dye (7), denaturated at 95 °C for 5 minutes and resolved on 7.5 % polyacrylamide gels (8 M Urea). The gels were quantified using a Molecular Dynamics Typhoon Phosphoimager and Image Quant Software. Primer extension stops observed in control reactions containing only native 30S subunits were used as negative control for the quantification procedures and subtracted from the corresponding stops in complete samples.

Overexpression and purification of elongation factors and PheRS

E. coli EF-G, EF-Tu, EF-Ts, Phe-RS and *T. thermophilus* EF-G carrying C-terminal His-tag have been overexpressed in *E. coli* M15 or BL21 (DE3) cells and purified using Ni-NTA agarose (*QIAGEN*) followed by Superdex 200 column chromatography (for *E. coli* EF-G only). The *T .thermophilus* EF-G purification procedure additionally involved a 20 minutes heating step at 70 °C prior to the Ni-

NTA agarose batch chromatography. Enzymes were dialyzed against buffer containing 50 mM Hepes/KOH pH 7.6, 100 mM KCl, 2 mM $MgCl_2$, 7 mM β-mercaptoethanol, 10% glycerol (for toeprinting assay) or 20 mM Tris/HCl pH 7.5, 100 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 10% glycerol (for *in vitro* translation), snap frozen and stored at -80°C.

Supplementary References

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