Supplementary Material: Materials and Methods

Strains, buffers and other reagents: Strain MY87 carrying a temperature sensitive allele of the $tRNA^{Trp}$ gene (*1*) (W3110, trpR, trpA, 9605-Am, His29-Am, metE, trpT190-ts) was a gift from Mike Yarus. Plasmid pRT33C (col E1, TetR) carrying $tRNA^{Asp}$ and $tRNA^{Trp}$ genes under control of the LacUV5 promoter was also a gift from M.Yarus, this plasmid originally carried a T33C subtitution in the TrpT gene which was reverted to wild-type by Quickchange (Stratagene). Plasmid pET24c containing His-tagged *E.coli* EF-Ts was a gift from Olke Uhlenbeck. All assays were carried out in buffer HiFi (50 mM Tris-HCl pH 7.5, 70 mM NH_4Cl, 30 mM KCl, 3.5 mM $MgCl_2$, 0.5 mM spermidine, 8 mM putrescine, 2 mM DTT) (*2*) at 20°C, except where noted. Ribosomes from MRE600 were harvested from cells in exponential growth phase, purified as previously described (*3*) and stored in HiFi buffer. Radioactive compounds were from Perkin Elmer. mRNAs were made by PCR and T7 transcription and contained an epsilon sequence (*4*), a Shine-Dalgarno sequence and a 7 nt spacer followed by the coding sequence AUG XXX UAA UUG CAG AAA AAA (where the second codon was either the cognate Trp codon UGG or a near-cognate one). Truncated mRNAs were from Dharmacon and were identical to those described except that they ended with the AUG codon.

EF-Tu and TrpRS cloning, expression and purification: EF-Tu was amplified by PCR from *E.coli* MRE600 genomic DNA with primers containing NarI and XhoI linkers. The digested PCR product was cloned into pPROEX HTb (Invitrogen) which adds an N-terminal His_6 tag followed by a TEV protease cleavage site. The sequence of the final product of purification and cleavage differs from wild-type EF-Tu by the addition of a Gly-Gly-Ala tripeptide at the N-terminus. EF-Tu carrying the H84A substitution shown

to inactivate the GTPase function (*5*) was made by Quickchange on this construct and expressed and purified in the same way. This mutant was tested for ribosome dependent GTPase activity and it was confirmed there was no significant hydrolysis after 30 min at 20°C. TrpRS from MRE600 was cloned in the same plasmid using the same sites as for EF-Tu. All coding regions of interest (tRNAs and proteins) were fully sequenced. Both enzymes were purified on NiNTA Superflow resin (Qiagen) following the manufacturer's procedure. EF-Tu storage buffer contained 20 μM GDP.

$tRNA^{Trp}$ expression and purification: Strain MY87 was transformed with plasmid coding for either wild-type or G24A $tRNA^{Trp}$ (mutation introduced by Quickchange, Stratagene). Overnight cultures were grown in LB with 15 μg/ml tetracycline, at 30°C, diluted to 1/100 typically in 6 liters of LB + Tet and grown for 6 hours at 42°C. The ts allele of $tRNA^{Trp}$ has been shown to be polyadenylated and degraded in cells growing at this temperature (*6*). Typically, 2-5% of total tRNA was $tRNA^{Trp}$ following this protocol. Cells were pelleted, washed once with water and resuspended in water (typically 100 ml). Then equal volume of water-saturated phenol was added and extraction proceeded for 2 hours at room temperature while rotating. The phenol phase was back-extracted and the aqueous phase re-extracted. Aqueous phases were pooled and 1/10 volume of 20% KOAc pH 5 and 2 volumes of EtOH were added. After 2 hours at -20°C samples were pelleted 10 min at 10,000 rpm. Pellets were resuspended in 0.1 M Tris-HCl pH 7.5 and the insoluble material re-pelleted. Samples were loaded on a pre-equilibrated DE52 gravity column, washed with 0.1 M Tris-HCl pH 7.5 until A_{260} returned to baseline and eluted with 0.1 M Tris-HCl pH 7.5 and 1 M NaCl. tRNAs were precipitated by adding 1.5 volumes of EtOH and centrifugation. Pellets were resuspended in water and loaded on a

C4 semi-preparative HPLC column equilibrated with buffer A (50 mM $Mg(OAc)_2$, 50 mM NH_4OAc, pH 5.3). tRNAs were eluted with a linear gradient from 0 to 50% buffer B (buffer A + 50% EtOH) in 60 min. Fractions were analyzed by aminoacylation with purified TrpRS and [^{14}C]-Trp (10 μM [^{14}C]-Trp, 5 mM ATP, 2 μM TrpRS in 10 mM Tris-HCl 8.0 and 10 mM $MgCl_2$, incubate 15 min at 37°C, TCA precipitate, filter and count). Fractions accepting [^{14}C]-Trp were pooled, aminoacylated, NaOAc pH 5 added to 0.3 M, phenol and chloroform extracted and EtOH precipitated. Pellets were resuspended in HPLC running buffer A and further purified on the C4 column using the same gradient. Fractions were analyzed by following [^{14}C] counts. Those containing $tRNA^{Trp}$ were pooled and concentrated for subsequent assays. Typical yields were 0.3-0.5 mgs of tRNA from 4-6 liters of cells.

GTP hydrolysis rate measurement: Ternary complex was prepared by incubating 5 μM $tRNA^{Trp}$ (wt or G24A), 20 μM EF-Tu, 15 nM EF-Ts, 50 μM [γ-^{32}P]-GTP, 3 mM phosphoenol pyruvate (PEP), 0.1 mg/ml pyruvate kinase (PK), 20 mM L-tryptophan, 2.5 mM ATP and 2 μM TrpRS in HiFi buffer for 1 hour at 37°C, and purified by gel filtration as described (*7, 8*). Fractions were analyzed by following [^{32}P] counts. 70S initiation complexes were formed by incubating 10 μM ribosomes, 30 μM mRNA (with different codons following the AUG), 12 μM fMet-$tRNA^{fMet}$, 2.5 μM each IF (1, 2 and 3) and 1.5 mM GTP in HiFi buffer for 1 hr at 37°C. This complex was then diluted with the same buffer to yield the desired concentrations for the ribosome titrations. Quench-flow assays were performed in a KinTek apparatus where equal volumes of each complex were rapidly mixed at 20°C and the reaction was quenched with 40% formic acid at the desired times. The fraction of [γ-^{32}P]-GTP hydrolyzed was analyzed by thin layer

chromatography on PEI cellulose plates (Selecto Scientific) in 0.5 M potassium phosphate pH 3.5 (plates were pre-run in water and allowed to dry before loading the samples). Rates were obtained by single exponential fitting using Kaleidagraph (Synergy Software). The concentration dependence of the rate was fit to a hyperbola from which the rate at saturation was calculated.

Peptide bond formation rate measurement and calculation of k_5 and k_7: Ternary complex was formed in the same way as above except that 1 mM GTP was used and the complex was not purified by gel filtration (assays with purified and non-purified complex were compared and the rates obtained were indistinguishable). 70S initiation complex was prepared as above, except that [^{35}S]-fMet-tRNA^{fMet} was used. Quench-flow reactions were quenched with 0.5 N KOH and products analyzed by TLC electrophoresis as previously described (*9*). The data was analyzed as above except that the rate of peptide bond formation for the wild-type tRNA^{Trp} on both near-cognate codons, and the rate for G24A tRNA^{Trp} on the first position mismatch were independent of ribosome concentration, and thus the rate constant was calculated as the average of the measured rates. The observed rate constants in this case correspond to the sum of k_5 and k_7 ($k_{obs}=k_5+k_7$) while the endpoint of the reaction represents the fraction of fMet that goes through the productive pathway ($Fxn=k_5/(k_5+k_7)$). From these two equations with two unknowns we can calculate the values of both rate constants. The errors in these values were calculated using standard error propagation.

Equilibrium binding measurement by filter binding: Purified tRNA^{Trp} was end-labeled with [α-^{32}P]-ATP and the CCA-repairing enzyme as previously described (*10*). Following phenol and chloroform extraction and EtOH precipitation the tRNA was

aminoacylated as described above, further extracted and precipitated and stored in 20 mM KOAc pH 5. Ternary complex was formed by incubating 60 nM EF-Tu H84A (*5*), 1 mM GTP, 1 mM PEP and 0.1 mg/ml PK in HiFi buffer with $MgCl_2$ added to 15 mM final concentration, for 45 min at 37°C to allow for GDP exchange. Aminoacylated [^{32}P]-$tRNA^{Trp}$ was added to 15 nM and incubation allowed to proceed for another 5 min at 37°C. Ribosome complexes with the different mRNAs were formed as above in HiFi buffer with $MgCl_2$ added to 15 mM final concentration and then diluted for the binding titration. Equal volumes (15 μl) of ternary complex and ribosome complex were mixed for 2 min at 20°C and 25 μl were filtered over nitrocellulose filters (Millipore) pre-soaked in the same buffer. Filters were washed with 4 ml of buffer, dried, dissolved in Filter-Count (Packard) and counted. Titrations with ribosomes programmed with the cognate Trp codon, UGG, were fit to a quadratic function while those using near-cognate codons were fit to a hyperbolic one. CPM values were normalized to the endpoint obtained for binding to the cognate mRNA, which was taken to be 100% bound. Titrations carried out at lower $MgCl_2$ concentrations resulted in similar K_d values for both tRNAs on the cognate Trp codon. At these $MgCl_2$ concentrations on near-cognate codons, the binding curves for both tRNAs were virtually identical though the endpoints were considerably lower. For this reason a concentration of 15 mM $MgCl_2$ was chosen for the experiments where binding to the 3rd and 1st position near-cognate codons reached ~95% and ~85% of the cognate endpoint, respectively.

References

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