Supporting Information

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SI Materials and Methods

Buffers. Buffer A: 20 mM Hepes-KOH, pH 7.5, 150 mM NH_4Ac, 4.5 mM $MgAc_2$, 4 mM β-mercaptoethanol, 0.05 mM spermine, and 2 mM spermidine. Buffer B: same as A, but with 20 mM $MgAc_2$. Buffer C: 20 mM Hepes-KOH, pH 7.5, 150 mM KAc, 14 mM $MgAc_2$, 4 mM β-mercaptoethanol, 0.05 mM spermine, and 2 mM spermidine (1). pH was adjusted at 0 °C.

Ribosome, Proteins, tRNAs, and mRNAs. The following materials were prepared as described previously: EF4 (2); tight-coupled ribosomes from *Escherichia coli* MRE600 cells; cloned *E. coli* His-tagged proteins EF-G, EF-Tu, IF1, IF2, and IF3; *E. coli* [^{35}S]fMet-tRNA^{fMet}, *E. coli* [^{35}S]fMet-tRNA^{fMet} (prf); *E. coli* [^{3}H]Phe-tRNA^{Phe} (3). Yeast Phe-tRNA^{Phe} (Cy5), yeast [^{3}H]-Phe-tRNA^{Phe} (Cy3), *E. coli* [^{3}H]-Lys-tRNA^{Lys} (Cy3), and *E. coli* [^{3}H]-Arg-tRNA^{Arg} (Cy3) were prepared as described (4, 5). L11$^{87\text{-}Cy3}$ ribosomes were prepared as described (4). Immobilization of ribosomes and other smFRET procedures were described (4). Protein concentrations were determined by Bradford assay (6). mRNA MFK, fluorescein-labeled mRNA Flu-mRNA014, and biotin-labeled mRNA MRF were purchased from Dharmacon with sequences 5′-GGG AAG GAG GUA AAA AUG UUU AAA CGU AAA UCU ACU-3′, 5′-GGG AAG GAG GUA AAA AUG UUU AAA CGU AA-Flu-3′, 5′-biotin-GGG AAU UCA AAA AUU UAA AAG UUA AUA AGG AUA CAU ACU AUG CGU UUC UUC CGU UUC UAU CGU UU-3′, respectively (initiator codon underlined). Pyrene-mRNA09 with sequence AAG GAG GUA AAA AUG UUU GCU was a kind gift from Simpson Joseph (University of California, San Diego, La Jolla, CA). Stock solutions of antibiotics were made up in water [spectinomycin (Sigma); viomycin (Research Diagnostics)].

Complex Formation. All the following complexes were made up in buffer A at 37 °C except as otherwise specified.

Initiation complex was formed by incubating ribosomes (2 μM) with mRNA MFK (8 μM) or Flu-mRNA014 (8 μM) or Pyrene-mRNA09 (8 μM) or biotin-labeled mRNA MRF (8 μM), IF1 (3 μM), IF2 (3 μM), IF3 (3 μM), GTP (1 mM), [^{35}S]-fMet-tRNA^{fMet} (3 μM), or [^{35}S]-fMet-tRNA^{fMet} (prf) (3 μM) for 25 min.

Ternary complex was formed by incubating EF-Tu (6 μM) with [^{3}H]-Phe-tRNA^{Phe} (6 μM) or [^{3}H]-Phe-tRNA^{Phe} (Cy5 16/17), GTP (1 mM), phosphoenolpyruvate (Roche Diagnostics) (1.5 mM), pyruvate kinase (Roche Diagnostics) (0.015 mg/mL) for 15 min.

Pretranslocation complex (PRE) complexes were formed by incubating initiation complex and ternary complex at 37 °C for 45 s. Then they were purified by ultracentrifugation through a 1.1 M sucrose cushion in buffer B (450,000 × *g*, 40 min, 4 °C). PRE complex concentration was calculated from the amount of ribosome-bound fMet-[^{3}H]-Phe-tRNA^{Phe}.

Posttranslocation complex (POST) complexes were formed by incubating ternary complex and initiation complex at 37 °C briefly for 45 s and then in the presence of EF-G (molar ratio of EF-G: ribosome was 0.2 : 1) and GTP (1 mM) at 37 °C for 10 min. Then they were purified by ultracentrifugation through a 1.1 M sucrose cushion in buffer A (450,000 × *g*, 40 min, 4 °C). POST complex concentration was calculated from the amount of ribosome-bound fMet-[^{3}H]-Phe-tRNA^{Phe}.

Fluorescence Measurements. All rate measurements and associated incubation steps were carried out at 25 °C in buffer A, except as otherwise indicated.

Stopped-flow fluorescence experiments were performed using either an SX.18MV stopped-flow spectrofluorometer (Applied Photophysics) or a KinTek stopped-flow spectrofluorometer model SF-2004. Fluorescein was excited at 460 nm and fluorescence was monitored using a 495-nm long-pass filter. For double incubation experiments, PRE or POST complexes were rapidly mixed with EF4•GTP and preincubated for various times prior to rapid mixing with EF-G•GTP and further incubation. The ratio of reagent volume from each syringe is 1 : 1 : 1. All the concentrations mentioned in the figure legends are the final concentrations after both mixing steps.

Steady-state fluorescence spectra and slow kinetics of fluorescence intensity change were measured on a photon-counting instrument (Fluorolog-3 spectrofluorometer, Horiba Jobin Yvon). For the steady-state measurements of pyrene-labeled mRNA, samples were excited at 343 nm and the emission spectrum from wavelength 360 to 430 nm was recorded. Typically three to five fluorescence traces were averaged for each result. The percentage change in fluorescence intensity was determined using the equation $= (I_{PRE}\text{-}I)/I_{PRE} * 100$, where I_{PRE} is the fluorescence intensity of PRE complex and I is the fluorescence intensity of the complex formed on either EF4 or EF-G addition. For slow kinetics performed using Flu-mRNA014, samples were excited at 460 nm and emission was monitored at 527 nm.

Ensemble FRET experiments were performed using a KinTek stopped-flow spectrofluorometer model SF-2004. Donor Cy3 was excited at 530 nm, and acceptor Cy5 fluorescence change was monitored at 680 ± 10 nm. Measurements were made in parallel on the DA (donor + acceptor), DU (donor only), and UA (acceptor only) samples for each ribosome complex. The signal for the corresponding blank sample containing unlabeled ribosomes and A site fMetPhe-tRNA^{Phe} was subtracted from each of the DU, DA, and UA samples (Fig. S5) to correct for light scattering and background fluorescence.

Total internal reflection fluorescence (TIRF) measurements on immobilized ribosomes. A custom-built objective-type TIRF microscope was based on a commercial inverted microscope (Eclipse Ti, Nikon) with a 1.49 N.A. 100× oil immersion objective (Apo TIRF; Nikon) (4). Alternating-laser excitation [ALEX, (7)] was performed. Only spots showing anticorrelation behavior between 532-nm laser-illuminated Cy3 and Cy5 emission channels, FRET changes in 532-nm laser-illuminated frames, and no significant changes in the 640-nm laser-illuminated frames (to eliminate photo-bleaching and blinking) were considered as displaying real FRET changes. No more than two FRET states were found in any single traces despite vigorous efforts to identify additional states. Traces with only one FRET state were termed nonfluctuating traces, whereas traces with two FRET states were termed fluctuating traces.

Quench-Flow Measurements. *Rapid quench experiments* were performed using a KinTek Chemical Quench-Flow Model RQF-3 machine. Puromycin reactions were quenched with 0.3 M NaAc solution at pH 5.0.

Double-mixing rapid quench experiments. PRE or POST complexes were rapidly mixed with the first reagent and preincubated for various times prior to rapid mixing with the second reagent and further incubation as described (3). The first mixture was diluted by 2.56 times in the second rapid mixing step. All the concentrations mentioned in the figure legends are the final concentrations after both mixing steps.

Rate Constant Estimation. Global fitting of data presented in Figs. 1 *A–D* and 4*E* was carried out using the program Scientist (MicroMath Research, LC). smFRET traces were analyzed by a Hidden Markov Model based software [HaMMy (8)].

1. Badu-Nkansah A, Sello JK (2010) Deletion of the elongation factor 4 gene (lepA) in Streptomyces coelicolor enhances the production of the calcium-dependent antibiotic. *FEMS Microbiol Lett* 311:147–151.
2. Qin Y, et al. (2006) The highly conserved LepA is a ribosomal elongation factor that back-translocates the ribosome. *Cell* 127:721–733.
3. Liu H, Pan D, Pech M, Cooperman BS (2010) Interrupted catalysis: The EF4 (LepA) effect on back-translocation. *J Mol Biol* 396:1043–1052.
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7. Kapanidis AN, et al. (2004) Fluorescence-aided molecule sorting: Analysis of structure and interactions by alternating-laser excitation of single molecules. *Proc Natl Acad Sci USA* 101:8936–8941.
8. McKinney SA, Joo C, Ha T (2006) Analysis of single-molecule FRET trajectories using hidden Markov modeling. *Biophys J* 91:1941–1951.

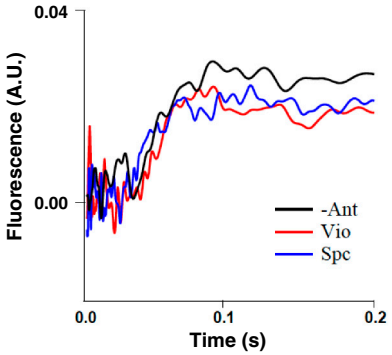

Fig. S1. Antibiotic effects on EF4 interaction with PRE complex. EF4•GTP (5 μM) was rapidly mixed with PRE complexes (0.1 μM) (black trace), PRE complexes (0.1 μM) with 1 mM viomycin (red trace), or PRE complexes (0.1 μM) with 3 mM spectinomycin (blue trace).

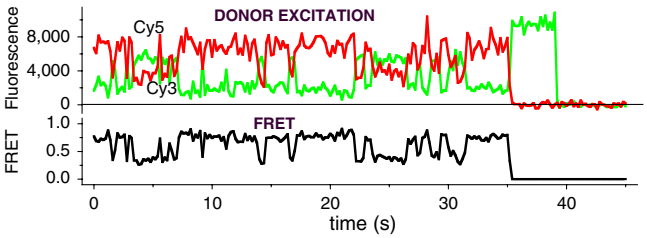

Fig. S2. Typical single molecule FRET trace showing fluctuation between classic (high FRET) and hybrid (low FRET) PRE (Lt) complexes containing $L11^{87\text{-}Cy3}$ and A-site fMetPhe-$tRNA^{Phe}$ (Cy5). Similar fluctuations were found for the PRE (tt) complexes.

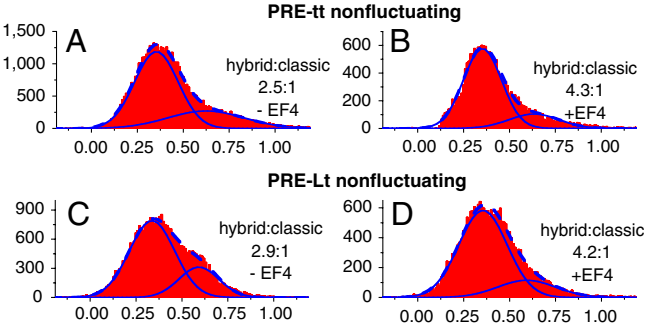

Fig. S3. Determinations of the influence of added EF4•GTP on the distribution of nonfluctuating ribosome complexes between classic and hybrid states. X axes show FRET values. Y axes show numbers of events. (*A*) PRE-tt complexes containing P-site $tRNA^{Arg}$ (Cy3) and A-site ArgPhe-$tRNA^{Phe}$ (Cy5). (*B*) Same as *A*, but following addition of EF4•GTP (2 μM). (*C*) PRE-Lt complexes containing $L11^{87\text{-}Cy3}$ ribosomes and A-site fMetPhe-$tRNA^{Phe}$ (Cy5). (*D*) Same as *C*, but following addition of EF4•GTP (2 μM).

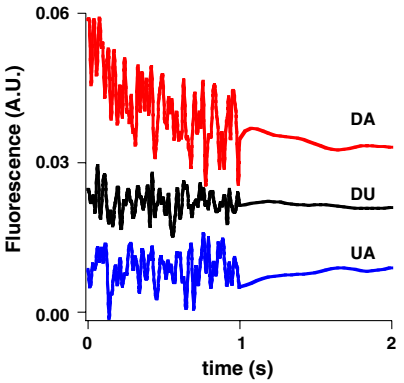

Fig. S4. Kinetics of ensemble FRET change. PRE-Lt complex (0.16 μM) containing $L11^{87\text{-}Cy3}$ and A-site fMetPhe-$tRNA^{Phe}$ (Cy5) (DA sample) was rapidly mixed with EF4•GTP (3 μM, after mixing) in buffer A at 25 °C, with excitation at 530 nm. Parallel experiments were carried out with the DU ($L11^{87\text{-}Cy3}$ and A-site fMetPhe-$tRNA^{Phe}$) and UA [unlabeled ribosomes and A-site fMetPhe-$tRNA^{Phe}$ (Cy5)] samples. Fluorescence intensities were monitored at the Cy5 emission wavelength (680 ± 10 nm). Traces are offset to directly compare the DA and UA traces. The corrected relative FRET change, presented in Fig. 4*E* and set equal to 1.0 at zero time, is calculated as $\Delta DA - (\Delta DU + \Delta UA)$, where Δ refers to the change in fluorescence intensity as a function of time, and the value at zero time is set equal to 1.0.

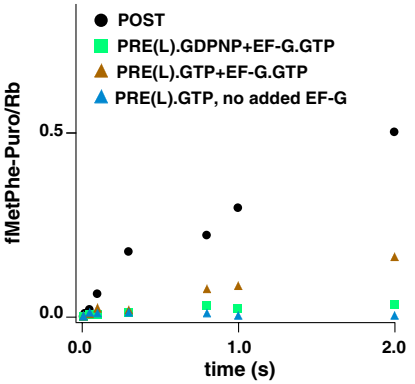

Fig. S5. EF-G conversion of PRE-L complex to POST complex. For all reactions shown, the final complexes (0.1 μM) were rapidly mixed with 3 mM puromycin for the indicated times, followed by quenching. All incubation and mixing steps were performed at 25 °C. PRE-like complex [PRE(L)] was formed by preincubating POST complex with EF4•GDPNP (1.25 μM) and $tRNA^{fMet}$ (0.15 μM) for 3 h. •, POST complex; ▲ (brown), PRE(L) complex was incubated with GTP (1 mM) for 45 min, then rapidly mixed with 3 mM puromycin and 5 μM EF-G•GTP; ▲ (blue), PRE(L) complex was incubated with GTP (1 mM) for 45 min, then rapidly mixed with 3 mM puromycin; ■, PRE(L) complex was incubated with GDPNP (1 mM) for 45 min, then rapidly mixed with 3 mM puromycin and 5 μM EF-G•GTP.

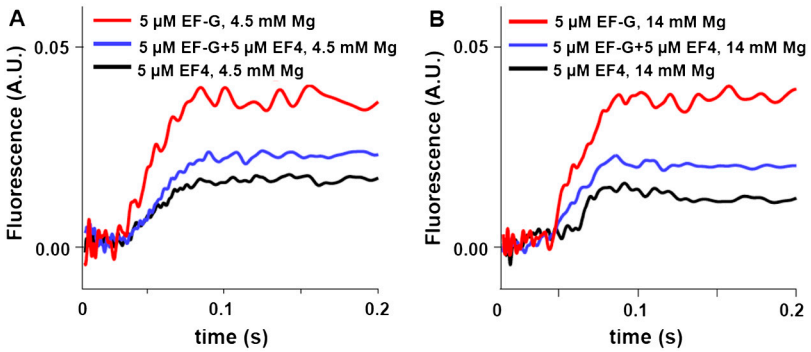

Fig. S6. EF4 dependent translocation under suboptimal (high) ionic strength conditions. PRE complexes programmed with Flu-mRNA014 (0.1 μM) were rapidly mixed in a stopped-flow spectrophotometer with EF-G•GTP, EF4•GTP or mixtures of EF-G•GTP and EF4•GTP at the indicated concentrations. (*A*) In 4.5 mM Mg^{2+} buffer (buffer A). (*B*) In 14 mM Mg^{2+} buffer (buffer C).

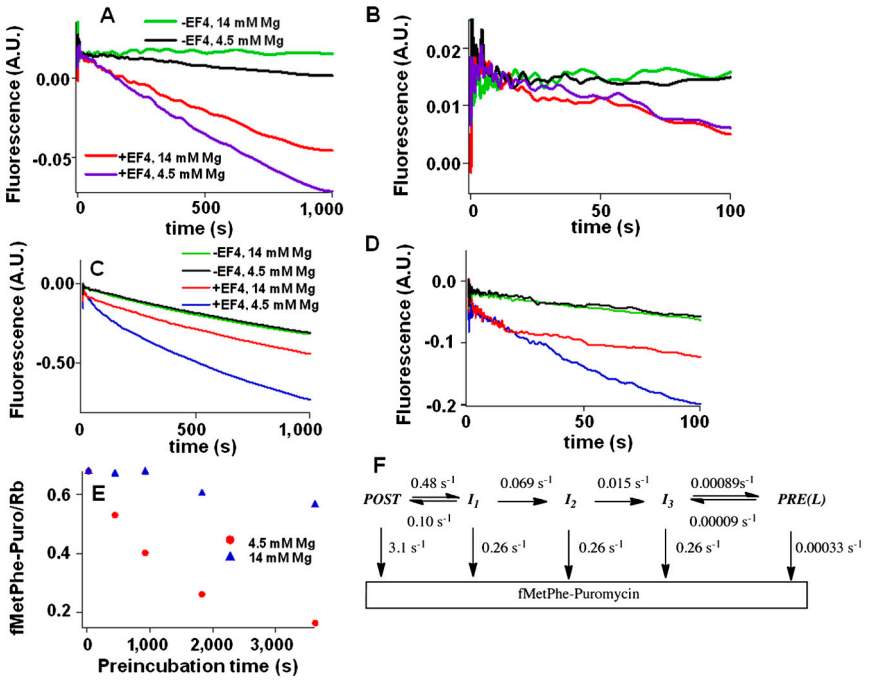

Fig. S7. EF4 dependent back translocation under suboptimal (high) ionic strength conditions. (*A* and *B*) POST complexes programmed with Flu-mRNA014 (0.1 μM) containing 0.15 μM added $tRNA^{fMet}$ were rapidly mixed with or without 3 μM EF4•GDPNP in 4.5 mM Mg^{2+} buffer (buffer A) and 14 mM Mg^{2+} buffer (buffer C) at different time scales. (*C* and *D*) 0.1 μM POST complexes containing $tRNA^{fMet}$ (prf) were rapidly mixed with or without 3 μM EF4•GDPNP in 4.5 mM Mg^{2+} buffer (buffer A) and 14 mM Mg^{2+} buffer (buffer C) at different time scales. (*E*) EF4-dependent back translocation measured by the change in puromycin reactivity of fMetPhe-$tRNA^{Phe}$. POST complex (0.1 μM) was preincubated for the times indicated with 0.15 μM *E. coli* $tRNA^{fMet}$ and 3 μM EF4·GDPNP in 4.5 mM Mg^{2+} buffer (buffer A, •) and 14 mM Mg^{2+} buffer (buffer C, ▲) prior to incubation with puromycin (5 mM) for 20 s prior to quenching. Loss of puromycin reactivity provides a direct measure of PRE(L) complex formation, as do the longer time fluorescence changes seen in parts *A* and *C* (3). (*F*) Quantitative kinetic scheme for EF4-catalyzed back translocation at 4.5 mM Mg^{2+} from ref. 3.