Altered tRNA dynamics during translocation on slippery mRNA as determinant of spontaneous ribosome frameshifting

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When reading consecutive mRNA codons, ribosomes move by exactly one triplet at a time to synthesize a correct protein. Some mRNA tracks, called slippery sequences, are prone to ribosomal frameshifting, because the same tRNA can read both 0- and -1-frame codon. Using smFRET we show that during EF-G-catalyzed translocation on slippery sequences a fraction of ribosomes spontaneously switches from rapid, accurate translation to a slow, frameshifting-prone translocation mode where the movements of peptidyl- and deacylated tRNA become uncoupled. While deacylated tRNA translocates rapidly, pept-tRNA continues to fluctuate between chimeric and posttranslocation states, which slows down the re-locking of the small ribosomal subunit head domain. After rapid release of deacylated tRNA, pept-tRNA gains unconstrained access to the -1-frame triplet, resulting in slippage followed by recruitment of the -1-frame aa-tRNA into the A site. Our data show how altered choreography of tRNA and ribosome movements reduces the translation fidelity of ribosomes translocating in a slow mode.
During translation elongation, the ribosome moves along the mRNA in steps of three nucleotides, i.e., one codon at a time, towards the mRNA 3′ end. The exact step size is essential, as it maintains the correct open reading frame until the ribosome encounters a stop codon that terminates translation. However, in some cases, ribosomes can switch from the original open reading frame (0 frame) into alternative −1, +1, +2 or −4 frames. Frameshifting usually occurs on “slippery” mRNA sequences which allow tRNAs to base pair with codons in both the original and an alternative frame. In programmed ribosome frameshifting, which is most prevalent in viruses, additional secondary structure elements in the mRNA facilitate frameshifting, resulting in two functional protein products that are usually essential for virus propagation1−7. In contrast, in most cases where slippage is spontaneous, −1 frameshifting results in out-of-frame decoding and termination at premature stop codons leading to synthesis of aberrant dysfunctional proteins8−10. The genomic abundance of slippery sequences is relatively high. In Escherichia coli, the slippery A AAA AAG sequence is found in 68 genes11 and in humans ~10% of all cellular mRNAs contain a slippery sequence12. However, the frequency of spontaneous frameshifting in vivo is as low as 10−4−10−5 during canonical translation13,14, even though some slippery sequences actually favor codon-anticodon pairing in the −1 frame15. This implies existence of a mechanism preventing the loss of the correct reading frame at slippery sites. Recent studies demonstrated that elongation factor G (EF-G) has a key role in reading frame maintenance during tRNA–mRNA translocation16−19.

Translocation is a complex process that entails movements of the ribosome, tRNAs, and mRNA. Pretranslocation (PRE) complexes comprise ribosomes with the mRNA-bound peptidyl-tRNA in the A site and deacylated tRNA in the P site. PRE complexes are dynamic and interconvert between two conformational states termed the non-rotated/classical state (or ground state 2)20−22. In the classical state, tRNAs reside in the A/A, P/P sites, respectively, both on the small (SSU) and large (LSU) ribosomal subunits. In the rotated/hybrid state, the SSU rotates with respect to the LSU and tRNAs move into hybrid (A/P, A/P*, and P/E) conformations, in which the tRNA anticodons remain in the A and P site, respectively, while the acceptor arms move towards the P and E site on the LSU19−21,22−23. The elbow region of the A/P-site tRNA is mobile and can adopt slightly different orientations, A/P and A/P*19,24,25. EF-G binding facilitates the movement of tRNAs into the hybrid states, as well as rotation of the ribosomal subunits relative to each other21,23,30,38−42. GTP hydrolysis by EF-G and the subsequent Pi release promote large-scale rearrangement of the complex that uncouples the movements of the head and body domains of the SSU. The tRNA anticodons and the mRNA move with the SSU head domain in forward direction, whereas the SSU body domain rotates backwards relative to LSU. As a result, tRNAs move from the hybrid into intermediate states called chimeric states (CHI or ap/P, pe/E), where the anticodons bind between the A and P and P and E sites on the SSU and the acceptor ends reside in the P and E site of the LSU, respectively19,23−25,38,41−43. Extensive analyses by time-resolved cryo-EM, single-molecule, and ensemble kinetics have shown that CHI states are authentic intermediates of translocation19,21,22,25. Subsequently, tRNAs move synchronously to post translocation states (P/P and E/E)26,38,41. At the end of translocation, the deacylated tRNA and EF-G dissociate from the ribosome and the SSU head domain swivels backwards locking pept-tRNA in the P site25,38,41,43.

Structural studies and kinetic analysis of translocation suggest how EF-G contributes to the reading frame maintenance. When ribosomes enter the CHI state, the stabilizing interactions between the tRNA–mRNA complex and 16 S rRNA on the SSU body domain are lost and instead the complex interacts with the residues at the tip of EF-G domain 4, in particular H583 and Q50719,23,45. In the absence of EF-G, the movements of the mRNA and tRNA anticodon become uncoupled and may lead to frameshifting18. Amino acid replacements at the tip of EF-G domain 4 promote −1 frameshifting on a slippery mRNA16,17. These replacements slow down translocation25,24,47 and alter the timing of translocation events, i.e., the backward movement of the SSU head from the swiveled to the non-swiveled state is delayed and uncoupled from the release of the deacylated tRNA from the E site19. These two steps occur simultaneously during translocation with the wild-type (wt) EF-G38,41 suggesting that rapid translocation and the EF-G-coordinated order of movements prevent spontaneous frameshifting.

While previous studies offered a conceptual framework for understanding reading frame maintenance, it remains unclear when and how spontaneous frameshifting occurs. Here we use smFRET to compare tRNA translocation pathways in the absence of frameshifting, i.e., on non-slippery mRNA, and at conditions where a significant fraction of ribosomes change from 0 to −1 frame, i.e., on a slippery mRNA and with frameshifting-promoting EF-G mutants. This approach allows us to identify potential heterogeneity within the ribosome population, to visualize local tRNA fluctuations, and to monitor how internal movements of the ribosome control frameshifting. We identify two different modes of ribosome progression along the mRNA, a fast and accurate mode, where rapid simultaneous movement of pept- and deacylated tRNA is coupled to the motions of the ribosome, and a slow, frameshifting-prone mode with uncoupled translocation of pept- and deacylated tRNA. In the slow mode, pept-tRNA is trapped in fluctuations between CHI and P/P states before accommodating in the P site, which occurs during back swivel of the SSU head domain. Decacylated tRNA translocates at normal speed and dissociates from the ribosome before the accommodation of pept-tRNA in the P site is completed. This opens a time window where a single pept-tRNA can reassign the reading frame according to the thermodynamic potential of the mRNA sequence. Ribosomes favor the fast mode during translocation with wild-type EF-G, but switch to a slow mode during translocation by frameshifting-promoting EF-G mutants, which suggests that a population of ribosomes with distinct kinetic features contributes to the loss of reading frame on a slippery mRNA.

Results

Translocation of peptidyl-tRNA and ribosome frameshifting. To dissect the mechanism of ribosome slippage, we first used smFRET to monitor EF-G-induced translocation of pept-tRNA from the A to the P site (Fig. 1a). We assembled PRE complexes on mRNA with or without a slippery sequence (Fig. 1b) and utilized FRET reporters attached to the ribosomal protein L11 (L11-Cy3) and pept-tRNALys-Cy5 to follow pept-tRNA movement during translocation of pept- and deacylated tRNA. In the slow mode, pept-tRNA is trapped in fluctuations between CHI and P/P states before accommodating in the P site, which occurs during back swivel of the SSU head domain. Decacylated tRNA translocates at normal speed and dissociates from the ribosome before the accommodation of pept-tRNA in the P site is completed. This opens a time window where a single pept-tRNA can reassigned the reading frame according to the thermodynamic potential of the mRNA sequence. Ribosomes favor the fast mode during translocation with wild-type EF-G, but switch to a slow mode during translocation by frameshifting-promoting EF-G mutants, which suggests that a population of ribosomes with distinct kinetic features contributes to the loss of reading frame on a slippery mRNA.

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**Fig. 1** EF-G-induced translocation of pept-tRNA. a Schematic of the smFRET experiment. Pept-tRNA^{Lys-Cy5} (red star) fluctuates between A/A, A/P, and A/P* states in the absence of EF-G. Upon EF-G addition, pept-tRNA moves into a transient CHI state before reaching the P/P state. The movement is monitored by change in FRET between pept-tRNA^{Lys-Cy5} and L11-Cy3 (green star). FRET values are assigned to PRE and POST states in independent experiments (Supplementary Fig. 1) and validated by distance measurements based on cryo-EM structures.19,24,25 b Coding sequences of mRNA constructs. Slippery sequence encodes fMet-Gly-Lys-Phe peptide in 0 frame and fMet-Gly-Lys-Val in -1 frame. Non-slippery mRNA that does not support frameshifting encodes only the 0-frame peptide fMet-Phe-Lys-Phe. c Contour plot showing distribution of FRET values during tRNA translocation on non-slippery mRNA. Trace shows fluctuations between FRET 0.8 (A/A, A/P) and 0.6 (A/P*) followed by rapid transition to FRET 0.2 (P/P). Black line shows the HMM fit of the data here and in all smFRET traces. d Contour plot showing distribution of FRET values during tRNA translocation on non-slippery mRNA. Transitions occur from either A/A and A/P or A/P* to P/P in less than 33 ms. Traces are synchronized to the first transition below FRET 0.5. Histogram at the right shows distribution of FRET values after synchronization. Data are from 5 independent experiments (N = 5). e Representative smFRET time trace showing tRNA translocation on slippery mRNA. Trace shows rapid transition from FRET 0.8 (A/A, A/P) to FRET 0.4 (CHI)23,46 followed by fluctuations between FRET 0.4 and 0.2 before adopting a long-lived FRET 0.2 state (P/P). f Contour plot showing distribution of FRET values during translocation on slippery mRNA. The contour plot contains mixture of trajectories. 81% of traces show direct transitions from A/A, A/P or A/P* to P/P. 19% of traces show transitions from A/A, A/P, or A/P* to CHI followed by fluctuations between CHI and P/P states before transition to long-lived P/P state. Traces are synchronized to the first transition below FRET 0.5. Histogram at the right shows distribution of FRET values after synchronization. Data are from 12 independent experiments (N = 12).

translocation rates ranging between 7 and 30 s⁻¹ at 22 °C.48–50 On the slippery mRNA, most ribosomes (81%) show tRNA trajectories similar to those on non-slippery mRNA. However, on a fraction of ribosomes (19%), the translocation trajectory appears different and pept-tRNA^{Lys-Cy5} transiently samples FRET 0.4 before reaching P/P (Fig. 1c, d). Previously, we and others have shown that FRET 0.4 corresponds to the CHI (ap/P) state where the anticodon stem-loop of the tRNA resides between A and P site on the SSU.23,44–46 During translocation on non-slippery mRNA, the CHI state is too transient to be captured (Fig. 1c, d). On the slippery mRNA, the movement of pept-tRNA^{Lys-Cy5} into CHI is rapid, but the exit from the CHI state is delayed due to enduring fluctuations between the CHI and P/P states before completing translocation (Fig. 1e, f).

We then performed analogous experiments using the slippery mRNA and EF-G mutants carrying single amino acid substitutions of residue Q507 at the tip of domain 4, EF-G(Q507A), EF-G(Q507N), and EF-G(Q507D) (Fig. 2a). Replacements of Q507 promote -1 frameshifting and the frameshifting efficiency depends on the type of amino acid substitution.16 With different mutants, we again find two ribosome populations, one where tRNAs move rapidly from PRE to POST and another in which tRNAs dwell in transitions between CHI and P/P states before reaching the POST state. The percentage of ribosomes with a delayed exit from the CHI state increases in the order EF-G(Q507A) < EF-G(Q507N) < EF-G(Q507D) (Fig. 2b–d). The variation in the fraction of rapidly translocating ribosome population is not due to ribosome binding defects of EF-G.
mutants\(^{16}\). EF-G wt and all Q507 variants stabilize the A/P* state upon binding to PRE prior to translocation (Supplementary Fig. 2a) and the transitions PRE to the CHI state are rapid in all cases \((k_{T1} \geq 5 \text{ s}^{-1}, \text{Fig. 1e})\). The prevalent fluctuations on the slowly translocating ribosomes are between CHI (FRET 0.4) and P/P (FRET 0.2) states (Fig. 2e, Supplementary Fig. 2b, Table 1). The overall translocation rate on ribosomes delayed in the CHI state is low \((\sim 0.2 \text{ s}^{-1})\) and similar with EF-G(wt) and Q507 mutants (Supplementary Fig. 2c, Table 1), which is two orders of magnitude slower compared to ribosomes that transit the CHI state rapidly. Notably, the biochemical frameshifting efficiency (determined in independent experiments by quantitative analysis of peptide products; Supplementary Fig. 3) correlates very well with the slow population among all translocating ribosomes (Fig. 1e, Supplementary Fig. 2b, Table 1). Frameshifting efficiencies were measured at 22 °C to match the conditions of the smFRET experiments. Shown are mean values with error bars representing the standard deviations. Black line indicates a linear fit with the slope of 1.3 ± 0.1, \(R^2 = 0.9982\). Frameshifting efficiencies are from three independent experiments \((N = 3)\). The percentage of traces with CHI states is derived from at least three independent experiments \((N = 12)\).
dequenching of the Cy3 fluorophore (Supplementary Fig. 4c, d) and presenting the 0-frame Phe codon in the A site. When Phe-tRNAPhe-Cy5 binds to POST2 complexes (Supplementary Fig. 5c–d), the accommodated Phe-tRNAPhe rapidly reacts with the pept-tRNA in the P site to form a peptide bond, resulting in a deacylated tRNALys in the P site and a pept-tRNAPhe-Cy5 in the A site. FRET population distribution analysis suggests that pept-tRNAPhe samples A/A, A/P and A/P states (Supplementary Fig. 5d) in a similar manner as seen for pept-tRNALys (Supplementary Fig. 1a–c). PRE2 converts to POST3 complex upon translocation by EF-G(wt) (Supplementary Fig. 4e; in the example shown in Supplementary Fig. 5b, c Cy5 photobleaches result in a stable high Cy3 signal). In contrast, when Val-tRNALys-Cy5 complementary to the −1-frame codon is added to POST2 complexes formed on non-slippery mRNA, we observe only short-lived FRET event (Supplementary Fig. 5e–h), showing Val-tRNALys as it makes multiple unsuccessful attempts to read a near-cognate 0-frame codon51. The dwell time of Val-tRNALys on POST2 complexes is two orders of magnitude lower than the dwell time of cognate Phe-tRNAPhe (k",Valseparable to the 0-frame codon in the A site. When Phe-tRNAPhe samples A/A, A/P and A/P states (Supplementary Fig. 5d) in a similar manner as seen for pept-tRNALys (Supplementary Fig. 1a–c). PRE2 converts to POST3 complex upon translocation by EF-G(wt) (Supplementary Fig. 4e; in the example shown in Supplementary Fig. 5b, c Cy5 photobleaches result in a stable high Cy3 signal). 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The dwell time of Val-tRNALys on POST2 complexes is two orders of magnitude lower than the dwell time of cognate Phe-tRNAPhe 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Fig. 3 Translocation of pept-tRNA<sup>Lys</sup> on slippery mRNA and incorporation of 0- and -1-frame aa-tRNAs. 

a Schematic of translocation on slippery mRNA with EF-G(wt). Pept-tRNA<sup>Lys</sup>-BHQ2 (black circle) moves from the A to the P site. The near-cognate -1-frame Val-tRNA<sup>Val</sup>-Cy5 (red star) samples POST2 complexes without accommodating in the A site. EF-G is added to immobilized PRE1 complexes together with the EF-Tu-GTP-Val-tRNA<sup>Val</sup>-Cy5 complex.

b Representative time trace of pept-tRNALys-BHQ2 movement and subsequent sampling of POST2 complexes by Val-tRNAVal-Cy5 (green label at the right Y-axis). Green labels at the right Y-axis indicate tRNALys conformational states monitored by Cy3 fluorescence, as assigned in Supplementary Fig. 4. Fluorescence, as assigned in Supplementary Fig. 4.

c Zoom-in into showing Cy3 and Cy5 FI and calculated FRET of the initial binding (IB) without accommodation of -1-frame Val-tRNAVal-Cy5 on POST2 complexes.

d Contour plot showing distribution of FRET values after Val-tRNAVal-Cy5 accommodation on POST2 complex, leading to CR and subsequent fluctuations between A/A, A/P and A/P* states.

Zoom-in into showing Cy3 and Cy5 FI and calculated FRET of Val-tRNAVal-Cy5 binding to POST2 complex, leading to CR and subsequent fluctuations between A/A, A/P and A/P* states.

Representative time trace of pept-tRNALys-BHQ2 translocation and incorporation of 0- and -1-frame aa-tRNAs. 

a Pept-tRNA<sup>Lys</sup>-BHQ2 (black circle) moves from the A to the P site. The near-cognate -1-frame Val-tRNA<sup>Val</sup>-Cy5 (red star) samples POST2 complexes without accommodating in the A site. EF-G is added to immobilized PRE1 complexes together with the EF-Tu-GTP-Val-tRNA<sup>Val</sup>-Cy5 complex. Red labels indicate conformational states of tRNAVal-Cy5.

b Representative time trace of pept-tRNALys-BHQ2 movement and subsequent sampling of POST2 complexes by Val-tRNAVal-Cy5 (red star) can bind to its cognate codon in -1-frame. 

c Zoom-in into showing Cy3 and Cy5 FI and calculated FRET of Val-tRNAVal-Cy5 binding to POST2 complex, leading to CR and subsequent fluctuations between A/A, A/P and A/P* states.

Zoom-in into showing Cy3 and Cy5 FI and calculated FRET of Val-tRNAVal-Cy5 binding to POST2 complex, leading to CR and subsequent fluctuations between A/A, A/P and A/P* states.

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e site
tRNALys does not base pair with the protein S13-Cy3 (green star). Translocation is induced by addition of EF-G to immobilized PRE complexes. FRET values corresponding to P, P/E and E states were determined in independent experiments (Supplementary Fig. 5 and 6). Ala-Lys-Lys-Phe in 0 frame and fMet-Ala-Lys-Lys-Val in EF-G(wt) showing step-wise transition from FRET 0.9 (P/P) to 0.6 (P/E) to 0.3 (E site) to 0.0 (dissociation).

Fig. 9 Translocation trajectory of deacylated tRNA. a Schematic of smFRET experiment monitoring movement of tRNA195-Cy5 (Cy5; red star) relative to protein S13-Cy3 (green star). Translocation is induced by addition of EF-G to immobilized PRE complexes. FRET values corresponding to P, P/E and E states were determined in independent experiments (Supplementary Fig. 5 and 6). b Coding sequences of mRNA constructs. Slippery sequence encodes fMet-Ala-Lys-Lys-Phe in 0 frame and fMet-Ala-Lys-Lys-Val in –1 frame. Non-slippery mRNA encodes only 0-frame fMet-Ala-Lys-Lys-Phe peptide because tRNA195 does not base pair with the –1-frame GAA. c Representative smFRET time trace of tRNA translocation on non-slippery mRNA in the presence of EF-G(wt) showing step-wise transition from FRET 0.9 (P/P) to 0.6 (P/E) to 0.3 (E site) to 0.0 (dissociation). d Contour plot showing distribution of FRET efficiencies during translocation on non-slippery mRNA by EF-G(wt). Transitions occur either from FRET 0.9 (P site) or 0.6 (P/E) to FRET 0.3 (E site). Traces are synchronized to the first transition below FRET 0.5. c Histogram at the right shows distribution of FRET values after synchronization. Inset shows rates and curve fits of tRNA dissociation from E site on slippery (closed circles) and non-slippery (open circles) mRNA (Supplementary Fig. 6b and Table 2). Normalization was performed by division by the number of transitions (n). Data are from four independent experiments (N = 4). e Representative smFRET time trace of tRNA translocation on slippery mRNA in the presence of EF-G(Q507D) showing step-wise transition from FRET 0.9 (P/P) to 0.6 (P/E) to 0.3 (E site) to 0.0 (dissociation), similar to EF-G(wt) (c). f Contour plot showing the distribution of FRET efficiencies during translocation on slippery mRNA by EF-G(Q507D). Transitions occur either from FRET 0.9 (P site) or 0.6 (P/E) to FRET 0.3 (E site). Inset shows rates and curve fits of tRNA dissociation from E site on slippery (closed circles) and non-slippery (open circles) mRNA. Normalization was performed by division by the number of transitions (n) (Supplementary Fig. 6c and Table 2). Data are from three independent experiments (N = 3).

FRET 0.3 state followed by the loss of FRET (Fig. 4c, d). We assign FRET 0.3 to the transient occupancy of the E site by deacylated tRNA; the subsequent transition to FRET 0 reflects the dissociation from the ribosome (Fig. 4a, Supplementary Fig. 9a). The translocation trajectory shows no apparent CHI state for the P-site tRNA. The overall reaction of tRNA translocation and dissociation is rapid (koff = 1 s−1, Fig. 3b–d, Table 2), in agreement with previous reports.38,41 Surprisingly, we observed the same translocation pattern on slippery and non-slippery mRNA with rapid transition of tRNA195-Cy5 to the E site followed by dissociation from the ribosome (Fig. 4d inset, Supplementary Fig. 9b, d, Table 2). Moreover, translocation with EF-G(wt) is not different from that with EF-G(Q507D), the mutant promoting the highest frameshifting efficiency and resulting in the lowest ensemble pept-tRNA translocation rate.16,17 (Fig. 4c, f, Supplementary Fig. 9c, d, Table 2). In all cases, translocation of deacylated tRNA is fast, directional, and irreversible. Notably, on ribosomes where pept-tRNA translocates slowly, translation and subsequent dissociation of deacylated tRNA is much faster than the movement of pept-tRNA (Tables 1 and 2), i.e., translocation of pept-tRNA from the A to the P site is not yet completed when deacylated tRNA has already cleared the E site. This indicates that movements of pept-tRNA are uncoupled from the displacement of deacylated tRNA on the fraction of ribosomes that undergo frameshifting, allowing pept-tRNA to more easily access the –1-frame codon after the E-site tRNA release.

Identification of key transitions leading to frameshifting. To further narrow down the timing of spontaneous –1
Table 2 Kinetics of deacylated trNA\textsuperscript{Lys} dissociation from the E site.

| EF-G       | Dissociation rate from E site, s\textsuperscript{−1} (N)\textsuperscript{a,b} | A AAA AAG (slippery) |
|------------|--------------------------------------------------------------------------------|----------------------|
| wt         | 0.9 ± 0.1 (196)                                                                | 1.0 ± 0.1 (169)      |
| Q507D      | 0.6 ± 0.1 (175)                                                                | 0.5 ± 0.1 (152)      |
| wt-GTPyS   | 0.2 ± 0.1 (205)                                                                | 0.3 ± 0.1 (173)      |
| wt-Spc     | n.d.                                                                            | 0.3 ± 0.1 (157)      |
| wt-fA      | <0.1 ± 0.1\textsuperscript{c} (169)                                             | n.d.                 |

\textsuperscript{a}N number of traces.
\textsuperscript{b}All rates were corrected for photobleaching of the FRET dyes (Methods) and are shown as mean±s.d. from at least three independent experiments. \textsuperscript{c}Rate was limited by the photobleaching of the FRET dyes.

Frameshifting, we studied translocation on slippery mRNA in the presence of EF-G(wt) and GTPyS, a slowly hydrolyzable GTP analog that binds to translational GTPases with similar affinity and orientation as GTP\textsuperscript{52}. Ensemble translocation rates are reduced by ~30-fold by replacing GTP with GTPyS, because tRNAs are trapped at the early steps of translocation (Fig. 5a).\textsuperscript{38,39,40} Although translocation rates with EF-G(wt)–GTPyS and EF-G(Q507D)–GTP are similar\textsuperscript{16,17}, the frameshifting efficiency on slippery mRNA is much lower than with EF-G(Q507D)–GTP, and is similar to that with EF-G(wt)–GTP (Fig. 5a). This result indicates that slow translocation alone does not explain the high frameshifting efficiency, which prompted us to analyze the pept-tRNA translocation trajectories in the presence of GTPyS (Fig. 5b–h). GTPyS did not affect the ability of EF-G to stabilize pept-tRNA in the A/P* state (Supplementary Fig. 10a). The majority of traces (73%) shows slow translocation while sampling the FRET 0.4 state (Fig. 5b, c, Table 1), which may be an authentic CHI or a CHI-like off-pathway state. Pept-tRNA fluctuates predominantly between A/P* and CHI, rather than between CHI and P/P states as observed with EF-G–GTP on slippery mRNA on the fraction of slow ribosomes (Fig. 5d, Supplementary Fig. 10b). The overall pept-tRNA translocation rate on a slippery mRNA is ~0.2 s\textsuperscript{−1} (Table 1), similar to that measured with GTPyS on a non-slippery sequence, 0.17 s\textsuperscript{−1}\textsuperscript{2}. Notably, also translocation of deacylated tRNA\textsuperscript{Lys}–GTPyS is slow (0.2–0.3 s\textsuperscript{−1}; Fig. 5e, f, Supplementary Fig. 10c, Tables 1 and 2), indicating synchronous displacement of the two tRNAs in the presence of EF-G–GTPyS. Deacylated tRNA dwells mostly in the E site, which may prevent the pept-tRNA from sampling the −1 frame codon and would explain why the reading frame is maintained.

We then used the antibiotic spectinomycin (Spc) as an alternative method to stall translocation at an early stage (Fig. 5a, Supplementary Fig. 11).\textsuperscript{24,41} Spc binds to h34 of 16 S rRNA connecting the head and the body domain of the SSU\textsuperscript{24,35}. Our smFRET translocation experiments show that the effect of Spc is similar to that of EF-G–GTPyS: pept-tRNA fluctuates between A/P* and a CHI-like state, whereas the dwell time of deacylated tRNA in the E site is prolonged; both tRNAs do not proceed to POST states and the efficiency of −1 frameshifting is low (Fig. 5a, Supplementary Fig. 11). These results suggest that although Spc binding to the ribosome does not affect GTP hydrolysis by EF-G, Spc blocks the conformational rearrangements that couple GTP hydrolysis and Pi release to tRNA movement. With respect to the mechanism of −1 frameshifting, these findings indicate that spontaneous ribosome slippage occurs with pept-tRNA in CHI state or attempting to move from CHI to the POST state, when deacylated tRNA has been released from the ribosome.

Movements of SSU head domain during translocation on slippery mRNA. To test the potential effect of ribosome dynamics, we monitored the SSU head domain movements using a validated FRET pair with labels on the SSU protein S13 (S13-Cy3) and the LSU protein L33 (L33-Cy5) (Fig. 6a).\textsuperscript{38,48} On PRE complexes in the absence of EF-G, we observe two inter-converting states with FRET 0.5 and 0.8, respectively, corresponding to non-rotated (N) and rotated–swiveled (S) states of the SSU head domain (Supplementary Fig. 12a–d). POST complexes are predominantly in the FRET 0.5 (N) state (Supplementary Fig. 12e–g). After the addition of EF-G to PRE complexes, the majority of ribosomes transiently populate the S state before adopting a long-lived N (POST) state (85% on non-slippery and 87% on slippery mRNA; Fig. 6b, Supplementary Fig. 13a). Notably, back swiveling of the SSU head domain in the presence of EF-G occurs at a rate similar to that on PRE complexes in the absence of EF-G (Supplementary Figs. 12d, 13b and Table 3), suggesting that back swiveling may occur spontaneously after EF-G dissociation from the ribosome. The transition rates from the S to long-lived N state are similar on non-slippery and slippery mRNA in the presence of EF-G(wt) (Supplementary Fig. 13b and Table 3) and agree with rates measured in previous ensemble and smFRET experiments.\textsuperscript{16,17,38,41,43} The minor population of complexes (15% on non-slippery mRNA and 13% on slippery mRNA) stayed in the S state and did not reach the N state during the time of the experiment.

When we induced translocation on slippery mRNA by EF-G(Q507D), the ratio of ribosomes showing fast and slow back swiveling is inversely correlated (13% fast and 87% slow, Fig. 6b, d). Notably, similar fractions of ribosomes show slow backward swiveling (Fig. 6d) and slow pept-tRNA translocation (Fig. 2d). The rate of SSU head domain closure (<0.1 s\textsuperscript{−1}, Supplementary Fig. 13c and Table 3) is lower than that of pept-tRNA translocation (0.3 s\textsuperscript{−1}, Table 1) or the dissociation of deacylated tRNA from the ribosome (0.6 s\textsuperscript{−1}, Table 2), suggesting that pept-tRNA continues to fluctuate between CHI and P/P states because the SSU remains in a swiveled conformation, while deacylated tRNA dissociates from the ribosome. It is important to note that, in contrast to the swiveling motion of the SSU head domain, the rotational movement of the SSU body domain with respect to the LSU most likely does not contribute appreciably to spontaneous frameshifting, as both forward and backward SSU body rotation precede the formation of the CHI state.\textsuperscript{19,25,38,56} In summary, our data show that slow translocation by EF-G(Q507D) stalls the SSU head domain in the swiveled state which renders ribosomes susceptible to spontaneous frameshifting due to delayed re-locking of pept-tRNA in the P site.

Discussion

Choreography of translocation on a slippery mRNA. The present data show how slippery sequences affect the choreography of translocation and how this leads to frameshifting. While on the non-slippery mRNA all ribosomes behave in a quasi-uniform way (within the time resolution of TIRF experiments) and translocate rapidly, on a slippery mRNA we identify two distinct ribosome populations, one that translocates rapidly, and another that is slow in completing pept-tRNA translocation (Fig. 7a, b). The fraction of such slow ribosomes in the population correlates with the frameshifting efficiency. On slow ribosomes, both tRNAs rapidly move from the A/P or A/P* and P/E into their respective CHI states. Decacylated tRNA is then rapidly released from the ribosome. Normally, pept-tRNA becomes locked in the P site upon backward movement of the SSU head domain. On the slippery sequence, the locking is delayed and pept-tRNA fluctuates between CHI and P/P states. If base pairing of the pept-
tRNA anticodon with the −1-frame codon is favored over the 0-frame codon, as is often the case with slippery sequences, these continuing fluctuations provide the time window for pept-tRNA to switch to the −1-frame, followed by incorporation of the −1-frame tRNA.

Residues at the tip of EF-G domain 4, in particular Q507, are important for reading frame maintenance. Recent cryo-EM structure suggests that in the A/P or A/P* state, EF-G domain 4 is flexible and most probably not involved in the stabilization of the codon-anticodon complex in the A site. The codon-anticodon duplex is supported by the interactions with the residues of 16S rRNA h44 in the SSU body domain. Upon moving to the CHI state, the contacts with the SSU body domain are disrupted and tRNA anticodon has a propensity to disconnect from its 0-frame codon. EF-G residues at the tip of domain 4 stabilize the codon-anticodon duplex in its correct geometry, thereby contributing to the reading frame maintenance.

The timing of events during spontaneous frameshifting shows similarities but also differences to −1 programmed ribosome frameshifting (−1PRF). A hallmark of −1PRF is the slowing down of translation at the slippery site facilitated by mRNA secondary structures, such as downstream stem loops or pseudoknots. During stalling, the SSU head domain is swiveled and the SSU body is rotated with respect to the LSU, suggesting a key role of SSU dynamics both in programmed and spontaneous −1 frameshifting. Single-molecule studies of −1PRF

Fig. 5 Translocation on slippery mRNA with EF-G(wt)—GTPγS. a Correlation between frameshifting and ensemble translocation rate. Frameshifting was measured at 37 °C, data are presented as mean ± s.d. from three independent experiments (N = 3). Translocation rates of EF-G(wt) and Q507 mutants are from ref. 16, of GTPγS (brown) from ref. 54, and of Spc (orange) from 31. b Above: schematic of smFRET experiment monitoring movement of pept-tRNA15S-Cy5 (red stars) relative to L11-Cy3 (green star) by EF-G(wt)—GTPγS (brown hexagon) binding to immobilized PRE complexes. Below: representative smFRET trace of pept-tRNA translocation by EF-G(wt)—GTPγS with fluctuations between FRET 0.8 (A/A, A/P) and 0.6 (A/P*) followed by fluctuations between FRET 0.4 (CHI) and 0.6. c Contour plot showing the distribution of FRET values during translocation of pept-tRNA on slippery mRNA by EF-G(wt)—GTPγS. Traces are synchronized to the first transition below FRET 0.5. Histogram at the right shows the distribution of FRET values after synchronization. Data are from six independent experiments (N = 6). d Transition frequencies between FRET states during pept-tRNA translocation on slippery mRNA by EF-G(wt)—GTPγS (Supplementary Fig. 7b and Table 1) and in the presence of Spc (Table 1). Data are presented as mean ± s.d. from 6 (N = 6, EF-G(wt)—GTPγS), or 3 (N = 3, Spc) independent experiments. e Above: schematic of smFRET experiment with tRNA15S-Cy5 (red star) moving relative to protein 513-Cy3 (green star) during translocation induced by addition of EF-G(wt)—GTPγS to immobilized PRE complexes. Below: representative smFRET trace of translocation by EF-G(wt)—GTPγS with step-wise transition from FRET 0.9 (P/P) to 0.6 (P/E) to 0.3 (E) to 0.0 (dissociation). f Contour plot showing the distribution of FRET values during translocation of pept-tRNA on slippery mRNA by EF-G(wt)—GTPγS. Inset shows rates and curve fit of tRNA dissociation from E site on slippery (closed circles) and non-slippery (open circles) mRNA (Supplementary Fig. 7c and Table 2). Normalization was performed by division by the number of transitions (n). Data are from 3 independent experiments (N = 3).
on *E. coli* dnaX mRNA using optical tweezers showed that stalled ribosomes make multiple translocation attempts sampling sequences upstream or downstream of the 0 frame61. In our study, fluctuations of pept-tRNA between CHI and P/P states allow ribosomes to explore alternative reading frames and eventually re-equilibrate in a reading frame that is thermodynamically favored before resuming translation. The difference between the spontaneous slippage and –1PRF concerns the timing of deacylated tRNA release, which is rapid during spontaneous frameshifting (this paper and ref. 16), but slow in the two examples of –1PRF where this was studied58,62. Thus, –1-frameshifting can occur by different mechanisms with one (during spontaneous frameshifting) or two (during programmed frameshifting) tRNAs bound, provided pept-tRNA is trapped in fluctuations between CHI and P/P.

Tetrameric slippery mRNA sequences such as CC[C/U]-[C/U] can induce ribosome frameshifting in +1-direction when tRNAs either lack post-transcriptional modifications or contain

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**Fig. 6 SSU head domain swiveling during translocation on slippery mRNA.** 

a Schematic of smFRET experiment. Movement of SSU head domain during translocation induced by addition of EF-G to immobilized PRE complexes monitored with FRET labels on ribosomal proteins S13 (S13-Cy3, green star) and L33 (L33-Cy5, red star). FRET values representing the non-swiveled (N) and swiveled (S) states are determined in independent experiments (Supplementary Fig. 9 and Table 3). 
b Representative smFRET time traces for SSU head movements during EF-G(wt)-induced translocation. The majority of traces (upper panel) show fluctuations between FRET 0.8 (S) and 0.5 (N) followed by a stable FRET 0.5 (N) state after translocation. 13% of traces (lower panel) show no transition to the N state in the time course of the experiment. Pie charts indicate the percentage of traces ending in N (white) or S (gray) state. Black vertical lines represent the synchronization point, i.e., the last transition to FRET 0.8 (S) (see below).
c Contour plot showing distribution of FRET values representing SSU head movement during translocation on slippery mRNA by EF-G(wt). Traces were synchronized to the last transition to FRET 0.8 (S). The duration of the last FRET 0.8 state is an estimate for the duration of translocation, because back swiveling occurs simultaneously with the dissociation of EF-G from the ribosome after translocation38. Histogram at the right shows distribution of FRET values after synchronization. Data are from four independent experiments (*N* = 4). 
d Representative smFRET time traces for SSU head domain movement during translocation on slippery mRNA with EF-G(Q507D). A small fraction of traces (upper panel) show fluctuations between FRET 0.8 (S) and 0.5 (N) and end in a stable FRET 0.5 (N) state after translocation. 87% of traces (lower panel) show no transition to the N state in the time course of the experiment. 
e Contour plot showing distribution of FRET values representing SSU head domain movement during translocation on slippery mRNA by EF-G(Q507D). Histogram at the right shows the distribution of FRET values after synchronization. Data are from three independent experiments (*N* = 3).
nucleotide insertions in their anticodon loops\textsuperscript{64–66}. SufB2, a +1-frameshifting-prone tRNA mutant containing a G37a insertion in the anticodon loop of yeast ProL tRNA\textsubscript{Pro}, uses triplet codon-anticodon-pairing in 0 frame, but shifts into the +1 frame in the process of translocation. smFRET experiments using L1 stalk movement as readout for translocation show that the transition of PRE to the POST state is much slower with SufB2 than with a canonical tRNA\textsubscript{Pro} in the A site\textsuperscript{66}. This suggests that slow tRNA movement correlates with +1 frameshifting, and hence slow translocation may be a hallmark for +1 or −1 frameshifting. It is not known which step of SufB2 translocation is slowed down, but cryo-EM suggests that the A-site tRNA shifts into the +1-frame soon after binding of EF-G–GDPCP\textsuperscript{65}. If GDPCP stalls translocation at the same stage as GTP\textsubscript{γS} (Fig. 5 and refs. 23,38,41), this would suggest that +1 frameshifting occurs early on the translocation pathway before CHI state formation, which would be clearly different to −1 frameshifting that occurs in the late phase of translocation after CHI state formation and during stabilization of the POST state.

**Slow gears of translocation.** Our results describe two alternative translocation pathways, one that ensures rapid coordinated movement of tRNAs in the correct reading frame, and the other which is slow and prone to −1 frameshifting (Fig. 7b). Recent force and fluorescence measurements suggested that ribosomes

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**Table 3 Kinetics of SSU head swiveling.**

| EF-G       | \(k_{S-N}\) s\(^{-1}\) (n)\(^a\),\(^b\) | G UUU AAG (non-slippy) | G GGA AAG (slippery) |
|------------|--------------------------------|------------------------|----------------------|
| no EF-G    | n.d.                             | 2.5 ± 0.2 (1975)        |                      |
| wt         | 2.2 ± 0.3 (149)                  | 1.9 ± 0.1 (124)         |                      |
| Q507D      | n.d.                             | <0.1 ± 0.3 (151)        |                      |

\(^a\) Number of transitions.

\(^b\) Rates are shown as mean ± s.d. from at least three independent experiments. \(R^2 ≥ 0.99\) in all cases.

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**Fig. 7 Translocation trajectories correlating with frameshifting.**

- **a** Pie charts comparing the frameshifting efficiency (gray) with the distribution of translocation rates for pept-tRNA (magenta) and deacylated tRNA (blue) and SSU head domain back rotation (green) on non-slippery and slippery mRNA with EF-G(wt) and EF-G(Q507D).
- **b** Kinetic model of translocation on slippery mRNA. Majority of ribosomes translocate in fast mode with tRNAs moving synchronously to the POST state; back swiveling of the SSU head domain completes translocation. A fraction of ribosomes translocates in slow mode where pept-tRNA is trapped fluctuating between CHI and P/P instead of moving to the POST state. Decacylated tRNA translocates rapidly and dissociates from the E site allowing pept-tRNA to sample 0- and −1-frame codons. SSU head closure is delayed due to prolonged fluctuations of pept-tRNA. Rates of the elemental reactions for pept-tRNA are indicated.

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can operate in two alternative (fast and slow) gears during translation in response to the mechanical barrier of an mRNA hairpin\(^{23}\). In this slow gear, unwinding of the hairpin occurred during forward rotation of the SSU head domain suggesting that delays in steps preceding tRNA movement into the CHI state were caused by the ribosome moving along the mRNA, but also in more subtle ways that may link specific mRNA sequences with alternative translocation outcomes. The structural basis as to why the ribosomes switch to the slow gear and the physiological importance of this switch for biological processes, such as the nascent protein folding or maintenance of the mRNA stability by ribosome loading, remains to be elucidated in future work.

**Methods**

All experiments were carried out in TAKM-Δ (50 mM Tris- HCl, pH 7.5 at 37°C, 70 mM NH₄Cl, 30 mM KCl, and 7 mM MgCl₂) at 22°C, unless stated otherwise.

Ribosomes from *E. coli*, 6-14HMet-tRNA\(^{15}\)Glu-tRNA\(^{13}\)Gly-tRNA\(^{11}\)Ala-tRNA\(^{18}\), Lys-tRNA\(^{19}\), Phe-tRNA\(^{20}\), Val-tRNA\(^{21}\), 14Clys-tRNA\(^{14}\)Cy5, initiation factors, and EF-Tu were prepared as described\(^{23}\). Ribosomes lacking 30 S and in the presence of the antibiotic Spc, but these fluctuations do not increase frameshifting. Thus, it appears that there are different ways for the ribosome to switch to, but frameshifting is promoted only at a particular step when pept-tRNA transits from CHI to P/P (Fig. 7b).

Our experiments show that at given translocation conditions, some ribosomes in a population take a fast route, whereas others switch into a slow mode. Previous molecular dynamics simulations revealed almost 500,000,000 possible kinetic sequences of intermediates during translocation and calculated the favorable route for tRNA movement\(^{23}\). Apparently, specific mRNA sequences can set the course for ribosomes to deviate from the designated route and change into alternative pathways (67 and our study). Thus, the sequence of the mRNA appears to modulate the pace of translation not only by posing a steric hindrance for the ribosome moving along the mRNA, but also in more subtle ways that may link specific mRNA sequences with alternative translocation outcomes. The structural basis as to why the ribosomes switch to the slow gear and the physiological importance of this switch for biological processes, such as the nascent protein folding or maintenance of the mRNA stability by ribosome loading, remains to be elucidated in future work.

**Frameshifting assay.** To form initiation complex (IC), 70S ribosomes were incubated with a 3-fold excess of mRNA, initiation factors and [14C]Met-tRNA\(^{15}\)Met and added to the 30 S complexes with 50 mM Tris- HCl, 70 mM NH₄Cl, 30 mM KCl, and 7 mM MgCl₂ for 30 min at 37°C. 70 S complexes were reconstituted through a 1.1 M sucrose cushion in TAKM buffer (50 mM tris-HCl pH 7.5, 70 mM NH₄Cl, 30 mM KCl, and 21 mM MgCl₂). Pellets were dissolved in TAKM buffer and the concentration was determined by absorption at 260 nm.

Translation was carried out by incubating purified *E. coli* tRNAs\(^{15}\)Glu, with a 100-fold excess of Cy5- or BHQ2-succinimidylster (GE Healthcare) dissolved in DMSO, and additionally by HPLC\(^{25,27}\).
generate the PRE complex carrying mFGK-tRNA\(^{35S}\)-Cy5 or mFKF-tRNA\(^{35S}\)-Cy5 in the A site and tRNA\(^{ Ala}\) or tRNA\(^{ Pho}\) in the P site. The same procedure was followed to prepare PRE complexes with mFGK-tRNA\(^{35S}\)-BHQ2 or mFKF-tRNA\(^{35S}\)-BHQ2. The PRE complex was immobilized in the coverslip as described below.

To prepare POST complexes carrying S13-Cy3 and mGK-tRNA\(^{35S}\)-Cy5 or mFKF-tRNA\(^{35S}\)-BHQ2 or mFKF-tRNA\(^{35S}\)-Pho in the P site, 30 S S13-Cy3 (2 μM) were incubated in TAKM\(_20\) at 37 °C for 30 min and used to form initiation complexes as described above (50 S-L33-Cy5 in 1.5-fold excess over 30 S, IFs, mRNA, and [\(^{35}\)P]Met-tRNA\(^{35S}\)) in 3-fold excess over 30 S). Ternary complexes were prepared as above using [\(^{35}\)C]Ala-tRNA\(^{35S}\)-Cy5 (10 μM), (1 μM), TC (5 μM) and EF–G–GTP (1 μM) were mixed together and incubated for 5 min at 37 °C. The POST complexes were purified by centrifugation through a 1.1 M sucrose cushion in TAKM\(_{20}\) (0.5 μM) added and incubated for 5 min at RT to form the PRE complex carrying mFGK-tRNA\(^{35S}\) in the A site and tRNA\(^{35S}\)-Cy5 in the P site.

To prepare POST complexes carrying S13-Cy3/L33-Cy5 and mGK-tRNA\(^{35S}\) or mFKF-tRNA\(^{35S}\)-BHQ2 or mFKF-tRNA\(^{35S}\)-Pho in the P site, 30 S S13-Cy3 were incubated in TAKM\(_{20}\) at 37 °C for 30 min and used to form initiation complexes as described above (50 S-L33-Cy5 in 1.5-fold excess over 30 S). Ternary complexes were prepared as above using [\(^{35}\)C]Gly-tRNA\(^{35S}\) or [\(^{35}\)C]Phe-tRNA\(^{35S}\). (1 μM), TC (5 μM), and EF–G–GTP (1 μM) were mixed together and incubated for 5 min at 37 °C. Post complexes were purified by centrifugation through a 1.1 M sucrose cushion in TAKM\(_{20}\), dissolved in TAKM, and the concentration was determined by [\(^{35}\)C]Ala radioactivity scintillation counting. POST complex (0.1 μM) was then mixed with a 3-fold excess of EF–Tu–GTP–Lys-tRNA\(^{35S}\)-Cy5, incubated for 5 min at 37 °C to generate the PRE IMAK complex, which was immobilized on the coverslip. Excess TC was removed by buffer exchange (10 volumes) with washing buffer. Subsequently, washing buffer containing EF–G–GTP (0.5 μM) was added and incubated for 5 min at RT to generate POST IMAK complex. Next, 0.3 μM unlabeled EF–Tu–GTP–Lys-tRNA\(^{35S}\) was added and incubated for 5 min at RT to form the PRE complex carrying IMAKK-tRNA\(^{35S}\)-Cy5 in the A site and tRNA\(^{35S}\)-Cy5 in the P site.

For all experiments, movies were recorded at a rate of 30.3 frames per second (33 ms per frame). Movies were recorded at a rate of 1 frame per second (100 ms per frame) in the experiment using S13-L33 FRET and EF–G–GQ (S057D).

Data analysis. Fluorescence time courses for donor (Cy3) and acceptor (Cy5) were extracted using custom-made Matlab (MathWorks) software according to published protocols. A semi-automated algorithm (Matlab) was used to select anti-correlated fluorescence traces exhibiting characteristic single fluorophore intensities. The bleed-through of Cy3 signal into the Cy5 channel was corrected using an experimentally determined coefficient of 0.13. The FRET efficiency was defined as the ratio of the measured emission fluorescence intensities, \(I_{\text{Cy3}}/ (I_{\text{Cy5}} + I_{\text{FRET}})\). Trajectories were truncated to remove photobleaching and photoblinking events. The set of all FRET traces for a given complex was compiled in a histogram, which was fitted to a sum of Gaussian functions. Matlab code using an unconstrained nonlinear minimization procedure (fminsearch, Matlab, R2011b) yields mean values and s.d. for the distribution of FRET states. Two-dimensional contour plots were generated from raw time-resolved FRET trajectories using a custom-made software. TAFRET trajectories were fitted by Hidden Markov model using the vbFRET software package (http://vbFRET.sourceforge.net) to generate the idealized trajectories revealing the number, sequence, and duration of FRET states. FRET changes in idealized trajectories that were smaller than the s.d. of the Gaussian distribution of the FRET states were not considered transitions because they could not be not distinguished from the noise. Dwell times of different FRET states were calculated from idealized trajectories. The dwell time distribution was fitted to an exponential function, \(y = y_0 + Ae^{-t/τ}\). The observed rates were corrected for the photobleaching of the Cy3 and Cy5 dyes and for the observation time according to \(k_{\text{obs,corrected}} = k_{\text{photobleach}} \times k_{\text{photobleach}} \times k_{\text{photobleach}}^{-1}/T\), where \(T\) observation time (33 s for most experiments, 100 s for S13-L33/EF–G–GQ (S057D) experiment). \(k_{\text{photobleach}} = 0.03 ± 0.01 s^{-1}\) for the experiments with EF–G. FRET traces were synchronized relative to the first transition to FRET ≤ 0.1 than the lowest value of the PRE state in the absence of EF–G, if not stated otherwise. To calculate translocation rates, the time distribution between the synchronization point and the last transition to the PRE state was fitted to an exponential function \(y = y_0 + Ae^{-t/τ}\). This part of the traces was also used to quantify the transition frequency, i.e., the number of transition between different states divided by the total number of transitions. GraphPad prism 8 software was used for the representation of smFRET data and fits of the data.

Coverslip preparation. Coverslips and objective slides were sonicated in 1 M KOH (em-CCD) camera (CCD-C9100-13, Hamamatsu, Japan). Color channels were assigned using HQ 605/40, HQ 680/30 (Chroma Technology). For most experiments, microscope slides were coated with double-sided sticky tape (em-CCD) camera (CCD-C9100-13, Hamamatsu, Japan). Coverslips and objective slides were sonicated in 1 M KOH for 5 min to immobilize the ribosomes on the surface through the hydroxyl group. Coverslips were incubated in TAKM\(_20\) at 37 °C for 30 min and used to form initiation complexes as described above (50 S-L33-Cy5 in 1.5-fold excess over 30 S). Ternary complexes were prepared as above using [\(^{35}\)C]Gly-tRNA\(^{35S}\) or [\(^{35}\)C]Phe-tRNA\(^{35S}\). (1 μM), TC (5 μM), and EF–G–GTP (1 μM) were mixed together and incubated for 5 min at 37 °C. The POST complex was purified by centrifugation through a 1.1 M sucrose cushion in TAKM\(_{20}\), dissolved in TAKM, and the concentration was determined by [\(^{35}\)C]Gly or [\(^{35}\)C]Phe radioactivity scintillation counting, respectively. POST complex (0.1 μM) was then mixed with a threefold excess of EF–Tu–Lys-tRNA\(^{35S}\)-GTP and incubated at 37 °C for 5 min to generate the PRE complex carrying mFGK-tRNA\(^{35S}\)-BHQ2 or mFKF-tRNA\(^{35S}\)-BHQ2 or mFKF-tRNA\(^{35S}\)-Pho in the A site and tRNA\(^{35S}\)-Pho in the P site. The PRE complex was immobilized in the coverslip as described below.

Sample preparation and TIRF microscopy. Purified ribosome complexes (0.1 μM) were diluted to 1 nM in buffer (TAKM\(_{20}\) complemented with 8 mM MOPS, 100 mM KCl, and 1 mM MgCl\(_2\)) and 1 mM nCitrulline (Thermo Scientific) for 5 min. Neuraminidase was removed by washing the flow chamber with 5-fold volume excess of the same buffer containing 1 mg/ml BSA. Ribosome complexes were added to the flow chamber and incubated for 5 min to immobilize the ribosomes on the surface through the mRiNA–bionubin–neuraminid interaction. Images were recorded after the addition of imaging buffer to the sample (same buffer with 2.5 mM protocatechuic acid, 50 mM protocatechuate-3,4-dioxygenase (Pseudomonas– Sigma-Aldrich), 2 mM Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) and 1 mM methylvio-logen (Sigma-Aldrich)). Cy3 and Cy5 fluorescence time courses during translo- cation were obtained by adding 0.1 μM EF–G and 1 mM GTP or GDP–F to the imaging buffer, which was added to PRE complexes immobilized on the PEG–Biotin-coated coverslips approximately 10 s before imaging. In the experiment with tRNA\(^{35S}\)-BHQ2, the imaging buffer was additionally complemented with 10 nM TC containing either Phe-tRNA\(^{35S}\)-Cy5 or Val-tRNA\(^{35S}\)-Cy5. In the experiment with FICyt3, FICy5, FICyt3 + FICy5, FICyt3 + FICy5 +stopped codon readthrough by the host restriction factor shiftless. Viruses 13, 1231 (2021).

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Author contributions

P.F. and A.P. performed experiments and data analysis. P.P., M.V.R., and S.A. designed experiments, interpreted the data, and wrote the paper.

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