Structural basis for +1 ribosomal frameshifting during EF-G-catalyzed translocation

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Frameshifting of mRNA during translation provides a strategy to expand the coding repertoire of cells and viruses. How and where in the elongation cycle +1-frameshifting occurs remains poorly understood. We describe seven ~3.5-Å-resolution cryo-EM structures of 70S ribosome complexes, allowing visualization of elongation and translocation by the GTPase elongation factor G (EF-G). Four structures with a +1-frameshifting-prone mRNA reveal that frameshifting takes place during translocation of tRNA and mRNA. Prior to EF-G binding, the pre-translocation complex features an in-frame tRNA-mRNA pairing in the A site. In the partially translocated structure with EF-G–GDPCP, the tRNA shifts to the +1-frame near the P site, rendering the freed mRNA base to bulge between the P and E sites and to stack on the 16S rRNA nucleotide G926. The ribosome remains frameshifted in the nearly post-translocation state. Our findings demonstrate that the ribosome and EF-G cooperate to induce +1 frameshifting during tRNA-mRNA translocation.
To accurately synthesize a protein, the ribosome maintains the mRNA reading frame by decoding and translocating one triplet codon at a time. Concurrent ~25 Å movement of the mRNA and tRNAs is catalyzed by the conserved translational GTPase EF-G in bacteria (EF2 in archaea and eukaryotes). After formation of a peptide bond, the peptidyl-tRNA, and decylated tRNA move from the A and P sites to the P and E sites, respectively. This translocation requires spontaneous and large-scale (~10°) inter-subunit rotation of the ribosome. Despite pronounced rearrangements of subunits and extensive motions of tRNA and mRNA at each elongation cycle, the ribosome maintains the correct reading frame through hundreds of codons.

Nevertheless, change of the reading frame, termed frameshifting, is common in viruses, bacteria, and eukaryotes, where it enables expansion of the coding repertoire and regulation of gene expression. During frameshifting, the translating ribosome switches to an alternative reading frame, either in the forward (+) or reverse (−) direction, i.e., skipping or re-reading one or more mRNA nucleotides, respectively. This work focuses on +1 frameshifting (+1FS), which is important for gene expression in various organisms. For example, +1FS controls the expression of the essential release factor 2 in bacteria, regulates metabolite-dependent enzyme expression, and leads to pathological expression of huntingtin in eukaryotes. +1FS can be amplified by dysregulation of ribosome quality control mechanisms, and/or oxyacetyl uridine (cmo5U34) and/or visualizing the possibility of rearrangements of a frameshifting complex at all slippage. Yet, the dynamics of the ribosome allow sampling of tRNA from an in-frame position at the A site to the position in the P site; and (3) stalling of the tRNA in the P site during decoding of a slippery sequence when the tRNA binds to the anticodon loop of tRNAs, exceeding the average frequency of spontaneous frameshifting at other sequences by two orders of magnitude. In vitro, mRNA CC[C/U]-N (N = A, C, G, U) sequences are even more prone to +1FS, achieving 70% efficiency. The CC[C/U]-N sequences code for proline (Pro) and are decoded by two isoacceptors of tRNA Pro in E. coli. The isoacceptor tRNA Pro (UGG), encoded by ProM, is essential for cell growth due to its ability to read all four Pro codons. It is highly prone to +1FS upon loss of the post-transcriptionally modified nucleotides 5-oxyacetyl uridine 34 (cmo5U34) and/or N1-methylation of guanosine 37 (m1G37). The isoacceptor tRNA Pro (UGG), encoded by ProL, is cognate to the CC[C/U] codon of the slippery motif, where +1FS can be induced upon loss of m1G37 and/or of the elongation factor EF-P.

Studies have proposed that +1FS by tRNA Pro (UGG) can occur during one of the three stages of the elongation cycle: (1) decoding of a slippery sequence when the tRNA binds to the ribosomal A site; (2) EF-G-catalyzed translocation of the tRNA from an in-frame position at the A site to the +1-frame position in the P site; or (3) stalling of the tRNA in the P site after translocation and/or EF-G dissociation. Crystal structures of anticodon stem-loops (ASLs) of other +1FS-prone tRNAs in the A site formed in the absence of elongation factors, argue against the shift during decoding, showing that steric hindrance in the decoding center prevents tRNA from slippage. Yet, the dynamics of the ribosome allow sampling of different structures, which may evade crystallization. Thus, the possibility of rearrangements of a frameshifting complex at all three elongation stages remain to be explored. To distinguish among the above three mechanisms, it is necessary to capture 70S translocation complexes that are formed with full-length aminoacyl-tRNAs and EF-G on a +1FS-prone mRNA.

In this work, we present cryo-EM structures of 70S complexes with full-length native E. coli tRNA Pro (UGG), formed with and without EF-G. The structures reveal differences between complexes with a non-frameshifting “control” mRNA and those containing a +1FS-prone mRNA. Unlike the ASLs in previous studies of +1FS, which contained an extra nucleotide next to the anticodon, native E. coli tRNA Pro (UGG) has a canonical anticodon loop. Here, we first describe two pre-translocation 70S complexes, containing a non-frameshifting mRNA codon motif C/C-A, or the frameshifting codon motif C/C-C-A, in the A site. Each complex is prepared with Met-tRNA Met in the P site and Pro-tRNA Pro (UGG) delivered by EF-Tu-GTP to the A site. To capture EF-G-catalyzed translocation states, we then add EF-G with non-hyrodylizable GTP analog GDP-CPP (S’-guanosyl-β,γ-methylene-triphosphate) to each pre-translocation complex and perform single-particle cryo-EM analyses (Methods). We use maximum-likelihood classification of cryo-EM data, which allows the separation of numerous functional and conformational states within a single sample. Our data classification reveals three elongation states in each complex (Supplementary Figs. 1, 2, and 4): (1) pre-translocation non-rotated 70S structures with tRNA Pro in the A site (I: non-frameshifting, and I-FS: frameshifting); (2) “mid-translocation” EF-G-bound structure, with tRNA Pro near the P site (II and II-FS); and (3) nearly fully translocated EF-G-bound state with tRNA Pro in the P site (III and III-FS). In addition, to visualize the pre-translocation ribosome before interaction with EF-G, we analyze the frameshifting complex formed without EF-G, which yielded a rotated pre-translocation 70S ribosome containing tRNA Pro in a hybrid A/P* conformation (Fig. 1b), supporting the notion that the ribosome is pre-disposed for +1FS before translocation, and that frameshifting is accomplished at an intermediate stage of EF-G-catalyzed translocation.

Results and discussion

E. coli tRNA Pro (UGG) has a propensity for +1FS in vivo and in vitro. While we previously showed that E. coli tRNA Pro (UGG) performs +1FS in vitro, it remained unknown whether the mRNA performs +1FS in cells, where all isoacceptors are present, and whether its post-transcriptional modifications impact +1FS. We addressed these questions in a cell-based reporter assay, in which a C1CX-X4 codon motif was inserted at the 2nd codon position of the lacZ gene next to the start codon AUG (Fig. 1a), such that a +1FS event was necessary to synthesize the full-length β-galactosidase (β-gal). The frequency of +1FS was measured as the ratio of β-gal in cells expressing the CXX-X reporter over cells expressing an in-frame insertion of the CCC or CCA codon to the lacZ reporter. We generated a non-FS C1CA-A3 reporter, where the CCA codon was cognate to tRNA Pro (UGG), and a +1FS CCC-A reporter, where the CCA4 codon in the +1-frame would be cognate to tRNA Pro (UGG). We also generated a +1FS CCC-C reporter, where the CCC codon was cognate to tRNA Pro (GGG).

In cells that expressed the CCA-A reporter and contained both tRNA Pro (UGG) and tRNA Pro (GGG), +1FS was suppressed to a background level (0.6%), which remained stable even upon deletion of tRNA Pro (GGG) (0.7%) (Fig. 1b). This indicates that tRNA Pro (UGG) is sufficient to decode the CCA codon of the reporter in cells. The loss of m1G37 on tRNA Pro (UGG) resulted in an elevated +1FS (1.7%) (Fig. 1b), supporting the notion that...
this tRNA isoacceptor is dependent on m1G37 to maintain the reading frame. By contrast, in cells that expressed the CCA-A reporter (Fig. 1c), expression of tRNAPro(UGG) in the absence of tRNAPro(GGG) resulted in a substantial increase in +1FS (4.2%) relative to those expressing both tRNAs (1.5%), and the +1FS frequency was further elevated by the loss of m1G37 (11.6%). These results are consistent with the notion that, while the CCA-A codon can be read by tRNAPro(UGG) through the cmo5U34-C3 wobble pairing, this pairing is unstable and is prone to +1FS to form a stable cmo5U34-A4 pairing. In cells that expressed the CCA-C reporter (Fig. 1d), loss of the cognate tRNAPro(GGG) increased +1FS from 0.9% to 2.0%, indicating the ability of tRNAPro(UGG) to read the 0-frame or +1-frame through cmo5U34-C3 or cmo5U34-C4 pairing. The further increase of +1FS upon loss of m1G37 (to 4.0% or 4.2%) confirmed the importance of this methylation in suppressing +1FS of tRNAPro(UGG).

We next used an in vitro translation assay to measure the efficiency of +1FS under different buffer conditions, varying the concentrations of Mg2+ and other constituents to affect the fidelity and efficiency of translation. We compared a high-fidelity (HF) buffer, which contains MgCl2 at the near-physiological 3.5 mM, and a cryo-EM buffer, which contains MgCl2 at 20 mM in the presence of spermine and spermidine as stabilizing reagents commonly used to capture ribosome complexes (see "Methods"). We began by testing frameshifting of the native-state tRNAPro(UGG) with natural post-transcriptional modifications. We formed an E. coli 70S initiation complex (70SIC) with fMet-tRNAMet in the P site and an mRNA containing the non-FS sequence AUG-CCA-AGU-U or the +1FS sequence AUG-CCC-AGU-U. We next mixed the 70SIC with an equimolar mixture of ternary complexes of EF-Tu-GDP with tRNAPro(UGG), Ser-tRNASer, and Val-tRNAVal in the presence of EF-G and GDP (Fig. 2a). Upon translocation and subsequent decoding, the resulting fMPV tripeptide would report on the amount of the 0-frame product, whereas fMPV would report on the +1FS product. As expected, the fMPV tripeptide was synthesized on the +1FS-prone CCC-A reporter but not on the CCA-A reporter (Fig. 2b–d). Measurement of the +1FS frequency, based on the fractional conversion of fMP to fMPV and fMPV, showed that the +1FS frequency was higher at 37 °C than at 20 °C (Fig. 2b, c), in keeping with the dependence of +1FS on the dynamics of the ribosome. The +1FS frequency was generally higher in the HF buffer than in the cryo-EM buffer, consistent with the notion that high Mg2+ concentrations are inhibitory to protein synthesis. Indeed, translation of the 0-frame fMPS decreased with increasing concentrations of Mg2+ in both the HF and cryo-EM buffers (Supplementary Fig. 5).

We next measured how post-transcriptional modifications in tRNAPro(UGG) contribute to +1FS by performing the assay with the fully modified native-state tRNAPro(UGG) or a modification-free in vitro transcript of tRNAPro(UGG). The unmodified tRNA resulted in increased +1FS on the CCA-A motif, but not on the CCA-A motif (compare Fig. 2e with 2c, d). This indicates that the absence of the cmo5 modification on U34 (the only post-transcriptionally modified nucleotide in the anticodon) likely destabilizes the wobble U34-C3 base pair and that this destabilization increases +1FS on the CCC-A motif. Notably, the transcript tRNA was slower in the fractional synthesis of the 0-frame fMPS at the CCA-A motif and failed to reach a plateau (Supplementary Fig. 6a, b), indicating inefficient interaction of the unmodified tRNA with the ribosome. These experiments demonstrate that our purified native-state tRNAPro(UGG) is an efficient substrate for protein synthesis and that it is capable of inducing +1FS both in cells and in functional assays in vitro.
Fig. 2 +1FS of E. coli tRNA\textsuperscript{Pro}(UGG) in a biochemical functional assay. a The reaction scheme of the functional assay to measure the yield of +1FS as the fractional conversion of fMP to IMPV. An E. coli 70SIC programmed with the non-slippery CCA-A reporter or the slippery CCC-A reporter was mixed with a TC containing EF-Tu-GTP with a native or transcript of tRNA\textsuperscript{Pro}(UGG), Val-tRNA\textsuperscript{*UAC, *UCU} (anticodon, *U = cmo\textsuperscript{5}U anticodon), and Ser-tRNA (GCU anticodon) in the HF or CE buffer containing the indicated MgCl\textsubscript{2} concentration. Each reaction was quenched after 5 min with 0.5 M KOH. Peptides were resolved by electrophoretic TLC and quantified by phosphor-imaging. b-f The fractional conversion of fMP to IMPV (pink) was reported for tRNA\textsuperscript{Pro}(UGG) in the native-state at the CCC-A codon motif at 20 °C (b); in the native-state at the CCA-A codon motif at 20 °C (c); in the native-state at the CCA-A codon motif at 20 °C (d); in the transcript-state at the CCA-A codon motif at 20 °C (e), and in the transcript-state at the CCC-A codon motif at 20 °C (f). All data are presented as mean ± SD. The bars in graphs b-f are SDs of three independent (n = 3) experiments; for datasets CCC-A transcript/HF(20),CE(3.5),CE(20); CCA-A transcript HF(3.5), HF(20),CE(3.5), n = 4.

mRNA frame is shifted in the EF-G-bound structures II-FS and III-FS. EF-G-GTP binds to the rotated conformation of pre-translocation ribosomes\textsuperscript{2,3,43,44}. Spontaneous reverse rotation of the 30S subunit in the presence of EF-G causes synchronous translocation of tRNA ASLs and mRNA codons within the 30S subunit, resulting in P/P and E/E states upon completion of the rotation\textsuperscript{45}. Previous structures of 70S\textsuperscript{tRNA•EF-G complexes captured 30S in rotated states that ranged from ~10 degrees to 0 degrees\textsuperscript{43,46–48}, revealing early (rotated) and late (non-rotated) stages of translocation. They show that domain IV of EF-G binds next to the translocating peptidyl-tRNA and sterically hinders its return to the A site on the 30S subunit upon reverse subunit rotation\textsuperscript{2,49,50}.

Our cryo-EM structures reveal two predominant translocation states with EF-G\textsuperscript{G-DP}: the partially rotated state (~5°) and the nearly non-rotated state (~1°, relative to the non-rotated pre-translocation structure I) (Figs. 4 and 5). The non-framing structures II and III closely resemble previously described mid-translocated\textsuperscript{17,48} (Fig. 4a–c) and post-translocated\textsuperscript{46} structures (Fig. 5a, b) formed with antibiotics. In the partially rotated state, the head of the 30S subunit is swiveled by ~16°, so the 30S peak is closer to the 50S subunit (Fig. 4a). The head swivel is coupled with tRNA ASL and mRNA translocation on the small subunit, allowing gradual translocation first relative to the 30S body and then the 30S head\textsuperscript{51}. In the head-swiveled Structure II, dipeptidyl fMP-tRNA\textsuperscript{Pro} is between the A and P sites of the 30S subunit (Fig. 4b). Here, the anticodon is only ~4 Å away from the P site of the body domain (measured at cmo\textsuperscript{5}U34), but it remains near the A site of the head domain due to the movement of the head in the...
Fig. 3 Cryo-EM structures of pre-translocation 70S formed with fMet-tRNA^{Met} (P site) and Pro-tRNA^{Pro} (A site). a Overall view of the 70S structure with non-frameshifting mRNA (CCA-A; Structure I-FS). Weaker density in the E-site than in the A and P sites suggests partial occupancy of E-tRNA (Methods). b Cryo-EM density (gray mesh) for codon-anticodon interaction between non-frameshifting mRNA and tRNA^{Pro} in the A site of Structure I. The view approximately corresponds to the boxed decoding center region (DC) in (a). The map was sharpened with a B-factor of -80 Å² and is shown at 2.5 σ. c Decoding center nucleotides G530 (in the shoulder region) and A1492-A1493 (in the body region) stabilize the codon-anticodon helix in Structure I. d Overall view of the 70S structure with the slippery mRNA (CCC-A; Structure I-FS). Weaker density in the E-site than in the A and P sites suggests partial occupancy of E-tRNA (see "Methods"). e Cryo-EM density (gray mesh) for codon-anticodon interaction between the slippery mRNA codon and tRNA^{Pro} in Structure I-FS. The map was sharpened with a B-factor of -80 Å² and is shown at 2.5 σ. f Partially open conformation of the 30S subunit due to the shifted G530 (in the shoulder region) in Structure I-FS relative to that in Structure I (16S shown in gray). Structural alignment was obtained by superposition of 16S ribosomal RNAs (rRNAs). In all panels, the 50S subunit is shown in cyan, 30S subunit in yellow, mRNA in blue, tRNA^{Pro} in green, and E-site tRNA in magenta.

direction of translocation. The acceptor arm is in the P site of the 50S subunit. Thus, the tRNA conformation is similar to the previously described chimeric ap/P conformation47 (denoting the anticodon at the A site of the 30S head and near the P site of the 30S body (ap), and the acceptor arm in the P site of the 50S subunit (P)). The nearly non-rotated Structure III features a small head swivel (~1°) and dipeptidyl-tRNA in the P site (Fig. 5a, b), resembling the non-rotated post-translocation ribosome46. Both the dipeptidyl-tRNA^{Pro} and the deacylated tRNA^{Met} are base paired with their respective mRNA codons in the P and E sites, respectively. In both structures II and III, domain IV of EF-G interacts with the ASL of the dipeptidyl-tRNA and the cognate CCA codon (Figs. 4b, c and 5b), consistent with the role of EF-G in stabilizing the codon-anticodon helix during translocation47 and after arrival of the codon-anticodon helix at the P site46. As in previous EF-G-bound structures with a catalytically inactive EF-G or with GTP mimics48,52–54, the switch loops in EF-G domain I are well resolved in Structures II and III, consistent with stabilization of the GTPase by GDPCP (Supplementary Fig. 9). By contrast, EF-G-GDPCP mediates frameshifting on the frameshift-prone CCC-A mRNA motif. In the mid-translocated Structure II-FS, the dipeptidyl-tRNA^{Pro} (Supplementary Fig. 7c) pairs with the mRNA in the +1-frame (C^2CA^4) between the A and P sites of the 30S subunit (Fig. 4d–f). Here, clearly resolved density demonstrates base-pairing of cmo5U34 of tRNA^{Pro} with A4 of the mRNA (Fig. 4e), although the cmo5 moiety of U34 is poorly resolved in this and other structures likely due to its conformational dynamics. The neighboring deacylated tRNA^{Met} is bound to the AUG codon near the E-site. Thus, +1FS results in a bulged mRNA nucleotide C1 between the E and P sites (Fig. 4e, g, i). C1 is sandwiched between the guanosine of the AUG codon and G926 of 16S rRNA. This stabilization allows mRNA compaction and accommodation of four mRNA nucleotides in the E-site, which normally accommodates three nucleotides. Due to frameshifting, tRNA^{Met} and tRNA^{Pro} are shifted away from each other; they are moved by 4 Å and 3 Å from their positions in the non-frameshifting Structure II, respectively (Fig. 4g). The shift of tRNA^{Pro} is compensated by the shift of EF-G loop II (His584-Asp587), critical for fast translocation55,56, whereas the rest of EF-G domain IV including loop I (Ser509-Gly511) is placed similarly to that in the non-frameshifting complex (Fig. 4h).

Previous crystallographic work suggested that the 16S rRNA nucleotides C1397 and A1503, which flank the A and E sites, respectively, prevent mRNA slippage by interacting with the bases of translocating mRNA38,37. These two nucleotides are part of the central region of the 30S head that is stabilized by numerous interactions, including the conserved 1399-1504 Watson–Crick base pair formed by nucleotides neighboring the "stoppers" C1397 and A1503. Our structures indicate that the positions and conformations of this head region, including C1397 and A1503, are nearly identical between the non-frameshifting Structure II and the frameshifted Structure II-FS (Supplementary Fig. 9). Thus, the compact and frameshifted mRNA can be
accommodated in the ribosomal mRNA tunnel during translocation without perturbing the conformations of the head nucleotides.

In the nearly translocated non-rotated Structure III-FS (Fig. 5c), the +1-frame CCA codon and dipeptidyl-tRNAPro are in the P site, while C1 and the AUG codon with the decacylated tRNAfMet are in the E site (Fig. 5d). To accommodate C1 in the E site, the E-site AUG codon and tRNAfMet are shifted by up to 3 Å (Fig. 5e). While most of tRNAfMet is well resolved, poor density for the ASL indicates destabilization of E-site codon-anticodon interactions. Weak C1 density suggests that C1 is detached from G926, which instead hydrogen-bonds with the phosphate group of the first nucleotide of the P-site codon (Fig. 5d, e). The P-site codon and tRNAPro are positioned nearly identically to those in the non-frameshifting Structure III (Fig. 5i). Thus, the frameshifted mRNA and peptidyl-tRNA are placed at the canonical P-site position at the end of the translocation trajectory, preparing the ribosome for the next elongation cycle on the new +1-frame of the mRNA.

Structural mechanism of +1 frameshifting. cryo-EM structures in this work provide the long-sought snapshots of +1 FS (Fig. 6), which are consistent with the recent biophysical work18 and other studies suggesting that frameshifting occurs during EF-G-catalyzed translocation. The use of the native E. coli tRNAPro (UGG) and visualization of EF-G-bound structures distinguishes this work from previous structural studies that were based on +1FS suppressor tRNAs with an expanded anticodon loop26,31–33 or on frameshifting-like complexes with a single tRNA58,59. To obtain a complete +1-FS-prone elongation complex with two tRNAs required for translocation, we placed a frameshifting mRNA sequence C1CC-A4 and tRNAPro(UGG)17 in the A site. The frameshifting ribosome complex therefore contains a wobble cmo5U34-C3 pair upon binding of tRNAPro to the C1CC-A4 sequence (Structure I-FS). Although the downstream A4 would have been a more favorable base-pairing partner for cmo5U34 of tRNAPro(UGG), there is no frameshifting upon decoding and peptidyl transfer (Structures I-FS and Irot-FS). Thus, the +1FS-prone pre-translocation complex maintains the 0-frame anticodon–codon pairing resembling that in canonical elongation complexes40 and crystal structures with +1FS suppressor tRNAs31–33. However, unlike the 0-frame complexes containing the cmo5U34-A3 base pair (Structure I, Fig. 6a) or previous complexes with suppressor tRNAs31–33, structure I-FS features an open 30S subunit, resembling transient decoding intermediates38. Here, G530 of 16S rRNA is shifted from its canonical position near the second base pair of the codon–anticodon helix37, thus
possibly destabilizing the labile three-base-pair codon–anticodon helix containing the cmo5U34-C3 pair upon efficient accommodation and peptidyl transfer. The pre-translocation ribosome therefore appears to predispose tRNAPro for sliding from its near-cognate codon CCC in the 0-frame to the cognate CCA codon in the +1-frame (Fig. 6b). Limited space in the A site, however, restricts the codon–anticodon dynamics and prevents slippage in this pre-translocation state.

In contrast to pre-translocation complexes, the mid-translocation complex with EF-G, and the highly swiveled 30S head features tRNAPro base paired with the +1-frame C2CA4 codon near the P site of the body and the A site of the head (Structure II-FS). This suggests that the ribosome switches to the +1-frame when tRNAPro and mRNA move from the decoding center, and that frameshifting is accomplished by the intermediate of EF-G-catalyzed translocation, at which the tRNA is nearly translocated along the 30S body. The complex remains frameshifted till the completion of translocation when tRNAPro is in the P site relative to both the body and head due to the reverse head swivel (Structure III-FS). Our work therefore suggests a structural mechanism (Fig. 6b), in which non-canonical pairing in the pre-translocation complex sets the stage for frameshifting by opening the 30S subunit and promoting frameshifting during EF-G-catalyzed translocation.

Our observation of destabilization of the pre-translocation complex and of EF-G-bound frameshifting structures is consistent with the high efficiency of 1FS on the mismatched CCC-A frameshifting codon motif shown in vitro and in cells (Fig. 1c). Other frameshifting sequences exist, however, which contain fully complementary codon–anticodon interactions in the 0- and +1-frames, including the CCC-C sequence decoded by tRNAPro (GGG)22, as demonstrated in Fig. 1d. In these cases, the pre-translocation complex most likely samples the canonical closed 30S conformation, in which the codon–anticodon helix is stabilized by the decoding center (as in Structure I). This frame stabilization must at least in part account for the lower efficiency of frameshifting on such sequences17 (Fig. 1d). Nevertheless, the low frequency with which 1FS occurs with such sequences indicates that the tRNA-mRNA interactions can be stochastically destabilized during translocation, when the 30S subunit, tRNAs, and mRNA rearrange. Indeed, recent 70S structures obtained without EF-G demonstrate mRNA frame destabilization upon 30S head swiveling. In a frameshift-like complex featuring a single tRNA and a swiveled 30S head, the bulged nucleotide between the E and P-site codons is stabilized by G92659, similarly to that in Structure II-FS. Furthermore, a recent crystal structure of a non-frameshifting complex with two tRNAs and swiveled 30S head revealed perturbation of the codon–anticodon interactions in the P site, despite full complementarity of the P-site tRNA with the 0-frame codon. While tRNA-mRNA pairing is unstable during head swiveling, EF-G maintains the reading frame in non-frameshifting complexes by interacting with both the tRNA anticodon and mRNA codon along the translocation trajectory (Structures II and III). By contrast, in the frameshifting-prone complexes, EF-G fails to support the codon–anticodon interactions that are transiently destabilized during translocation along with the 30S subunit (such as the CCC-A motif in this study) and allows slippage into the +1 frame that is fully complementary to...
tRNA. Upon slippage, EF-G and 30S residues can stabilize the new frame at the final stages of translocation (Structures II-FS and III-FS; Fig. 6b). We cannot exclude an alternative scenario, in which cellular frameshifting occurs during or after EF-G GDP dissociation from the ribosome in a head-swiveled conformation60. In the cryo-EM sample equilibrated with EF-G GDP, frameshifting could have initiated on fully translocated ribosomes (as in III-FS), which spontaneously reverted to the frameshifted mid-translocation states with stalled EF-G (II-FS). Either scenario is consistent with the prevalence of frameshifting in the stalled cryo-EM structures, which contrasts the small fraction of frameshifting during dynamic translocation with EF-G GTP (Fig. 2), emphasizing the key role of timely association and dissociation of EF-G in mRNA frame maintenance.

Methods
Cell-based assays for +1FS. The E. coli lacZ plasmid in pKK223-3, which we developed previously22, was modified by QuikChange mutagenesis to contain a CCX-X motif following the start codon AUG of the IPTG-inducible reporter gene. Control plasmids containing the in-frame insertion of CCA or CCC were made in parallel. Primers used for mutagenesis to insert a slippery or non-slippery motif in the lacZ plasmid are listed in Supplementary Table 1. The measured β-gal activity from a reporter was normalized by the activity of the corresponding CCA or CCC control plasmid to calculate the +1FS frequency of the reporter. To determine the effect of m1G37 on the frequency of +1FS, each reporter and the corresponding control plasmid was expressed in an E. coli trmD-KO/JM109 strain, where trmD was eliminated from the chromosome and cell survival was maintained by arabinose (Ara)-controlled expression of the human counterpart trm5 from the pACYC plasmid. This E. coli trmD-KO/JM109 strain was made by transducing the P1 lysate of a previously described E. coli trmD-KO/MG165522 into JM109. To determine the contribution of the tRNAPro(GGG) isoacceptor to +1FS, a proL-deletion mutant of E. coli trmD-KO/JM109 was made so that only the proM tRNAPro(UGG) isoacceptor was active for decoding the CCA-A or CCC-A codon motif in a reporter. This proL-deletion mutant of E. coli trmD-KO/MG1655 was made by replacing proL on the chromosome with a Kan marker using the P1 lysate of a previously described E. coli proL-KO strain18, followed by removing the Kan marker with pCP20.

To measure the frequency of +1FS, the E. coli trmD-KO/JM109 strain maintained by trm5 was transformed with a lacZ reporter plasmid. A single colony of each strain was grown overnight in Luria-Bertani (LB) medium in the presence of 0.2% Ara at 37 °C, then inoculated 1:100 to fresh LB with or without 0.2% Ara to generate an m1G37+ or m1G37− condition, respectively. After 1-h growth at 37 °C, during which Trm5 was depleted and m1G37 level reduced in the m1G37− condition, 0.4 mM IPTG was added to turn on the lacZ gene, and cells were grown for additional 4 h at 37 °C. Cells were harvested and the β-gal activity was measured.

Fig. 6 The mechanism of +1 frameshifting. a Schematic of canonical ribosomal translocation by EF-G and ribosome rearrangements. b Schematic of ribosomal translocation by EF-G resulting in +1 frameshifting. The second rows in a, b show local rearrangements of mRNA-tRNA and positions of the decoding-center nucleotide G530 and P-site nucleotide G926 of the 30S subunit. The color scheme is as in Fig. 4.
as a Miller Unit\(^2\). The +1S efficiency (\%) was calculated by determining the \(\beta\)-gal activity of a reporter construct relative to that of the corresponding in-frame reference construct. We used CCA for CCA-A and CCC for CCC-A and CCC-C for reference.

### Biochemical assays for +1S
Two mRNAs used in the biochemical study were prepared by in vitro transcription with \(T7\) RNA polymerase to place the test sequence CCX-X after the start codon. The non-slippery mRNA was prepared by in vitro transcription with \(T7\) RNA polymerase to place the test identical sequence context. Native whereas the slippery mRNA contained the slippery CCC-A motif in an otherwise CAC] contained the non-slippery CCA-A codon motif after the AUG start codon, GAG GUA AAA AUG CCA AGU UAU AAG CAC CAC CAC CAC CAC CAC

Full-length E. coli were loaded in Buffer C and eluted with a linear gradient of Buffer D (Buffer C with 0.7 M KCl, 10 mM MgCl\(_2\), 0.5 mM EDTA, 6 mM \(\beta\)ME) and concentrated with an ultrafiltration unit using a 10-kDa cutoff membrane (Millipore). The concentrated protein was flash-frozen in liquid nitrogen and stored at \(-80^\circ\)C.

### Preparation of charged tRNAs, and mRNA sequences for cryo-EM
\(E. coli\) mRNA was purchased from Chemical Block. Native \(E. coli\) mRNA (\(U\)GG) was overexpressed in \(E. coli\) from an IPTG-inducible \(p\) promoter gene carried by \(p\)KK223-3. Total mRNA was isolated using differential centrifugation and \(p\) mRNA was isolated using a complementary biotinylated oligonucleotide attached to streptavidin-sepharose yielding approximately 40 nmoles \(p\) mRNA from 1 liter of culture. \(E. coli\) mRNA (\(U\)GG) was aminated in the charging buffer (50 mM HEPES-KOH pH 7.5, 50 mM KCl, 10 mM MgCl\(_2\), 0.5 M DTT) in the presence of 40 \(\mu\)M L-proline, 2 \(\mu\)M prolyl-tRNA synthetase (ProRS), 0.625 mM ATP and 15 \(\mu\)M elongation factor \(E\) Tu (purified as in our recent work) or the mixture was incubated for 10 min at 37°C. To stabilize the charged \(p\) mRNA and form the ternary complex for the elongation reaction 0.25 mM ATP, 15 \(\mu\)M elongation factor \(E\) Tu and 80 \(\mu\)M GTP was added to the mixture. The mixture was incubated for 3 min at 37°C.

### Preparation of 70S translocation complexes with or without EF-G
70S translocation complexes with or without EF-G were prepared as follows, separately for the slippery and non-slippery mRNAs. In each, 0.33 \(\mu\)M 30S subunits (all concentrations specified for the final solution) were pre-activated at 42°C for 5 min in the ribosome-reconstitution buffer (20 mM HEPES-KOH pH 7.5, 20 mM NaCl, 20 mM MgCl\(_2\), 50 mM KCl, 50 mM NH\(_4\)Cl, 20 mM HEPES-KOH, pH 7.5) supplemented with 0.5 mM GTP. TCS were formed by first incubating EF-G at 37°C for 15 min in the TCS buffer (Buffer B, 0.5 mM GTP, followed by the addition of native or transcript Pro-tRNA(\(U\)GG), transcript Ser-tRNA(\(U\)AC), and native Val-tRNA(\(U\)AC) for 15 min in an ice bath. Prior to mixing equal volumes of the TCS and 70S complexes, the MgCl\(_2\) concentration of each was kept at 3.5 mM or adjusted upward to 7–20 mM as indicated. Reactions were carried out at 37°C in the presence and containing or removing 0.5 mM spermidine, 1.25 \(\mu\)M 3-(\(\beta\)-mercaptoethanol) (\(\beta\)ME), and 0.005 mM spermine, 6 mM KCl, 30 mM MgCl\(_2\), 1 mM dithiothreitol, 30 mM KCl, 70 mM NH\(_4\)Cl, 50 mM Tris-HCl, pH 7.5) or the cryo-EM-5 \(\beta\)G (3.5 mM MgCl\(_2\), 0.05 mM spermidine, 2 mM spermine, 6 mM 2-mercaptoethanol, 120 mM NaCl, 20 mM HEPES-KOH, pH 7.5) supplemented with 0.5 mM GTP. TCS were formed by first incubating EF-G for 30 min at 37°C then cooled down to room temperature, resulting in 70S translocation complexes with or without EF-G.

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summarized in Supplementary Fig. 1. FREALIGNX was used for all steps of particle alignment, refinement, and final reconstruction steps, and FREALIGN v9.11 was used for classification steps\textsuperscript{57}. Classification from FREALIGNX to FREALIGN for classification was performed by removing a column 12, which contains phase shift information (not applicable as no phase plate was used) and adding an absolute magnitude value. Reverse conversion from FREALIGN to FREALIGNX for refinement was performed automatically by FREALIGNX. The frameshifting 70S ribosome (62,193 particles) was initially aligned to a ribosome reference (PDB 5U9F)\textsuperscript{68} using 5 cycles of mode 3 (global search) alignment including data in the resolution range from 300 to 30 Å until the convergence of the average score. Subsequently, the 4x-binned stack was aligned against the common reference resulting from mode 1 (refine), including gradually increasing resolution limits (5 cycles per each resolution limit; 18-12-10-8 Å) up to 8 Å. 3D density reconstruction was obtained using 60% of particles with highest scores. The refined parameters were used for classification of the 2x-binned stack into eight classes in 50 cycles using the resolution range of 300–8 Å. In the following steps, the 4x-binned stack was replaced by the 2x-binned image stack, which was successively aligned against the common reference using mode 1 (refine), including gradually increasing resolution limits (5 cycles per each resolution limit; 18-12-10-8 Å) up to 8 Å. 3D density reconstruction was obtained using 60% of particles with highest scores. The refined parameters were used for classification of the 2x-binned stack into eight classes in 50 cycles using the resolution range of 300–8 Å. This classification revealed six high-resolution classes, one low-resolution (junk) class, and one class representing only 50S subunit (Supplementary Fig. 1a). The particles assigned to the high-resolution 70S classes were extracted from the 2x-binned stack (with >50% occupancy and scores >0) using merge_classes.exe (part of the FREALIGNX distribution), resulting in a stack containing 41,382 particles. Classification of this stack was performed for 50 cycles using a focused spherical mask between the A and P sites (mask center coordinates: $x = 191.1 \text{ Å, } y = 224.7 \text{ Å, } z = 159.6 \text{ Å and 30 Å radius, as implemented in FREALIGNX}$. This sub-classification into four classes yielded two high-resolution classes, one low-resolution class, and one class representing only 50S subunit (Structure I). For the classes of interest (Structure I, 4263 particles; Structure II, 3179 particles; Structure III, 4612 particles), particles with >50% occupancy and scores >0 were extracted from the 2x-binned stack. Refinement to 6 Å resolution using mode 1 (refine), including gradually increasing resolution limits (5 cycles per each resolution limit; 18-12-10-8 Å) up to 8 Å. 3D density reconstruction was obtained using 60% of particles with highest scores. The refined parameters were used for classification of the 2x-binned stack into eight classes in 50 cycles using the resolution range of 300–8 Å. This classification revealed 11 high-resolution classes, two low-resolution (junk) classes, and three classes representing only 50S subunit (Supplementary Fig. 3a). The particles assigned to the high-resolution 70S classes were extracted from the 2x-binned stack (with >50% occupancy and scores >0) using merge_classes.exe (part of the FREALIGNX distribution), resulting in a stack containing 25,345 particles. Classification of this stack was performed for 50 cycles using a focused spherical mask in the P site of the ribosome (mask center coordinates: $x = 178.0 \text{ Å, } y = 224.9 \text{ Å, } z = 176.9 \text{Å and 30 Å radius, as implemented in FREALIGNX}$. This sub-classification into eight classes yielded one high-resolution class, which contained a rotated ribosome with one tRNA. The particles assigned to the high-resolution 70S class were extracted from the 2x-binned stack (with >50% occupancy and scores >0) using merge_classes.exe (part of the FREALIGNX distribution), resulting in a stack containing 25,345 particles. Classification of this stack was performed for 50 cycles using a focused spherical mask in the P site of the ribosome (mask center coordinates: $x = 178.0 \text{ Å, } y = 224.9 \text{ Å, } z = 178.1 \text{ Å and 40 Å radius}$. This sub-classification into eight classes yielded one high-resolution class, which contained both rRNAs and ribosome in the rotated state (Structure I-FS). For the class of interest (Structure I-FS, 3,658 particles), particles with >50% occupancy and scores >0 were extracted from the 2x-binned stack. Refinement to 6 Å resolution using mode 1 (5 cycles) of the respective 1x-binned stack using 59% of particles with highest scores resulted in ~3.2 Å (Structure I-FS), ~3.2 Å (Structure II-FS), and ~3.3 Å (Structure III-FS) maps (FSC = 0.143).

For the rotated pre-translocation frameshifting 70S mRNA(CCA)-Met-tRNA\textsubscript{Met}-Pro-tRNA\textsubscript{Pro}(UGG)-EF-G-GDP,PCP translocation complex, a dataset of 2591 movies containing 178,117 particles was collected on a Titan Krios (FEI) microscope (operating at 300 kV) equipped with the K3 camera system (Gatan), with ~0.8 to ~2.0 μm defocus. Each exposure was acquired with continuous frame streaming at 25 frames, yielding a total dose of 40.2 e\textsuperscript{-}/Å\textsuperscript{2}. The nominal magnification was 105,000 and the calibrated super-resolution pixel size at the specimen level was 0.415 Å. The dataset was otherwise collected and processed in the same way as that for non-frameshifting or frameshifting complex with EF-G-GDP,PCP. All movies were used for further analysis after inspection of the averages and the power spectra computed by CTFIND4 within cisTEM. The stack and particle parameter files were assembled in cisTEM with the binnings of 1x, 2x and 4x (box size of 490 for unbinned stack). Data classification was summarized in Supplementary Fig. 3. FREALIGNX was used for all steps of particle alignment, refinement and final reconstruction steps and FREALIGN v9.11 was used for 3D classification steps\textsuperscript{57}. The 4x-binned image stack (178,117 particles) was initially aligned against a ribosome reference (PDB 5U9F)\textsuperscript{65} using 5 cycles of mode 3 (global search) alignment including data in the resolution range of 300–8 Å. This classification revealed 11 high-resolution classes, two low-resolution (junk) classes, and three classes representing only 50S subunit (Supplementary Fig. 3a). The particles assigned to the high-resolution 70S classes were extracted from the 2x-binned stack (with >50% occupancy and scores >0) using merge_classes.exe (part of the FREALIGNX distribution), resulting in a stack containing 25,345 particles. Classification of this stack was performed for 50 cycles using a focused spherical mask in the P site of the ribosome (mask center coordinates: $x = 170.7 \text{ Å, } y = 222.4 \text{ Å, } z = 176.9 \text{ Å and 30 Å radius, as implemented in FREALIGNX}$. This sub-classification into eight classes yielded one high-resolution class, which contained both tRNAs and EF-G (Structure II and III); and one high-resolution class, which contained both tRNAs and EF-G (Structure II and III). This sub-classification into eight classes yielded one high-resolution class, which contained both tRNAs and EF-G (Structure II and III). The particles assigned to the high-resolution 70S class were extracted from the 2x-binned stack (with >50% occupancy and scores >0) using merge_classes.exe (part of the FREALIGNX distribution), resulting in a stack containing 25,345 particles. Classification of this stack was performed for 50 cycles using a focused spherical mask in the P site of the ribosome (mask center coordinates: $x = 178.0 \text{Å, } y = 224.9 \text{ Å, } z = 178.1 \text{ Å and 40 Å radius}$. This sub-classification into eight classes yielded one high-resolution class, which contained both tRNAs and EF-G (Structure II-FS). For the class of interest (Structure II-FS, 3,658 particles), particles with >50% occupancy and scores >0 were extracted from the 2x-binned stack. Refinement to 6 Å resolution using mode 1 (5 cycles) of the respective 1x-binned stack using 59% of particles with highest scores resulted in ~3.2 Å (Structure II-FS) map (FSC = 0.138).

The maps (Structure I, II, III, I-FS, II-FS, and III-FS) were filtered for structure refinements, by blicores and blicfilt from the Bsoft package\textsuperscript{86}. To this end, a mask was created for each map by low-pass filtering the map to 30 Å in Bsoft, then binarizing, expanding by three pixels and applying a three-pixel Gaussian edge in Bsoft. The map was roll corrected at 20° for each map. In each case, the resolution criterion was FSC with cutoff of 0.143. The output of blicores was used to filter maps according to local resolution using blicfilt (Supplementary Fig. 4). A range of B-factor values from –50 to –120 Å\textsuperscript{2} was tested for blicore maps to achieve optimal balance between higher/lower-resolution regions. Maps sharpened to –80 Å\textsuperscript{2} (the B-factor in the middle of the map) were used for model building and structure refinements. FSC curves were calculated by FREALIGN for even and odd particle half-sets.

**Model building and refinement.** Reported cryo-EM structure of *E. coli* 70S-Met-tRNA\textsubscript{Met}-Phe-tRNA\textsubscript{Pro}+EF-Tu+GDP,PCP complex (PDB 5UYM), including EF-Tu and tRNAs, was used as a starting model for structure refinement. The structure of EF-G from PDB 4V7D was used as a starting model, and switch regions were generated by homology modeling from PDB 4V9P. The structure of tRNA\textsubscript{Pro} (UGG) was created by homology modeling (according to tRNA\textsubscript{Met} (UGG) sequence) using blicores-blicfilt (Bsoft) and blicores-pycr (CGG) using blicores-blicfilt (Bsoft) and blicores-pycr (CGG).

Initial protein and ribosome domain fitting into cryo-EM maps was performed using Chimera\textsuperscript{87}, followed by manual modeling using PyMOL. The links between
the domains and parts of the domains that were not well defined in the cryo-EM maps (e.g., loops of EF-G) were not modeled.

All structures were refined by real-space simulated-annealing refinement using atomic electron scattering factors in RSRef72. Secondary-structure restraints, comprising hydrogen-bonding restraints for ribosomal proteins and base-pairing restraints for RNA molecules, were employed. Refinement parameters, such as the relative weighting of stereochemical restraints and experimental energy term, were optimized to produce the stereochemically optimal models that closely agree with the corresponding maps. In the final stage, the structures were refined using phenix.real_space_refine73, followed by a round of refinement in RSRef applying harmonic restraints to preserve protein backbone geometry. Real-space R-factor (RSRef refinement) and correlation coefficient (model-to-map fit—Phenix refinement) were closely monitored to prevent the overfitting of the models to the corresponding maps. The refined structural models closely agree with the corresponding maps, as indicated by low real-space R-factors and high correlation coefficients (Supplementary Table 2). FSC between the final models and maps, and cross-validation half-map FSCs were calculated using Phenix74, demonstrating good agreement between the structural models and maps (Supplementary Fig. 4).

The resulting models have good stereochemical parameters, including low deviation from ideal bond lengths and angles, low number of macromolecular backbone outliers etc., as shown in Supplementary Table 2. Structure quality was validated using MolProbity75.

Structure superpositions and distance calculations were performed in PyMOL.

To calculate the degree of the 30S body rotation or head rotation (swivel) between two 70S structures, the 23S rRNAs or 16S rRNAs of the 30S body were aligned using PyMOL, and the angle was measured in Chimera. These degrees of rotation (30S body/subunit rotation and 30S head rotation) for Structures II, III, Irot-FS, II-FS, and III-FS are reported relative to the classical non-rotated Structures I and F-S, respectively. Figures were prepared in PyMOL and Chimera.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability.** The EM density maps generated in this study have been deposited in the EMDB under accession codes EMD-22669 (Structure I); EMD-22670 (Structure II); EMD-22671 (Structure III); EMD-22672 (Structure I-FS); EMD-23528 (Structure Irot-FS); EMD-22673 (Structure II-FS); EMD-22674 (Structure III-FS). The atomic coordinates generated in this study have been deposited in the PDB under the accession codes 7K50 (Structure I); 7K51 (Structure II); 7K52 (Structure III); 7K53 (Structure I FS); 7L10 (Structure Irot-FS); 7K54 (Structure II-FS); 7K55 (Structure III-FS). Source data are provided with this paper.

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Competing interests

The authors declare no competing interests.

Additional information

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