Thermodynamic control of −1 programmed ribosomal frameshifting

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mRNA contexts containing a ‘slippery’ sequence and a downstream secondary structure element stall the progression of the ribosome along the mRNA and induce its movement into the −1 reading frame. In this study we build a thermodynamic model based on Bayesian statistics to explain how −1 programmed ribosome frameshifting can work. As training sets for the model, we measured frameshifting efficiencies on 64 dnaX mRNA sequence variants in vitro and also used 21 published in vivo efficiencies. With the obtained free-energy difference between mRNA-tRNA base pairs in the 0 and −1 frames, the frameshifting efficiency of a given sequence can be reproduced and predicted from the tRNA−mRNA base pairing in the two frames. Our results further explain how modifications in the tRNA anticodon modulate frameshifting and show how the ribosome tunes the strength of the base-pair interactions.

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Ribosome frameshifting is a reoccurring event in the translation process of proteins that occurs when the ribosome moves to different reading frames along the mRNA, changing the sequence of the synthesized protein. Frameshifting can occur due to various mechanisms, including the presence of slippery sequences, which are short nucleotide sequences that facilitate reverse movement of the ribosome.

Frameshifting is a rare event that occurs in vivo, but it is important because it allows ribosomes to accommodate frameshifting events without altering the primary structure of the protein. This is particularly relevant for the translation of genes that encode proteins with multiple reading frames, such as the lambda phage and the E. coli dnaX gene.

In this work, we show that frameshifting can be quantitatively explained by a simple thermodynamic model based on the energy difference of the mRNA–tRNA base-pairing energies for 64 variants of slippery sequences containing Lys (AAA/G) and Phe (UUA/U) codons. The free-energy differences of tRNA base-pairing energies in the 0-frame and −1-frame enabled us to reproduce FS for mRNA sequences used in the model and to predict efficiencies for other slippery sequences. The results explain the experimental results and suggest that the frameshifting efficiency is controlled by the relative thermodynamic stability of the codon–anticodon interactions in the 0-frame and −1-frame.
To test whether the branch point of frameshifting is the same on other slippery site variants, in particular those where frameshifting involves non-Watson–Crick codon–anticodon interactions in the −1-frame, we determined the rates of frameshifting for three slippery sequence variants, A1G, A1U, and A4G. With these mRNAs, the presence of the downstream stem-looop did not affect the incorporation rate for Lys2, but reduced the rate of the 0-frame Phe incorporation. This indicated that the point of slippage remains unchanged with the mRNA variants (Supplementary Figs. 2d, e, 3d, Supplementary Table 2).

Analogous to the A1 AAA4 AAA7 slippery sequence, A1G, A1U, and A4G mRNA variants maintained very efficient frameshifting as shown by the high rate of Val incorporation (Supplementary Figs. 2b, c, 3b).

Another observation suggesting that frameshifting takes place at the translocation step is that with the wild-type dnaX sequence, the presence or absence of tRNAs decoding the Phe (0-frame) or Val (−1-frame) codons downstream the slippery sequence does not change the FS. This indicates that the commitment to the new reading frame occurs before the Val or Phe codon is available for decoding in the A site, that is, at the preceding translocation step. Similarly, for the A1G, A1U and A4G variants the incorporation efficiency is not affected by the presence of Val-tRNA and Phe-tRNA encoding the overlapping (G UUC) codons (Supplementary Figs. 2b, c and 3b). For the A1G construct, if tRNA slippage occurred on the first codon (AAG4) while the second codon was free, the second codon would be decoded in the −1-frame (G4AA) by Glu-tRNAa, resulting in a MAKE peptide. However, in the presence of Glu-tRNAa, the MAKK peptide is still predominant (Supplementary Fig. 3b), indicating that slippage takes place after the decoding of the second codon by tRNAa. In the presence of all aa-tRNAs, we observe only about 5% of the frameshifting peptide containing Glu (MAKEV) (Supplementary Fig. 3d), rendering an alternative frameshift pathway, e.g., stimulated by aa-tRNA depletion, unlikely under given conditions.

Free-energy model of −1PRF. Upon frameshifting, the mRNA–tRNA base pairs change depending on the slippery sequence (Fig. 1a). We asked whether the FSs can be explained by differences in the interaction free-energy between the base pairs involved in the 0-frame and −1-frame duplex. If all individual base-pair free-energy differences ΔGbp were known, and assuming thermodynamic equilibration during the frameshifting, the FS
of a given mRNA sequence could be calculated from the total free-energy difference \( \Delta G \) for all anticodon–codon positions in the 0-frame and -1-frame, \( \Delta G = \exp(-\Delta G/(k_B T))/[1 + \exp(-\Delta G/(k_B T))] \) with Boltzmann factor \( k_B \) and temperature \( T = 310 \) K. However, initially we face the inverse problem, as the FS values were known from experiments (Fig. 1b), whereas the base-pair free-energy differences were unknown. To tackle this inverse problem, we used Bayesian statistics to obtain the individual free-energy differences that best fit all measured FSs (see the “Methods” section).

The ribosome provides structurally different environments for the mRNA–tRNA interactions at the first and the second slippery codons, which likely have an effect on the base-pair free energy. To take into account this effect of the different environments on the base-pair free energies of the tRNA, we used 16 \( \Delta G_{bp} \) variables for all base-pair changes at position of each codon, of which the 14 \( \Delta G_{bp} \) variables turned out to be independent. Although frameshifting occurs in the intermediate state of translocation, most likely in the ap/P or pe/E chimera hybrid state, for ease of notation we indicate the interactions for the first and second codon as P and A, respectively. The position of a base pair (X-Y) within the codon is indicated by a number (1, 2, 3) and the first base (X) denotes the codon base and the second the anticodon base (Y).

First, we tested whether the free-energy model with the underlying assumptions and the set of parameters is able to reproduce the measured FS values. To that end, we used all 64 measured FS values (Fig. 1b) to obtain base-pair free-energy differences for 10 single base-pair changes in the P site (Fig. 2a) and in the A site (Fig. 2b). The remaining six base-pair changes only occur in pairs in the sequences used here (Fig. 2c). For these changes, we obtained the free-energy differences of changing two base pairs at the same time. Positive free-energy differences indicate that the free energy of base pairing in the -1-frame is larger than in the 0-frame, i.e., that base pairing in the -1-frame is less favorable than in the 0-frame. Accordingly, the larger free energy of the -1 frame renders frameshifting less likely, and thus FS < 50% is expected. Next, we calculated distributions of FS values (FS_{model}) from the obtained variation of free-energy differences using the ratio of Boltzmann distributions shown above (see the “Methods” section). Despite the fact that our model only contains 14 free parameters, the calculated efficiencies agree well with the measured efficiencies with a root mean square deviation (rmsd) of 2.5% between the mean FS_{model} and the FS_{experiment} (Fig. 2d).

Second, we tested if the free-energy model is also able to predict FS values for mRNA variants that were not used to obtain the free-energy differences. To this aim, we omitted one mRNA variant from the dataset and derived a new free-energy model. This set was subsequently used to predict the efficiency of the omitted variant. We repeated this cross-validation procedure for each mRNA variant and good agreement with the measured efficiencies was seen (Fig. 2e, rmsd 4.1%), underscoring that the model is indeed predictive.

One critical assumption underlying our thermodynamic equilibrium model is that the FS is determined only by the free-energy difference of tRNA binding in -1-frame vs. 0-frame. This is only possible if frameshifting is significantly faster than the completion of translocation. In such a case, the kinetic
partitioning between frameshifting and translocation would be negligible and the probability of the ribosome to switch into −1-frame would be predominantly thermodynamically controlled. This assumption is plausible, because translocation is slowed down considerably by the mRNA stem-loop, but as the elemental rate of tRNA slippage is not known, the extent to which frameshifting is affected by kinetics is unclear. To challenge this assumption, we included a kinetic factor \( \kappa \) into our model such that \( F_{\text{kinetic}} = FS \cdot (1 - \kappa) \), where \( FS \) is obtained from the ratio of Boltzmann distributions (see the “Methods” section; Fig. 3a). For the kinetic factor, we assumed that a free-energy barrier limits the rate of tRNA slippage from the 0-frame to the −1-frame and back, i.e., the slippage is slower than translocation. The mean kinetic factor for all mRNA variants was included within our Bayesian approach as a nuisance parameter. Similar as above, the probability of base-pair free-energy differences was obtained for all 64 sequences, now additionally including \( \kappa \). The resulting rmse value (2.5%) is similar to that from the model without the kinetic contribution. Further, the most probable mean kinetic factor \( \kappa \) was found to be below 0.1% (Fig. 3b), which indicates that the kinetics of frameshifting does not markedly affect the FS in this experimental system. Independent estimations of frameshifting kinetics suggest that the rate of slippage into −1-frame is \( 10^{-1} \) s\(^{-1}\) on the original dnaX slippery sequence and \( 3 \) s\(^{-1}\) on the \( \Delta \)G sequence (B.-Z. Peng, L. V. Bock, R. Bellardinelli, F. Peske, H. Grubmüller, and M. V. Rodnina, unpublished data). In comparison, the step that limits the completion of translocation was estimated to \( 0.1 - 0.5 \) s\(^{-1}\) (refs. 19,20). This supports the notion that, with the frameshifting secondary structure element on the mRNA, completion of translation is sufficiently slow to allow the tRNAs to re-pair with their thermodynamically favored codons.

In summary, these results show that the FS values are consistent with—and can even be predicted by—a thermodynamic model that is based on only two assumptions, (1) that all FS values are determined solely by the free-energy differences between the 0-frame and −1-frame and (2) that the total free-energy difference is the sum of the individual free-energy differences for each mRNA–tRNA base-pair change upon frameshifting, i.e., that the coupling of free-energy changes of base pairs is small.

Free-energy model applied to an independent dataset. Tsuchihashi et al. reported a large set of FS values for 23 variants of the dnaX mRNA in vivo, which enabled us to test the free-energy model of frameshifting against an independent dataset. The alternative dataset by Sharma et al. could not be used, as the values for FS on the native dnaX slippery sequence are inconsistent with those of refs. 20,26, thereby precluding meaningful comparisons. We used 21 FS values from the Tsuchihashi dataset (Supplementary Table 3) to obtain probabilities for free-energy differences, excluding the G·A variant, which was considered unreliable by the authors due to the possibility of transcriptional slippage, and the 123C variant which encodes a proline and may reflect proline-specific stalling effects. The 21 mRNA variants not only include seven variants that have been tested in this work, but also sequences that do not preserve the Lys or Phe codon identity, because their P-site and A-site codons encode different amino acids in the 0-frame. As a consequence, a larger set of 26 individual base-pair changes upon −1-frameshifting can be considered in the model. Notably, we did not include any information about the experimental FS values obtained in this work (Fig. 1b), so using the ‘Tsuchihashi dataset provides an independent test for the model in vivo. The green histograms in Fig. 2a–c show the probability distributions for the free-energy differences obtained from both datasets, and Fig. 4a show the probability distributions for the remaining base-pair changes. We obtained free-energy differences for six base-pairs in the P site (Figs. 2a and 4a) and for three in the A site (Figs. 2b and 4b), as well as for nine combinations of P-site and A-site base pairs (Figs. 2c and 4c).

The FS values calculated from the free-energy differences are in excellent agreement with the measured FS values, with an rmse of 2.6% (Fig. 4d). Notably, the probability distributions obtained using the Tsuchihashi et al. dataset are much broader than those obtained from our data set (Fig. 2a–c). For some base-pair changes, the probability density extends towards large free-energy differences, indicating that FS values only provide a lower boundary, e.g., for P2 G·C −→ A·C (Fig. 4a, top panel). A lower boundary arises from the close to 0% FS values, for which the corresponding \( \Delta G \) values increase without limit, such that no upper boundary is obtained. Overall, the broader distributions reflect a higher uncertainty of the free-energy differences obtained from the Tsuchihashi et al. data set than to those derived from our dataset—mostly due to the smaller number and larger experimental uncertainties of the FS values used to obtain free-energy differences for a larger number of base-pair changes.

Free-energy differences of base-pair changes. As expected, all free-energy differences \( \Delta G_{\text{bp}} \) for changing a Watson–Crick base pair (A·U or U·A) into a mismatched base pair (C·U, U·U, G·U, A·A, C·A, G·A) are positive (Fig. 2a, b, Supplementary Table 4). Following this notion, more mismatches introduced in the −1 frame imply lower FS. Interestingly, changing the Watson–Crick A·U base pair to pyrimidine-pyrimidine (C·U or U·U) base pairs comes with the highest energetic penalty, whereas changing the Watson–Crick base pair to a mismatched purine-purine base pair (A·U −→ G·U) or to a mismatched pyrimidine-purine (U·A −→ C·A) has a lower penalty. The lowest penalty comes with the change to purine-purine base pairs (A·A or G·A). It is possible that in the codon–anticodon helix two pyrimidines are too far apart, while a pair of larger bases can form contacts, albeit not as well as the Watson–Crick base pairs. For the sequences investigated here, the only base-pair change with a negative \( \Delta G_{\text{bp}} \) is G·S to A·S in the wobble position of the A-site codon (A3). The nucleotide S is a modified U (mmn5s2U) which interacts more strongly with A than with G; \( \Delta G_{\text{bp}} = -2.9 \) or \(-4.6 \) kJ/mol from our in vitro dataset and the Tsuchihashi et al. dataset, respectively (Fig. 2b, Supplementary Table 4). For the similar modified nucleotide mmn5U, which lacks the s2 modification, the interaction with A is also stronger than with G and
ΔG<sub>pp</sub> is similar, −3.8 kJ/mol (Fig. 4a, Supplementary Table 4; note that the positive values shown therein are for the A → G change, whereas in Fig. 2b the change is in opposite direction, G → A). The similar free-energy differences of mm<sup>3</sup>s<sup>2</sup>U and mm<sup>3</sup>U suggest that the s<sup>2</sup> modification of U does not play a large role in the base-pair free energy. This is different with yeast tRNA<sub>lys</sub>, where mcms<sup>3</sup>-modified tRNA<sub>lys</sub> lacking the s<sup>2</sup> group has a lower affinity of binding to the cognate codon AAA than the fully modified tRNA<sub>lys</sub>. The difference may be related to the existence of two tRNA isoacceptors in yeast dedicated to reading of AAA or AAG codon each, whereas in E. coli one tRNA isoacceptor reads both codons.

tRNA<sub>phe</sub> with the 3′ AAG<sup>5</sup> anticodon is able to decode codons UUC and UUU through C·G Watson–Crick or U·G wobble base pairing at the A3 codon position. The free-energy difference between the two base pairs (A3 C·G → U·G) is 3.4 ± 0.1 kJ/mol (Fig. 2b, Supplementary Table 4) which agrees well with the values of 6.2 ± 3.0 or 1.3 ± 2.5 kJ/mol obtained from free-energy molecular-dynamics simulations with two different starting structures.

The nucleotide queosine (Q) is a modified G. Changing C·Q to A·Q or U·Q to A·Q has similar free-energy costs (Fig. 4b), indicating that the base-pair free energies of C·Q and U·Q are similar. Queosine is found in the 3′ UUQ<sup>5</sup> anticodon of tRNA<sub>asn</sub> which decodes both AAC and AAU codons. In agreement with the similar free energies, FS is similar on U<sub>1</sub> UUA<sub>4</sub> AAC/U<sub>7</sub> mRNA variants (2% and 3%)<sup>39</sup>. In the absence of the modification (3′ UUG<sup>5</sup> anticodon), the FS of the AAC<sub>7</sub> variant is lower (1%) than for the AAU<sub>7</sub> variant (5–6%)<sup>39</sup>, as expected from the result that the U·G wobble base pair is weaker than the C·G base pair.

In the free-energy differences also explain the unexpected observation that mutations A<sub>3</sub>G and A<sub>3</sub>G retain a surprisingly high FS despite the fact that they involve a mismatch in the −1-frame. The A<sub>3</sub>G sequence (G<sub>1</sub> AAA<sub>4</sub> AAG<sub>7</sub>) undergoes two-base-pair changes upon shifting to the −1-frame. The first change introduces an unfavorable mismatch in the first position of the first codon, P1 A·U → G·U, which comes with a free-energy penalty of 5.1 kJ/mol (Supplementary Table 4, ΔG<sub>pp</sub>, obtained from our dataset). The second base-pair change, the A3 G·S → A·S at the A-site wobble position, however, reduces the free-energy difference by −2.9 kJ/mol. Therefore, the total energetic cost ΔG between the frames is only 2.2 kJ/mol, which, using the ratio of Boltzmann probabilities, results in a FS of 30% that is close to the measured value of 28%. The sequence of A<sub>1</sub>G (A<sub>1</sub> AAG<sub>4</sub> AAG<sub>7</sub>) also introduces one G·U mismatch (A1 position) upon frameshifting, but has an even higher measured FS of 44%. Our model attributes this higher FS to a favorable G·S → A·S base-pair change in the A3 position (−2.9 kJ/mol), which almost neutralizes the unfavorable changes in P3 and A1 (G·S → A·S and A·U → G·U, respectively) of 3.3 kJ/mol to a total free energy cost ΔG = 0.4 kJ/mol, which results in a FS of 46% in agreement with the measured value of 44%.

The environment of the codon–anticodon base pairs is different in the P and A sites, which prompted us to compare the individual free-energy differences in the P and A sites, respectively. The free-energy differences for A·U → U·U and U·A → A·A, which have been obtained directly (Figs. 2a, b and 5), are similar in the P and A sites. The free-energy differences for C·A → C·U and G·A → G·U were not obtained directly, but inferred from a combination of free-energy differences under the assumption that the A·U and U·A base pairs have the same free energy (see the “Methods” section). The resulting free-energy differences for C·A → C·U are similar in the P and A sites (Fig. 5). The G·A → G·U change appears somewhat less favorable in the P site than in the A site by about 1 kJ/mol, but this difference could also arise from a non-isotericity of the A·U base pair. Overall,
these results suggest that the base-pair free energies in the first position of the codon–anticodon helix are similar in the P-site and A-site environment.

Base-pair interactions in solution and on the ribosome. To further investigate the effect of the ribosome on the mRNA–tRNA base pairs, we compared the free-energy differences of the base pairs obtained from our model, $\Delta G_{\text{bpb}}$, with those calculated from free-energy molecular dynamics simulations for isolated base pairs in solution, $\Delta G_{\text{sol}}$ (Fig. 6a, b). The conformations of several Watson–Crick and mismatched mRNA–tRNA base pairs have been obtained by X-ray crystallography27,34, which allows us to check whether the predicted free-energy differences are reflected in the conformational differences. The G·S → A·S change is favorable both in solution and in the A1 codon position on the ribosome, but it is slightly more favorable in solution (Fig. 6a). The G·S and A·S base pairs both have two H-bonds on the ribosome27 and in solution40, but the base-pair conformations differ (Fig. 6c, left panel), which agrees well with the different free-energy differences. Interestingly, the sulfur atom (yellow) of the modified nucleotide mnm5s2U (S) is not directly involved in the base pairing on the ribosome, which also agrees with the observation that mnm5U, which lacks the sulfur atom, shows similar free-energy differences as mnm5s2U (see above).

Base-pair changes introducing mismatches C·G → U·G and A·U → C·U are unfavorable both in solution and on the ribosome (Fig. 6a). For the C·G → U·G change, the A-site conformations of the base pairs34 are similar to those in solution (Fig. 6c, middle panel) and indicate a loss of one H-bond, which again agrees with the positive free-energy differences $\Delta G_{\text{bpb}}$ and $\Delta G_{\text{sol}}$. In contrast, the A·U → U·U base-pair change is favorable in solution, but unfavorable on the ribosome (Fig. 6a). This result agrees with the structural data which shows that for the U·U base pair a Watson–Crick-like conformation is enforced in the A site, which prevents H-bond formation27, while in solution two H-bonds can form, stabilizing the base pair (Fig. 6c, right panel).

In the P site, base-pair changes to U·U are favorable in solution, but unfavorable on the ribosome, which suggests that also in the P site the favorable non-Watson–Crick conformation is prevented (Fig. 6). The base pairs A·U and G·U have similar free energies in solution and the G·U engages in a non-Watson–Crick conformation40. In the P site, in contrast, the G·U base pair is predicted to be weaker than the A·U base pair, which agrees with the observation that Watson–Crick-like conformation are also enforced in the P site41. Overall, these comparisons indicate that the predicted free-energy differences agree with qualitative expectations solely based on structural studies, thus providing further and independent support for our free-energy model.

Discussion

The present work provides the thermodynamic framework for understanding PRF. We show that, when translocation is slowed down by an mRNA secondary structure element downstream of the slippery site, the propensity of a tRNA to frameshift on a given slippery codon is largely determined by the free energies of base pairing, and the FS can be confidently predicted based solely on the combinations of base pairs at a given frameshift site. For the presented system, kinetic effects do not contribute to frame-shifting, indicating that the stalling time is sufficiently long to allow equilibration via frequent re-crossings of the free-energy barriers between the two reading frames. As a result, only the free-energy difference between the frames governs the FS.
long pause of the ribosome at an mRNA secondary structure element may serve to achieve sufficient equilibration and thereby contribute to enhance the FS. For shorter startling times, the pause would become too short for the barrier to be overcome sufficiently often, and thus one would predict the FS to decrease with increasing translation rate. In this non-equilibrium case, the free-energy model that includes the kinetic contribution should provide good estimates for the rates of recoding. It would be interesting to see whether these rates differ for different tRNA species or for modified vs. non-modified tRNAs, which would indicate that certain tRNAs in the cell are more prone to frameshifting.

Our model also shows that for the studied mRNA variants under the given conditions, the free-energy differences of the individual base-pair changes are additive and appear not to be energetically coupled. The observation that the decoding center is in the same conformation with bound cognate or near-cognate tRNAs suggests that the free-energy difference between the two cases stems from the base-pair interactions27,34,41,42, providing a possible reason for the absence of coupling. In all of the studied mRNA variants, the base pairs in second codon positions remain unchanged upon frameshifting (Fig. 1a). However, if base pairs at the first and second codon positions would be changed at the same time, the helix conformation might change markedly, rendering a coupling between base-pair changes more likely. This effect can be tested by applying the free-energy model to a larger set of FS values for mRNA variants including sequences that lead to position 1 and 2 mismatches. Furthermore, the Watson–Crick-like conformations of U–G mismatches34,41,42 imply that the free-energy difference of changing a C–G to U–G in the first two codon positions is different from that in the third positions, where wobble base pairs are tolerated34. It was suggested that either tautomerization or ionization of the bases allow the U–G base pairs to adopt the Watson–Crick-like conformation41. Our free-energy model applied to frameshifting efficiencies of mRNA variants which entail the C–G to U–G base-pair changes in different codon positions would provide access to the corresponding free-energy differences.

In summary, the thermodynamic model of frameshifting can be applied to larger datasets and hence allows for testing for—and even predicting—possible kinetic contributions of other cis and trans-acting frameshift stimulatory RNA elements. Furthermore, besides being a powerful tool to predict recoding efficiencies on a given frameshift site, our model should be able to distinguish between sequence patterns that allow efficient alternative frame translation in genomes. This way, in combination with bioinformatics, the free-energy model may also become a useful tool for further exploration of genomes for potential frameshifting sites.

**Methods**

**Translation assays.** We used a variant of the original dnaX frameshifting site as described previously39. The mRNAs were prepared by in vitro transcription with T7 RNA polymerase34,35 and purified using the RNasey midi kit (Qiagen) following the manufacturer’s recommendations. All mRNAs had the same length and contained the native stem-loop structure, but differed in the slippery site sequence, as shown in Supplementary Table 1. Ribosomes from E. coli MRE 600, E. coli TF, E. coli EF-G, and initiation factors, were prepared according to detailed protocols37,43–45. Met-tRNA\textsubscript{Met} tRNA\textsubscript{A1}, Val-tRNA\textsubscript{Val}, Glu-tRNA\textsubscript{Glu}, Asp-tRNA\textsubscript{Asp}, and Leu-tRNA\textsubscript{Leu}(CUN) were prepared as described previously68,69. Ala-tRNA\textsubscript{Ala} and Gin-tRNA\textsubscript{Gin} were prepared by hydrophobic tagging69. Aminoacyl-tRNAs (aa-tRNAs) were precipitated with ethanol and dissolved in water.

Concentrations of aa-tRNAs were determined photometrically by absorbance measurements at 260 nm and by liquid–liquid scintillation counting where applicable (Ultima Gold XR, Perkin Elmer).

The experiments were carried out in buffer A (30 mM Tris–HCl [pH 7.5], 70 mM NH\textsubscript{4}Cl, 30 mM KCl, 7 mM MgCl\textsubscript{2}) supplemented with GTP (1 mM) at 37°C. To prepare initiation complexes, 70S ribosomes (1 μM) were incubated with GTP (1 mM) under conditions optimal for initiation. Initiation complexes were purified by centrifugation through a sucrose cushion (1.1 M) in buffer A. Ternary complexes were prepared by incubating EF-Tu (two-fold excess over aa-tRNA) with GTP (1 mM), poly(U) (3 μM), and the respective tRNA (0.1 mg/ml) in buffer A for 15 min and then with the purified aa-tRNAs for 1 min. Translation experiments were performed in buffer A at 37°C either as end-point experiments (60–120 s incubation) by hand or using a KinTek RQF3 quench-flow apparatus. Translation experiments were carried out by rapidly mixing initiation complexes (0.2 μM) with the respective ternary complexes as indicated (1 μM) and EF-G (2 μM) with GTP (1 mM). Reactions were quenched by the addition of KOH (0.5 M), and peptides were released by incubation for 30 min at 37°C. After neutralization with acetic acid, samples were analyzed by HPLC (1HPLC was equipped with a 100 μm x 20 cm Lichrosorb RP8 HPLC column; Merck) using a gradient mixture in 0.1% heptafluorobutyratic acid (HFBA) in order to separate the basic (MAK) and KAPK peptides or in 0.1% trifluoroacetic acid. The elution times of the reaction products were established using a set of model peptides synthesized in vitro, in which one of the amino acids is [14C]-labeled10. The extent of product formation was determined from the ratio of [14C]Met in the respective peak to the total [1H]Met activity in the eluate. Quantification of [1H]MetAlaLys[14C]GluVal was based on the amount of [14C]Glu activity in peptides. FS was calculated from the end points of in vitro translation experiments; the values are mean ± s.d. (n = 3 or more independent experiments). Times were cultured by numerical integration using Matlab software according to the model shown in Supplementary Fig. 1c. An analogous model was used to evaluate peptide synthesis in the presence and absence of Glu-tRNA\textsubscript{Glu}. The fraction of non-progressing ribosomes was taken into account by drop-off parameters in the model. Standard deviations of rates were determined by numerical integration using in-built software routines assuming 95% confidence limit. All kinetic experiments were repeated at least twice.

**FS as a function of base-pair free-energy differences.** For the −1 frameshifting of the dnaX mRNA, a stem loop downstream of the slippery sequence is essential26. The stem loop has been proposed to pause the ribosome, thereby increasing the time the tRNAs interact with the slippery sequence codons30. If the pausing time is long enough, frameshifting efficiency, FS, i.e., the probability of ending up in the −1-frame is lower than that of the 0-frame and −1-frame. To test if the free-energy differences suffice to explain the measured FS values, we introduce a free-energy model of frameshifting.

Given are frameshifting efficiencies FS\textsubscript{experiment} = (FS\textsubscript{experiment,0} − FS\textsubscript{experiment,−1}) measured for N different mRNA sequences (Fig. 1, Supplementary Table 1). Here, we analyze N = 64 different mRNA variants.

Upon frameshifting, the mRNA–tRNA base-pairing nucleotides change and therefore the free energy of the mRNA–tRNA interaction changes. Let us assume that the efficiency FS of the ith mRNA variant solely depends on the difference between the free energy of the −1-frame Fi, and of the 0-frame G0. In this case, the efficiencies can be determined by the ratio of Boltzmann probabilities

\[
FS = \frac{\exp \left( \frac{-G_i}{kT} \right)}{1 + \exp \left( \frac{-G_i}{kT} \right)} \Delta G_i = G_i - G_0, \quad FS_{\text{experiment,0}} = \frac{G_0}{kT} \ln \left( \frac{FS_{\text{experiment,0}}}{1 - FS_{\text{experiment,0}}} \right). \tag{1}
\]

Hence, if ΔG\textsubscript{i} for the ith mRNA variant is negative, i.e., the free energy of the −1-frame is lower than that of the 0-frame, the corresponding FS is larger than 50%.

During dnaX−1 frameshifting, the two tRNAs, which were located in the ribosomal P and A sites prior to translocation, are interacting with the first and second positions of the mRNA, respectively. Due to the shift of the reading frame, the base pairs in the codons may change, depending on the sequence (compare Fig. 1a). Each codon consists of three base pairs which are denoted by either P1, P2, P3 or A1, A2, A3, where the letter corresponds to the P-site or A-site codon and the number corresponds to the position of the base pair in the codon.

The second assumption, which will be tested using the free-energy model, is that the free-energy difference ΔG\textsubscript{i} only arises from the free-energy differences of the base pairs at the individual positions present in the 0-frame and −1-frame, and further, that these are additive. Base-pair changes are denoted by their codon position and the two base pairs, e.g., P1 A→U−C corresponds to changing the first base pair A of the 0-frame to U of the −1-frame. In the N = 64 mRNA sequences, n = 16 base-pair changes are found: at the first positions of both codons (P1 and A1), six base-pair changes each (A→U−G, A→U−C, A→G−U, U−C, U−G), at the second positions (P2 and A2), two base-pair changes each (C→G−A, C→G−U). The base pairs at the second positions (P2 and A2) remain unchanged in frameshifting for all mRNA variants and therefore do not contribute to the free-energy differences ΔG\textsubscript{i}. Here, S denotes the modified nucleotide m\textsubscript{1}G\textsubscript{2}U, which is present in the anticodon of tRNA\textsubscript{Gln} (Supplementary Fig. 4).

The estimated free-energy difference ΔG\textsubscript{frameshifting} between the −1-frame and the −1-frame for the ith mRNA sequence can then be calculated from the sum of the individual free-energy differences ΔG\textsubscript{pairs} of the base-pair combinations

\[
\Delta G_{\text{frameshifting}} = \sum_{i=1}^{N} \Delta G_i \cdot M_i = \sum_{i=1}^{N} \Delta G_i \cdot M_i \cdot \Delta G_{\text{pairs}}. \tag{2}
\]

where M\textsubscript{i} is 1 if the free-energy difference ΔG\textsubscript{i} contributes to ΔG\textsubscript{j} −1 if −ΔG\textsubscript{j}.
contributes to ΔGp and if ΔGbp does not contribute to ΔGp. For a compact notation, the base-pair free-energy differences are combined into a vector

\[ ΔG_b = [ΔG_{bp}; ΔG_{bp}; \cdots; ΔG_{bp}]^T, \]

\[ = [ΔG(P1A \ U \ U - C \ U); ΔG(P1A \ U \ U - U \ U); \cdots; ΔG(A3C \ G - U \ G)]^T. \]

Writing all the estimated free-energy differences ΔG\text{est} of the N mRNA sequences as a vector,

\[ ΔG_{\text{est}} = (ΔG_{\text{est}1}; ΔG_{\text{est}2}; \cdots; ΔG_{\text{est}N})^T, \]

Equation (2) can be expressed as a matrix multiplication, ΔG\text{est} = M ⋅ ΔGp, where M is an N x n matrix with entries M_{ij}. From the estimated free-energy differences ΔG\text{est}, using Eq. (1), we can calculate the frameshift efficiencies ΔG\text{model} which are now a function of the base-pair free-energy differences.

**Metropolis with Bayesian Inference.** The aim is to find the individual base-pair free-energy differences ΔGbp, that best reproduce the measured frameshift efficiencies ΔG\text{experiment}. Using Bayesian inference, the probability for the base-pair free-energies is

\[ P(ΔG_{bp}|ΔG_{\text{experiment}}) ∝ P(ΔG_{\text{experiment}}|ΔG_{bp}) \cdot P(ΔG_{bp}), \]

where P(ΔG\text{est}) is the prior probability of the base-pair free-energy differences, and P(ΔG\text{experiment}|ΔG\text{est}) is the probability of observing specific frameshift efficiencies for a given base-pair free-energy differences ΔG\text{est}.

\[ P(ΔG_{\text{experiment}}|ΔG_{bp}) = \prod_{i=1}^{n} \frac{1}{\sqrt{2πσ^2_{\text{experiment},i}}} \exp \left( -\frac{(FE_{\text{experiment},i} - FE_{\text{model},i})^2}{2σ^2_{\text{experiment},i}} \right), \]

where σ\text{experiment,i} is the standard deviation of FE\text{experiment,i} obtained from repeated measurements (Fig. 1b, Supplementary Table 1), and FE\text{model,i} is the ith entry of the vector of frameshift efficiencies FE\text{model} estimated from ΔG\text{est} using Eqs. (1) and (2). The prior distribution P(ΔG\text{est}) of the vector of base-pair free-energy differences ΔG\text{est} is

\[ P(ΔG_{\text{est}}) = \prod_{i=1}^{N} P(ΔG_{bp,i}), \]

where P(ΔG\text{est}) is the prior distribution of free-energy difference of the jth base pair. This prior distribution was chosen to be a uniform distribution between -25 and 25 kJ/mol.

P(ΔG\text{est}|ΔG\text{experiment}) was sampled using the Metropolis Monte Carlo algorithm in two independent calculations with 10^6 steps. To that aim, we used the function

\[ f(ΔG_{\text{experiment}}|ΔG_{bp}) = P(ΔG_{\text{experiment}}|ΔG_{bp}) \cdot P(ΔG_{bp}), \]

which is proportional to the desired probability distribution (compare to Eq. (4)).

The initial free-energy difference values ΔG\text{est} were set to 0 kJ/mol and the function f was evaluated. For each Metropolis step, n sub-steps were carried out. For each subset j, first, a new value for the ΔG\text{est} was drawn from a normal distribution centered on the current value with a standard deviation of 0.2 kJ/mol. Then, the function f was evaluated with the new ΔG\text{est} and the ratio a of the new and previous value of f was used as the acceptance ratio: If a > 1, the new ΔG\text{est} was accepted. If a < 1, a random number u between 0 and 1 was drawn and the new ΔG\text{est} value was accepted if u ≤ a and rejected otherwise.

**Determination of independent free-energy differences.** The mean μ and standard deviation σ of the probability distributions of the base-pair free-energy differences ΔG\text{est}, obtained from Metropolis sampling of P(ΔG\text{est}|ΔG\text{experiment}), are shown in Supplementary Fig. 5a. For 10 of the 16 base-pair combinations σ is small (Supplementary Fig. 5a, green background) showing that the ΔG\text{est} of these combinations is well determined.

For the remaining six base-pair combinations with large σ values, the absolute ΔG\text{est} values are not determined, but their ΔG\text{est} values sampled during the calculations show strong mutual correlations (Supplementary Fig. 6a). The ΔG\text{est} values of the three base-pair changes P3 G → U, A1 A/U → C/U, and A1 U/A → G/A show strong positive pairwise correlations. The same is observed for the other three base-pair changes P3 C/G → U/G, A1 A/U → C/U, and A1 U/A → G/A. Since strong positive correlations for pairs of ΔG\text{est} values means that the difference between them is determined, we ran additional Metropolis sampling calculations, now with ΔG\text{est} set to zero for P3 G/S → A/S (Supplementary Fig. 5b). As expected from the correlation, the σ of the ΔG\text{est} values for A1 A/U → G/U and A1 U/A → G/A is reduced. Analogously, setting ΔG\text{est} of P3 C/G → U/G to zero, leads to small σ values for A1 A/U → C/U and A1 U/A → G/A (Supplementary Fig. 5c). Setting both ΔG\text{est} values to zero at the same time, results in low σ values for all base-pair combinations (Supplementary Fig. 5d). This result enabled us to determine probability distribution for ΔG\text{est} values for pairs of base-pairs (Fig. 2c).

To check the consistency of the free-energy model, we recalculated the FE values, using Eqs. (1) and (2), from all the ΔG\text{est} values after omitting the first 20% of the Metropolis steps. The rmsd between the measured FE\text{experiment} and those obtained from the model FE\text{model} was 2.52% for all of these cases (Supplementary Fig. 5a–d, Fig. 2d). To further test whether the FE\text{model} values have converged, we first extracted intervals of the sampled ΔG\text{est} values from the first 1000 Metropolis steps (Supplementary Fig. 7a, number of steps). For each interval, the first 20% Metropolis steps were omitted and then, the rmsd between the FE\text{model} values and the mean FE\text{model} values obtained from the ΔG\text{est} values was calculated. For the two independent calculations, the rmsds drops to the same value showing that the FE values have converged (Supplementary Fig. 7a).

For cross validation, iteratively, each mRNA variant was selected and the distributions of ΔG\text{est} were calculated from the FE values of all other mRNA variants. Next, the FE for the selected mRNA variant was predicted from the obtained ΔG\text{est} values (Fig. 2e). The rmsd between the predicted and the measured FE values as a function of Metropolis steps is shown in Supplementary Fig. 7b and converges to 4.1%.

**Estimation of the kinetic contribution to frameshifting.** So far, the frameshifting efficiency was assumed to be solely determined by the free-energy difference between the 0-frame and the −1-frame AG. Next, to challenge this assumption and to test if the frameshifting efficiency also depends on kinetics, we expanded the model by a term that describes kinetic effects. Given the two states, 0-frame and −1-frame, and the rates between these states, k_{0→1} and k_{1→0}, the master equation for the time-dependent probability of being in the −1-frame P_{−1}(t) can be written as

\[ \frac{dP_{−1}(t)}{dt} = k_{0→1} [1 − P_{−1}(t)] − k_{1→0} P_{−1}(t). \]

Assuming that the system starts in the 0-frame and, hence, P_{−1}(0) = 0, the solution of the master equation is given by

\[ P_{−1}(t) = \frac{k_{0→1}}{k_{0→1} + k_{1→0}} \left[ 1 − \exp(-(k_{0→1} + k_{1→0}) t) \right]. \]

With transition rates given by Arrhenius’s law

\[ k_{0→1} = A \exp \left( \frac{−ΔG^2}{k_b T} \right), \]

\[ k_{1→0} = A \exp \left( \frac{ΔG^2 − ΔG^2}{k_b T} \right), \]

with barrier height ΔG^2 and attempt frequency A, the probability of being in the −1-frame reads

\[ P_{−1}(t) = FS \left[ 1 − \exp \left(−(k_{0→1} + k_{1→0}) t\right) \right], \]

where FS is the equilibrium frameshift efficiency as described in Eq. (1). Obviously, for t → ∞, the probability P_{−1}(t) converges to P_{−1}(t → ∞) = FS, the equilibrium efficiency. The time window for transitions between the two frames is limited and ends with the completion of tRNA translocation. We define the kinetic factor κ as \[ κ = \exp \left(−\frac{ΔG^2}{k_b T}\right) \cdot \left[ 1 + \exp \left(\frac{ΔG^2}{k_b T}\right) \right]. \]

This model of the possible non-equilibrium dynamics of frameshifting leaves

\[ C = A \exp \left(\frac{ΔG^2}{k_b T}\right), \]

as the only remaining unknown parameter. The kinetic factor κ is different for different mRNA variants, depending on the ΔG which in turn depends on the base-pair free-energy differences.

Since we are interested in the kinetic contribution to frameshifting, we performed additional Metropolis sampling calculations, with the mean κ of all kinetic factors included as a nuisance parameter. The probability for the base-pair free-energies is then

\[ P(ΔG_{bp}|ΔG_{\text{experiment}}) ∝ P(ΔG_{\text{experiment}}|ΔG_{bp}) \cdot P(ΔG_{bp}) \cdot P(κ), \]

where P(κ) is the prior distribution of κ for which a uniform distribution between 0 and 1 was chosen. To sample the probability, in each Metropolis step, the parameter C is calculated from κ which results in the mean kinetic factor κ is calculated and from that the κ value for each sequence.

Two independent calculations were carried out with 10^6 Metropolis steps each, as described above, now with an additional sub step for drawing a new value for κ from a normal distribution centered on the current value of κ with σ = 0.001. The obtained probability distribution of κ is shown in Fig. 3b.

**Independent set of FS values.** To further test the validity of the free-energy model, we applied it to an independent data set of 21 FS values previously published by Tsushihashi et al. (Supplementary Table 3). The
Table 1 Base-pair changes contributing to the codon-anticodon interactions in the mRNA sequences of the Tsuichihashi data set

| Base-pair position | Base-pair change |
|--------------------|------------------|
| P1                 | A-U → C-U        |
| P2                 | A-U → U-U        |
| P3                 | G-S → A-S        |
| A1                 | A-U → U-U        |
| A2                 | G-S → A-S        |
| A3                 | G-A → G         |

Modified nucleotides: S = mmmU; U = mmmU; Q = queuosine; V = cmoU; M = ααU; C = 2-lysidine

Changes highlighted in bold are present in our data set as well (compare Fig. 2a–c).

codon–anticodon base-pair interactions of the 21 mRNA variants, can be described by 26 free-energy differences (Table 1). Metropolis sampling of P (ΔGΔP(FSexperiment)) was carried out as described above (without kinetic factor), now for the 26 free-energy differences and 21 FS values. In the first step (Supplementary Fig. 8a), the ΔGΔP of 9 base-pair changes have a low standard deviation σ, showing that their free-energy differences are determined by the Tsuichihashi FS data set (green histograms in Figs. 2a, b, and 3a, b). In steps 2–9 (Supplementary Fig. 8b–i), iteratively, the ΔGΔP of a base-pair change that had a large σ in all previous steps was set to 0 kJ/mol. Monitoring for which base-pair changes, the σ values were reduced, we could identify which ΔGΔP values of pairs of base-pair changes were determined (Figs. 2c and 3c). Finally, in step 10, all the ΔGΔP values set to 0 kJ/mol in steps 2–9 were set to 0 kJ/mol at the same time (Supplementary Fig. 8f). For all steps 1–10, the rmsd for the measured FS values and those obtained from the model was found to be between 2.51% and 2.59%. For the Metropolis calculations of step 10 (Supplementary Fig. 8f), the rmsd was calculated as a function of Metropolis steps (Supplementary Fig. 7c) showing the convergence of the Metropolis sampling.

Comparison of P-site and A-site base pairs. The environment of the base pairs is different in the ribosomal P and A sites. The free-energy differences for C-A → C-U and G-A → G-U could not be obtained directly from the set of FS values. Under the assumption that A-U and A-A base pairs have the same free energy, the free-energy difference for P1 C-A → C-U was obtained by subtracting ΔGΔP of P1 U-A → C-A from the P1 A-U → C-U (Fig. 2a, fifth and first rows). For the corresponding A-site free-energy difference, the ΔGΔP of a pair of base-pair changes P3 C-G → U-G and A1 U-U → A-A (Fig. 3c, fourth row) was subtracted from that of another pair P3 C-G → U-G and A1 U-U → C-U (Fig. 3c, third row). For G-A → G-U, we used the ΔGΔP of Fig. 2a, third and sixth rows, for the P site, and Fig. 2c, first and second rows, for the A site.

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

Data availability

The authors declare that the data supporting the findings of this study are available within the paper and its supplementary information files.

Code availability

The code used for Monte Carlo sampling and analysis is available from the corresponding author upon request.

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Author contributions

N.C. and N.K. prepared materials and conducted experiments, N.C. conducted rapid kinetic measurements. L.V.B. built and analyzed the free-energy model. L.V.B., N.C., N.K., F.P., M.V.R., and H.G. discussed the results and wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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