High-Resolution DNA Dual-Rulers Reveal a New Intermediate State in Ribosomal Frameshifting

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1. **Sequences of the mRNA and DNA rulers**

All DNA probes and 5′-biotinylated mRNA were purchased from Integrated DNA Technologies (IDT, idtdna.com). 3′-biotinylated mRNA was from Horizon Discovery (horizondiscovery.com). The sequence of the mRNA containing the GA\_G motif was 5′-Bio-C AAC UGU UAA UUA AAU UAA AUU AAA AAG GAA AUA AAA AUG UUU GAA AAA AAG UAC GUA AAU CUA CUG CUG AAC UC-3′ (Bio: biotin functionalized). The slippery motif that causes frameshifting is underlined.

Sequences of the DNA rulers are listed below. The ribosome complexes were 5′-biotinylated unless specially marked. The bases in bold are complementary with the mRNA. TEG: linker from IDT.

To probe Pre:

5′-probe:  (R5-pre-12):  3′-A ATT TAA TTT TT A₅₀/TEGBio/-5′

(R5-pre-14):  3′-A ATT TAA TTT TTC C T₅₀/TEGBio/-5′

3′-probe:  (R3-pre-14):  3′-G CAT TTA GAT GAC GTG AAC T/TEGBio/5′

For force calibration:

3′-probe:  (Cal-12):  3′-T TTA GAT GAC GA/TEGBio/5′

(Cal-13):  3′-AT TTA GAT GAC GA/TEGBio/5′

(Cal-14):  3′-CAT TTA GAT GAC GA/TEGBio/5′

To probe Post:

5′-probe:  (R5-post-14):  3′-ATT TAA TTT TTC CT T₅₀/TEGBio/-5′

3′-probe:  (R3-post-14):  3′-G CAT TTA GAT GAC GAG AAC TC/TEGBio/5′

To probe 3′-biotinylated Post:

5′-probe:  (R5-post-13):  3′/-TEGBio/TTA AAT ATT TAA TTT TTC C -5′

(R5-post-14b):  3′/-TEGBio/ TTA AAT ATT TAA TTT TTC CT -5′
2. Experimental procedures

2.1 Preparation of the ribosome complexes

We first prepared the MFE-post complex. All the mixtures were in TAM10 buffer, which consisted of 20 mM Tris-HCl (pH 7.5), 10 mM MgAc2, 30 mM NH4Cl, 70 mM KCl, 5 mM EDTA, and 7 mM 2-mercaptoethanol (βME). Three mixtures were prepared: initiation ribosome complex mix, EF-Tu mix without EF-G(TuG), and aminoacylation mix. The ribosome mix contained 1 μM ribosome, 1.5 μM each of IF1, IF2, IF3, 2 μM of mRNA, 4 μM of charged fMet-tRNA\(^{f\text{Met}}\), and 4 mM of GTP. The TuG mix contained 4 μM EF-Tu, 0.4 μM EF-Ts, 4 mM GTP, 4 mM PEP, 2 μM EF-G, and 0.02 mg/mL pyruvate kinase. The aminoacylation mix contained 100 mM Tris (pH 7.8), 20 mM MgAc2, 1 mM EDTA, 4 mM ATP, 7 mM βME, 2 μM LysRS (tRNA\(^{\text{Lys}}\) synthetase), 5% V of S-100 (tRNA aminoacylation synthetase), 2 \(A_{260}\)/mL tRNA\(^{\text{Lys}}\), 2 \(A_{260}\)/mL tRNA\(^{\text{Glu}}\), 0.25 mM phenylalanine, and 0.25 mM glutamic acid. The ribosome complex MFE-post were made by mixing the initiation complex mix, EF-Tu mix with EF-G, and the aminoacylation mix with volume ratio of 1:2:2 and incubated at 37 °C for 30 min. The product was processed for gradient ultracentrifugation at 120,000×g with 1.1 M sucrose cushion overnight. The pellet of the ribosome complexes was then collected and dissolved in TAM10 buffer. All chemicals were purchased from MilliporeSigma unless specified otherwise.

Then the MFEK-pre ribosome complex was prepared from the MFE-post ribosome complex. Here, two mixtures were prepared: EF-Tu mix without EF-G (Tu0G) and aminoacylation mix. The Tu0G mix contained 4 μM EF-Tu, 0.4 μM EF-Ts, 4 mM GTP, 4 mM PEP, and 0.02 mg/mL pyruvate kinase. The aminoacylation mix contained 100 mM Tris (pH 7.8), 20 mM MgAc2, 1 mM EDTA, 4 mM ATP, 7 mM βME, 2 μM LysRS (tRNA\(^{\text{Lys}}\) synthetase), 2 \(A_{260}\)/ml tRNA\(^{\text{Lys}}\), and 0.25 mM lysine. The ribosome complex MFEK-pre were made by mixing the MFE-post complex mix, EF-Tu mix without EF-G, and the aminoacylation mix with volume ratio of 1:2:2 and incubated at room temperature for 15 min. The product was processed for gradient ultracentrifugation at 120,000×g with 1.1 M sucrose cushion overnight. The pellet of the ribosome complexes was then collected and dissolved in TAM10 buffer.

To synthesize the Post complex without antibiotics, 1 μM Pre complex was incubated with 2 μM EF-
G, 4 mM GTP, 4 mM PEP, and 0.02 mg/ml pyruvate kinase at 37 °C for 30 min.

To synthesize Post with fusidic acid (FA), 1 µM Pre complex was incubated with 0.50 mM FA, 2 µM EF-G, 4 mM GTP, 4 mM PEP, and 0.02 mg/ml pyruvate kinase at 37 °C for 30 min. The mixture was diluted before adding to sample wells, and the final concentration of FA was 0.25 mM or 25 µM.

2.2 Sample immobilization and magnetic labeling

The sample well, with a bottom surface area of 4×3 mm² that was coated with biotin, was incubated with 0.25 mg/mL streptavidin for 50 min. After rinsing off the extra streptavidin, the ribosome complexes were immobilized on the surface via the 5'- or 3'-end biotin on the mRNA by incubating for 1 h. Then 1 µM probing DNA was added and incubated overnight to form duplexes with the uncovered mRNA. Afterward, streptavidin-coated magnetic beads (M280, Invitrogen) were introduced into the sample well and incubated for 2 h. Before the force measurements, the sample was magnetized for 2 min using a permanent magnet (~0.5 T) and centrifuged (5427R, Eppendorf) at 820×g for 20 min to remove non-specific bound magnetic particles.

2.3 Force spectroscopy measurements

The super-resolution force spectroscopy (SURFS) technique was invented in our group. Details have been published elsewhere (Jia, H., Tsai, T.-W. & Xu, S. Appl. Phys. Lett. 113, 193702, 2018). Briefly, it uses ultrasound to generate acoustic radiation force on the magnetic beads labeled on the DNA-mRNA duplexes, and employs an atomic magnetometer to measure the sample’s magnetic signal (Supporting Figure S1). The ultrasound amplitude is gradually increased; at each amplitude, the sample’s magnetic signal is obtained. When the acoustic radiation force exceeds the dissociation force of the duplex, an abrupt decrease in magnetic signal will be observed. This is because after the magnetic beads dissociate from the surface, their magnetic dipoles will become random due to Brownian motion. Therefore, the dissociation force of the DNA-mRNA duplexes can be determined from the force spectra. Because the duplexes with different bp numbers show discrete dissociation forces under our experimental condition, the length of the duplexes can be precisely determined from the dissociation force. In this work, each force spectrum was repeated at least three times to ensure reproducibility. The force resolution was
typically between 0.9 pN (at ~20 pN) and 1.6 pN (at ~50 pN). The key components of our SURFS technique studying ribosomal frameshifting are shown in Figure S1.

**Figure S1.** SURFS for studying ribosomal frameshifting. The sample was placed on the sample holder that was driven by a linear motor. The piezo disk produced acoustic radiation force on the sample via the magnetic bead. An atomic magnetometer was used for detection. The call-out box shows the detailed sample construct, which included immobilized ribosome complex with mRNA, duplex formed between the DNA ruler and exposed mRNA, and magnetic bead on the DNA ruler.
3. **Dissociation force as a function of duplex base pair (bp)**

DNA rulers with different lengths (Cal-12, Cal-13, and Cal-14) were used to form 12-, 13-, and 14-bp duplexes with the mRNA, respectively. Their dissociation forces were measured to be 22.6, 34.2, and 46.5 pN, respectively. These force values were used as the basis to assign the number of bp between the DNA rulers and the mRNA on the ribosome complexes.

![Figure S2. Force spectra of DNA-mRNA duplexes with different lengths.](image_url)
4. Formation of Post(-1*) at a lower fusidic acid concentration

The concentration of fusidic acid was 25 μM. The DNA ruler was similar to the one used in Figure 2 in terms of the bp numbers: Post(0) would form a 14-bp duplex with this DNA, whereas Post(-1*) would form a 12-bp duplex. The force spectrum showed two dissociation forces, 50.3 pN for Post(0) and 30.9 pN for Post(-1*). Both values are in excellent agreement with the results in Figure 2d.

**Figure S3.** Formation of Post(-1*) at a lower fusidic acid concentration. (a) The probing scheme with DNA ruler R5-post-14b. (b) Force spectra.
5. Reproducibility of the sub-nucleotide difference between Post(-1*) and Post(-1)

Three independent force spectra were obtained using the schemes in Figure 4. The excellent overlapping among them confirmed the reproducibility of the sub-nucleotide difference between Post(-1*) and Post(-1).

Figure S4. Three repeated measurements to confirm the sub-nucleotide difference between Post(-1*) and Post(-1).