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

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Ribosomal frameshifting is an important pathway used by many viruses for protein synthesis that involves mRNA translocation of various numbers of nucleotides. Resolving the mRNA positions with subnucleotide precision will provide critical mechanistic information that is difficult to obtain with current techniques. We report a method of high-resolution DNA rulers with subnucleotide precision and the discovery of new frameshifting intermediate states on mRNA containing a GA_7G motif. Two intermediate states were observed with the aid of fusidic acid, one at the "0" reading frame and the other near the "−1" reading frame, in contrast to the "−2" and "−1" frameshifting products found in the absence of the antibiotic. We termed the new near-"−1" intermediate the Post(−1*) state because it was shifted by approximately half a nucleotide compared to the normal "−1" reading frame at the 5′-end. This indicates a ribosome conformation that is different from the conventional model of three reading frames. Our work reveals uniquely precise mRNA motions and subtle conformational changes that will complement structural and fluorescence studies.

Introduction

Probing the precise motion of nucleic acids is essential to studying the mechanism of their many biological functions. One of the most important cases is the ribosomal translocation during protein synthesis, in which the ribosome usually moves 3 nt per step on the messenger RNA (mRNA).[1,2] Frameshifting occurs when the ribosome moves by 1, 2, or 4 nt, termed as "−2", "−1", and "+1" frameshifting, respectively, which are important for many viral infectiousness.[3–5] Technical approaches for revealing translocation mechanism include X-ray crystallography, cryo-electron microscopy (cryo-EM), FRET), and kinetics methods.[6–12] Detailed mechanistic insights have been obtained by trapping various intermediate states via mutations and antibiotics. However, direct information regarding the mRNA positions on a slippery sequence that causes frameshifting, thereby branching of different reading frames, remains limited.

We recently developed DNA rulers based on force-induced remnant magnetization spectroscopy (FIRMS).[13] With 2–4 pN force resolution, we were able to probe the mRNA positions with single-nt resolution and observed a looped mRNA conformation trapped by antibiotics. Meanwhile on the technical front, we invented super-resolution force spectroscopy (SURFS) by implementing acoustic radiation force, which was automatable and more precise than the centrifugal force used in FIRMS.[14] With 0.5 pN force resolution, we observed subnucleotide conformational difference between two different ribosome states within one translocation step.[15] Compared to other force-based techniques for ribosome research, mainly atomic force microscopy (AFM) and optical tweezers,[16,17] our approaches have the unique advantages of resolving different reading frames within the same sample, and probing both the moving edges of the ribosome.

Here, we report high-resolution DNA rulers based on SURFS that can resolve multiple intermediate states with subnucleotide precision for the first time. Using an mRNA containing the GA_7G slippery motif, in which both "−1" and "−2" frameshifting complexes were previously identified by FIRMS,[18] we observed another intermediate state with fusidic acid, which is an antibiotic to lock the EF–G conformational change after GTP hydrolysis. Our results showed that two products were obtained. One finished 3 nt translocation we named the "0" state to represent no frameshifting event. The other was the slightly compressed "−1*" state, in which the mRNA on the entrance side and the ribosome trailing edge was less exposed than the regular "−1" state. This report demonstrates a new method with unprecedented resolution in studying molecular motors.

Results

The design of the DNA rulers and their validation are shown in Figure 1. Detailed experimental procedures are described in the Supporting Information and Supporting Figure S1. The ribosome-mRNA complex was immobilized on the surface at the 5′-end of the mRNA. DNAs with sequences complementing either the 5′- or 3′-ends of the mRNA were labeled with magnetic beads (Figure 1a). They would form duplexes of different numbers of base pairs with the mRNA, depending on the ribosome position. In each experiment, only one DNA ruler was

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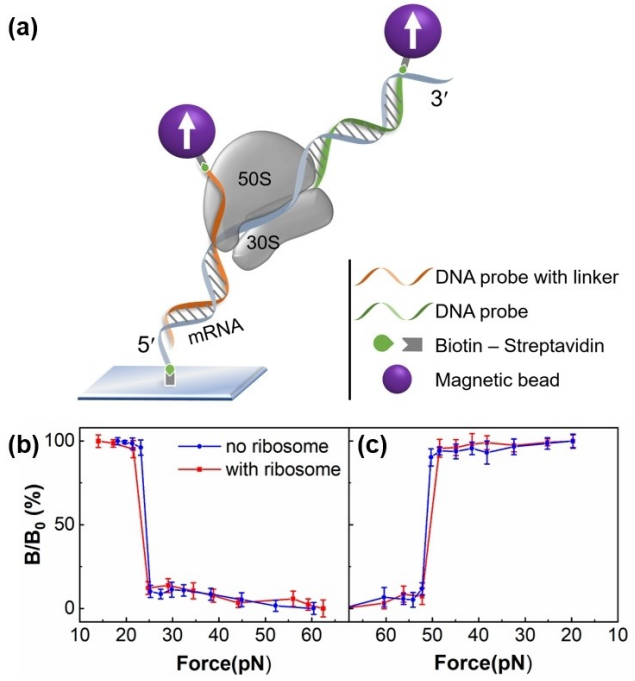

Figure 1. Principle and calibration of high-resolution DNA rulers for ribosomal frameshifting. a) Detection scheme. b), c) Force spectra of the DNA-mRNA duplexes with and without the ribosome, from the 5′- and 3′-ends, respectively.

used. The 5′-end DNA rulers contained a 50-nt linker to overcome the steric hindrance between the surface and the ribosome.[13] The precise lengths of the mRNA uncovered by the ribosome were determined by the dissociation forces of the DNA-mRNA duplexes. Therefore, the ribosome footprints will be deduced. The correlation of the duplex dissociation force and the ribosome's coverage on the mRNA has been demonstrated earlier, in which we found that the ribosome covers 27 nt in the pre- and post- states, while the first mRNA nt out of the leading edge (3′-end) is +12 from the first nucleotide in the P-site.[13,18]

Force spectra of the duplexes were obtained by measuring the magnetic signal of the sample as a function of acoustic radiation force exerted on the duplexes via the magnetic bead, in which the dissociation force was indicated by a decrease of the magnetic signal. Figure 1b shows the force spectra of DNA rulers that formed 12-bp duplex with the 5′-end mRNA, in the presence and absence of the ribosome in the pre-translocation state (Pre). The sequences are shown in the Supporting Information. The dissociation forces are 23.2 ± 0.9 and 24.1 ± 0.9 pN, respectively, which are the same within the uncertainty. Figure 1c shows the spectra of the DNA ruler that formed 14-bp duplexes with the 3′-end mRNA, with and without the ribosome. The dissociation forces are nearly identical, at 50.4 ± 1.6 and 51.3 ± 1.6 pN, respectively. The results confirmed that the ribosome itself does not interfere with the dissociation forces of the DNA-mRNA duplexes. In addition, we have performed force calibration for 12-, 13-, 14-bp DNA-mRNA duplexes (Figure S2), which yielded dissociation forces of 24.3, 36.8, and 50.1 pN, respectively, with typical uncertainties between 0.9 and 1.6 pN. Therefore, the mRNA positions during frameshifting can be precisely measured.

Figure 2 shows the results of ribosomal frameshifting on the GA_7G motif. The post-translocation products (Post) were obtained by incubating the pre-translocation complex (Pre) with EF–G and GTP, in the absence and presence of fusidic acid, with details provided in the Supporting Information. The 5′-end DNA ruler, R5-post-14, was designed to form 12-, 13-, and 14-bp duplexes with the post-complexes with 1-, 2- and 3-nt movements, which are named Post(−2), Post(−1), and Post(0), respectively (Figure 2a). The negative numbers indicate the frameshifting position. The 3′-end ruler, R3-post-14, was designed similarly, which would form 14-, 13-, and 12-bp duplexes with Post(−2), Post(−1), and Post(0), respectively. The 12–15 bp duplexes were designed such that the dissociation forces stayed within the optimal detection range (ca. 10–70 pN) of SURFS.[14] By probing both ends of the mRNA, we obtained the precise frameshifting steps, which was a unique advantage of our method. Figure 2b and c show the results of frameshifting without fusidic acid. On the 5′-end, we observed dissociation forces at 22.2 and 36.6 pN, corresponding to 12 and 13 bp duplexes respectively. On the 3′-end, we observed forces at 50.4 and 36.6 pN, corresponding to 14 and 13 bp duplexes. Therefore, both Post(−2) and Post(−1) were formed, but not Post(0). Five repeated measurements yielded a standard deviation of 1.2 pN for both the 14 and 13 bp duplexes, demonstrating high reproducibility of our assay. The result of observing both Post(−2) and Post(−1) are consistent with our previous study,[18] but here with much better resolution. In

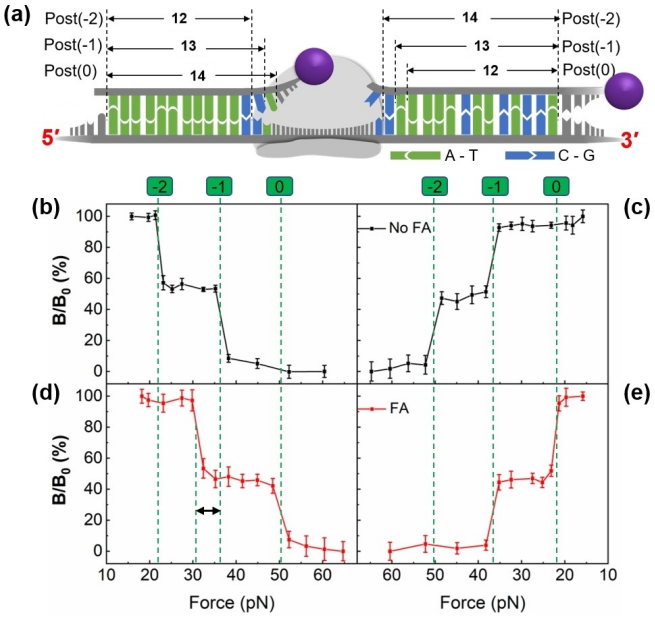

Figure 2. Resolving multiple frameshifting intermediates. a) Probing scheme for resolving the three reading frames from both the 5′- and 3′-ends. b), c) Force spectra in the absence of fusidic acid (FA), from the 5′- and 3′-end, respectively. Both Post(−2) and Post(−1) were revealed, but not Post(0). d), e) Force spectra in the presence of fusidic acid, which showed the formation of Post(−1*) and Post(0), from the 5′- and 3′-end, respectively. The double arrow in d indicates the force gap between "−1" and the new "−1*" states.

addition, we confirmed the frameshifting products at the 5′-end of the mRNA for the first time.

To investigate the possible frameshifting intermediates, we performed the same experiments in the presence of fusidic acid at 0.25 mM (Figure 2d and e). In contrast to the Post(−2) and Post(−1) products without fusidic acid, we observed the Post(0) state, indicated by dissociation forces of 50.2 pN in Figure 2d and 22.4 pN in Figure 2e, and a new intermediate state that differs from all the three reading frames. Its dissociation force of 30.9 pN at the 5′-end indicates its movement at the trailing edge was between 1–2 nt but closer to 2 nt, whereas the force of 36.9 pN in Figure 2e indicated normal "−1" frameshifting movement at the leading edge. We thus term this state as Post (−1*). Notably, no Post(−2) was observed in either edge. In addition, we investigated the effect of fusidic acid at a much lower concentration of 25 μM. The values are almost identical to the data in Figure 2d which showed two dissociation forces of 30.9 and 50.3 pN, corresponding to Post(−1*) and Post(0), respectively (Figure S3).

To verify that these results were due to fusidic acid effects on EF–G, we probed the Pre complex in the presence of fusidic acid only, using the DNA rulers showed in Figure 1. The results are shown in Figure 3. Both panels indicate a single ribosome population. The dissociation forces were 24.4 and 50.4 pN, for the 5′- and 3′- ends, respectively. Compared to the values of 23.2 and 50.4 pN obtained in Figure 1 where no fusidic acid was used, we concluded that fusidic acid alone did not cause mRNA movement or aberration in duplex dissociation forces. Therefore, the changes observed in Figure 2 were due to the FA-trapped intermediate states during EF–G catalyzed translocation.

To confirm that the subnucleotide difference at the 5′-end mRNA between Post(−1*) and Post(−1) was real, we designed a different immobilization method and a new DNA ruler (R5-post-13 in the Supporting Information). As shown in Figure 4a, the ribosome complex was immobilized via the 3′- end biotin. This eliminated the potential steric effect in between the ribosome, magnetic bead, and the surface when the 5′-end immobilization method was used. The DNA ruler would form exactly 13 bp duplexes with both Post(−1) and Post(0) due to its limited length. If Post(−1*) was identical to Post(−1), we would only observe a single dissociation force corresponding to the 13-bp duplex. A coarse force scan shown in Figure 4b exhibited two different mRNA positions, both of which were different from the 12-bp duplex which corresponded to Post(−2). A fine scan shown in Figure 4c exhibited two well-resolved dissociation forces, at 30.5 and 37.4 pN respectively. The force difference of 6.9 pN is significantly greater than the force uncertainty, and substantially less than the 14.7 pN difference for a whole nucleotide step between Post(−2) and Post(−1). The fine scan was repeated three times to ensure reproducibility (Figure S4). Therefore, we confirmed that Post(−1*) is a new intermediate state during ribosomal frameshifting on the GA_7G motif.

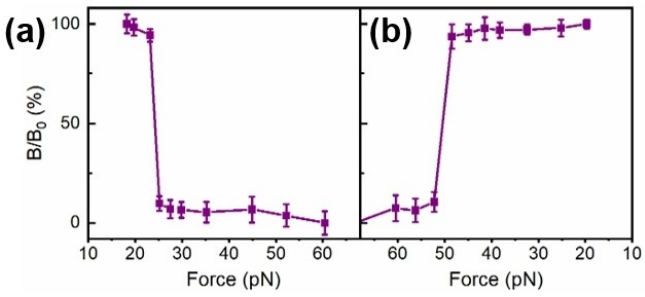

Figure 3. The effect of fusidic acid on the Pre complex. Force spectra of the a) 5′-end duplex and b) 3′-end duplex. The spectra showed no difference from the corresponding ones without fusidic acid (Figure 1).

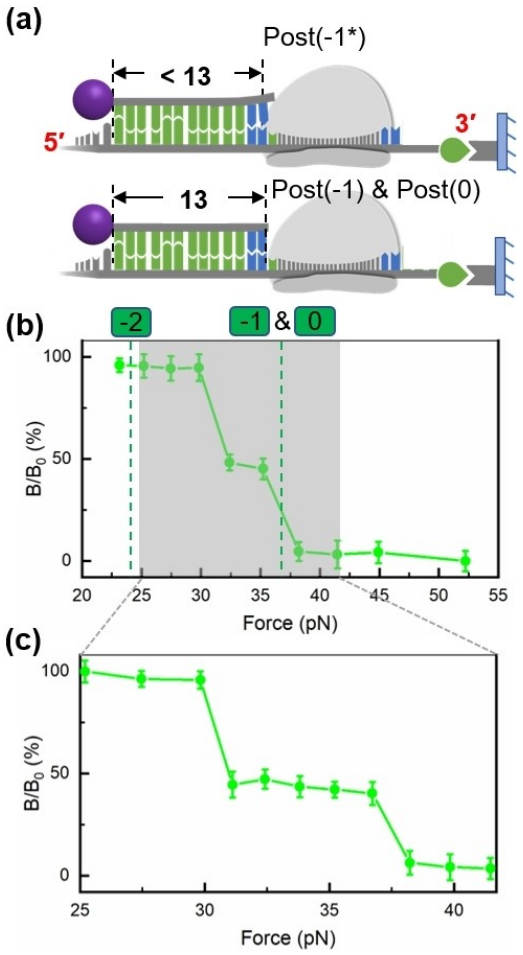

Figure 4. Verification of the Post(−1*) state, which differs by a subnucleotide in 5′-end mRNA position from the conventional "−1" reading frame. a) Probing scheme. b) Coarse force spectrum. c) Fine force spectrum.

Discussion

We compare our results with other mechanistic studies in the literature. Fusidic acid has been commonly used to trap intermediate states during translocation for structural, fluorescence, and kinetic methods. For example, Zhou et al. observed two conformational changes between the two subunits of the ribosome during translocation inhibited by

fusidic acid.[6] Rodnina and co-workers revealed that EF–G engagement trapped by fusidic acid prevented the ribosome small subunit backward transitions.[8] Based on detailed kinetic studies, Ehrenberg and co-workers deduced that fusidic acid functions in three different translocation stages.[10,11] Our results confirm that there are possibly multiple translocation steps that are affected by fusidic acid. Therefore, various subtle products can be formed at different mRNA motifs. In the case of the GA_7G motif that exhibits both "−1" and "−2" frameshifting, fusidic acid caused uneven movement of the mRNA, resulting in two different intermediates of Post(−1*) and Post(0). The subnucleotide difference between the new Post(−1*) state and Post(−1) can only be resolved with our high-resolution DNA rulers.

Our force-based DNA rulers can find broad applications in studying nucleic acid motions during their functions in protein synthesis. The effects of antibiotics on altering the ribosome reading frames can be precisely resolved. Consequently, different proteins will be synthesized. Due to the common applications of frameshifting in viral infections, such as HIV, simarteriviruses, and the current SARS-CoV-2,[19–21] our method can be used to screen for antibiotics to achieve manipulation of promoting or inhibiting the desired reading frames.

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Conflict of Interests

The authors declare no conflict of interests.

Keywords: antibiotics · atomic magnetometer · DNA rulers · force spectroscopy · ribosomal frameshift

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