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**Single-Molecule Mechanical (Un)folding of RNA Hairpins:
Effects of Single A-U to A·C Pair Substitutions and Single Proton
Binding and Implications for mRNA Structure-Induced –1
Ribosomal Frameshifting**

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Abstract

A wobble A·C pair can be protonated at near physiological pH to form a more stable wobble A⁺·C pair. Here, we constructed an RNA hairpin (rHP) and three mutants with one A-U base pair substituted with an A·C mismatch on the top (near the loop, U22C), middle (U25C) and bottom (U29C) positions of the stem, respectively. Our results on single-molecule mechanical (un)folding using optical tweezers reveal the destabilization effect of A-U to A·C pair substitution, and protonation-dependent enhancement of mechanical stability facilitated through an increased folding rate, or decreased unfolding rate, or both. Our data show that protonation may occur rapidly upon the formation of apparent mechanical folding transition state. Furthermore, we measured the bulk -1 ribosomal frameshifting efficiencies of the hairpins by a cell-free translation assay. For the mRNA hairpins studied, -1 frameshifting efficiency correlates with mechanical unfolding force at equilibrium and folding rate at around 15 pN. U29C has a frameshifting efficiency similar to that of rHP (~2%). Accordingly, the bottom 2-4 base pairs of U29C may not form under a stretching force at pH 7.3, which is consistent with the fact that the bottom base pairs of the hairpins may be disrupted by ribosome at the slippery site. U22C and U25C have a similar frameshifting efficiency (~1%), indicating that both unfolding and folding rates of an mRNA hairpin in a crowded environment may affect frameshifting. Our data indicate that mechanical (un)folding of RNA hairpins may mimic how mRNAs unfold and fold in the presence of translating ribosomes.

INTRODUCTION

RNAs form complex and dynamic structures for various biological functions such as serving as templates for the synthesis of proteins and nucleic acids, and as regulators or catalytic centers for transcription, pre-mRNA splicing, and protein synthesis.¹ RNA secondary structures are typically composed of double-stranded stems and various loops, which can further assemble into tertiary structures.²⁻⁴ The RNA stems are stabilized by canonical base pairs including A-U, G-C, and G-U.^{3,5,6} Formation of various non-canonical base pairs such as G-A, U-U, U-C, and A-C pairs⁷⁻¹⁴ contributes to the complex structures and dynamics of RNA. Non-canonical base pairs, in turn, are often involved in RNA tertiary interactions and facilitate binding to proteins, small molecules, and ions.

Shifting of pK_a values and protonation dependent conformational dynamics in RNAs¹⁵⁻²³ are important for the catalytic activities of ribozymes,^{18,19,24-26} small molecule-RNA interactions,^{27,28} protein-RNA assembly,²⁹⁻³¹ and regulation of protein expression.^{32,33} A wobble A-C pair (cis Watson-Crick/Watson-Crick³⁴) (**Figure 1a**) is structurally similar to a wobble G-U pair,^{35,36} with the N1 nitrogen atom of A often having a pK_a near neutrality primarily driven by the formation of a wobble A⁺-C pair with one extra hydrogen bond.^{16,18,19,37-39}

The sequence and structural contexts and environmental factors affect the formation of wobble or non-wobble A-C pairs. For example, an “open” A-C mismatch with no hydrogen bonding interactions and a wobble A-C pair are found in the acceptor stem and anticodon loop of tRNAs, respectively.⁴⁰⁻⁴² A wobble A-C pair also exists in ribozymes such as *Neurospora Varkud* satellite (VS) ribozyme,^{26,43} and the artificial leadzyme with the self-cleavage activity stimulated by micromolar concentrations of lead ions.¹⁸ An RNA duplex containing an A-C pair (compared to A-U, A-A, and A-G pairs) is a more favored substrate for the RNA editing enzyme adenosine deaminase acting on RNA (ADAR).^{44,45} A wobble A-C pair found in the pre-catalytic conformation of intramolecular stem-loop of U6 snRNA

may be critical for regulating the binding and positioning of a catalytic magnesium ion and the dynamic assembly of spliceosomal complexes.⁴⁶⁻⁴⁹ A substitution of an A-U pair with an A-C pair resulting from genetic mutations in the microtubule-associated tau pre-mRNA splice site hairpin may cause dysregulation of alternative splicing and thus brain disorders such as frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17).^{50,51}

Figure 1. Wobble A·C and A⁺·C base pairs and 32-mer hairpins studied in this paper. (a) Chemical structures of wobble A·C and A⁺·C base pairs. (b) RNA constructs. U22C, U25C and U29C are mutants with the single A·C mismatch at the top, middle, and bottom position of the stem, respectively. The mutated residues are shown in red colour. The open and closed arrows indicate the approximate apparent two-state mechanical unfolding and folding transition state positions, respectively, revealed in this work.

Thermal unfolding studies for relatively small model RNAs and DNAs reveal that pH dependent wobble A⁺·C pair formation enhances folding stability.^{9,16,19,52} Single-molecule manipulation using optical tweezers allows the characterization of RNA mechanical

(un)folding at near-physiological buffer conditions at room temperature, and may provide deeper insights into the complex folding free energy landscapes⁵³⁻⁵⁵ and the effects of protonation. In addition, mechanical (un)folding may better mimic how RNA structures (un)fold in physiological conditions.^{13,56-69} For example, during ribosomal translation, mRNA structures in the coding regions need to be unfolded into single strand form at the mRNA entrance tunnel of the small subunit of ribosome.^{70,71} The force exerted by ribosome during translation elongation is about 12-20 pN.^{72,73}

Minus-one programmed ribosomal frameshifting is a mechanism utilized by cells and viruses to regulate the expression of multiple proteins from one mRNA as well as mRNA half-life.⁷⁴⁻⁷⁸ Minus-one programmed ribosomal frameshifting can be stimulated by an mRNA structure (hairpin or pseudoknot) located downstream a slippery site (e.g., U UUU UUA), where ribosome may be temporarily stalled. A backward slippage of ribosomal reading frame by one nucleotide may release the tension and result in the translation at -1 frame.^{66,67,78-85} The mechanical stability of the downstream mRNA structure shows positive correlation with -1 frameshifting efficiency.^{66,67,81} However, other factors may also affect -1 frameshifting efficiency.⁸⁶

Here, we combined traditional ensemble UV-absorbance-detected thermal melting and single-molecule mechanical (un)folding methods to unveil how the structural stability and (un)folding dynamics of a series of 32-mer RNA hairpins (**Figure 1b**) are affected by (i) a single A-U to A·C pair substitution at varied stem positions and (ii) the protonation of the single A·C pairs by varying the buffer pH. Furthermore, we used an *in vitro* translation assay to test how the single A-U to A·C pair substitutions in mRNA hairpins affect -1 ribosomal frameshifting.⁷⁴⁻⁷⁸

MATERIALS AND METHODS

Thermal melting experiments

We constructed a 32-mer RNA hairpin (rHP) and three mutants with one A-U base pair substituted with an A-C mismatch on the top (U22C), middle (U25C) and bottom (U29C) position of the stem, respectively, for the thermal melting experiments (**Figure 1b**). The 32-mer RNA oligonucleotides (**Figure 1b**) were purchased from Sigma with HPLC-purification. The 32-mer hairpins have the sequences of 5'-UAGAGAGAGAAAGUUUCGACUUUCUCUCUCUA-3' (rHP), 5'-UAGAGAGAGAAAGUUUCGACUCUCUCUCUCUA-3' (U22C), 5'-UAGAGAGAGAAAGUUUCGACUUUCCCUCUCUA-3' (U25C), and 5'-UAGAGAGAGAAAGUUUCGACUUUCUCUCCCUA-3' (U29C). All thermal melting studies for the RNA hairpins were carried out using a Shimadzu 2550 spectrophotometer. The absorption at 260 nm was recorded. The six thermal melting buffers with the pH ranging from 5.0 to 8.0 are 200 mM NaCl, 20 mM NaOAc, 0.1 mM EDTA, pH 5.0; 200 mM NaCl, 20 mM MES, 0.1 mM EDTA, pH 5.5 or 6.0; and 200 mM NaCl, 20 mM HEPES, 0.1 mM EDTA, pH 6.5, 7.3, or pH 8.0. NaOAc, MES and HEPES are used because their pK_a values change insignificantly when temperature varies. The sample concentrations used in the UV-absorbance-detected thermal melting studies were 3 μ M. The temperature was firstly held at 95 $^{\circ}$ C for 15 minutes, then decreased from 95 $^{\circ}$ C to 20 $^{\circ}$ C and increased back to 95 $^{\circ}$ C at a ramp rate of 0.5 $^{\circ}$ C/min. The thermal melting experiments were repeated three times. The normalized melting curves were obtained by dividing the measured absorption values at 260 nm by the lowest adsorption value in each curve. The first derivatives of the thermal curves were fit to Gaussian functions to extract the thermal melting temperatures.

Optical tweezers experiments

The single-molecule constructs suitable for optical tweezer experiment were constructed as previously reported.^{61,66} The DNA sequences were inserted into p2luc vector as reported previously.⁸⁷ The mutated sequences for the constructs with an A-C mismatch were constructed by site directed mutagenesis. The constructed vectors were transformed into *E.*

coli competent cells (DH5 α). The plasmids were purified from the bacterial cell cultures. The sequences of all the constructs were verified by Applied Biosystems highest capacity-based genetic analyzer platforms (1st BASE).

With properly designed primers, the duplex DNAs of about 1170 base pairs (bp) containing a T7 promoter were amplified by PCR. *In vitro* transcription by T7 RNA polymerase was carried out to transcribe the RNAs. A 583-bp DNA handle B and a 521-bp DNA handle A flanking the 32-mer sequences of interest were also generated by PCR. The RNA containing the 32-nucleotide (nt) native hairpin sequences, flanked by a 583-nt upstream sequence together with a 4-nt linker and a 521-nt downstream sequence together with a 4-nt linker, were then annealed with complementary DNA strands to form RNA/DNA hybrid handle B and handle A, respectively. The 3' end of the DNA strand of the RNA/DNA hybrid handle A was labelled by introducing biotin-modified dUTP (Biotin-16-dUTP, Roche) using T4 DNA polymerase. The 5' end of the DNA strand of handle B was labelled by digoxigenin-labelled primer during PCR. The streptavidin coated polystyrene beads with a diameter of $\sim 1.8 \mu\text{m}$ were purchased from SpheroTech. The anti-digoxigenin coated beads were synthesized by cross-linking the anti-digoxigenin antibody to the protein G-coated beads with a diameter of $\sim 3 \mu\text{m}$. The protein G-coated beads were synthesized by coating the recombinant protein G (Thermo Scientific) on the carboxyl coated polystyrene beads (SpheroTech) using EDC (Sigma) and sulfo-NHS (Thermo Scientific). Well-annealed DNA/RNA hybrids are then linked to both beads to form a tether for the mechanical pulling experiments.

The single-molecule mechanical pulling experiments were performed using a dual-beam Minitweezers with a dual-laser optical trap generated by two well aligned and highly focused laser beams.^{73,88,89} During the pulling experiment, handle B was attached to an anti-digoxigenin coated polystyrene bead through digoxigenin-anti-digoxigenin interaction and handle A was attached to a streptavidin coated polystyrene bead through biotin-streptavidin

interaction. The anti-digoxigenin-coated polystyrene bead was trapped in an optical trap and the streptavidin-coated polystyrene bead was held on a micropipette by suction. We used three different pulling modes (force-ramp, constant-force, and passive modes) for the characterization of the mechanical (un)folding dynamics. The force was varied by moving the trap at 100 nm/s during the force-ramp experiments. In constant-force experiments, the force was maintained at a pre-set value by an electronic feedback with a response time of 5 ms. In a passive mode, there is no electronic feedback. The constant trap stiffness was about 0.07 pN/nm. The data acquisition rate was 1000 Hz. All the mechanical pulling experiments were carried out at a constant temperature of 22 ± 1 °C. The optical tweezers experiments were carried out in four buffers: (i) 200 mM NaCl, 20 mM MES, 0.1 mM EDTA, pH 5.0; (ii) 200 mM NaCl, 20 mM HEPES, 0.1 mM EDTA, pH 6.5; (iii) 200 mM NaCl, 20 mM HEPES, 0.1 mM EDTA, pH 7.3; and (iv) 200 mM NaCl, 20 mM HEPES, 0.1 mM EDTA, pH 8.0.

***In vitro* ribosomal frameshifting assay**

The plasmids used for the frameshifting assay are the same as those for mechanical pulling studies. Briefly, we inserted in between the *Renilla* luciferase (RLuc) and firefly luciferase (FLuc) reporter genes of p2luc vector^{87,90,91} the target DNA sequences of experimental rHP (5'-GAT CCT TTT TTA GGG TAG AGA GAG AAA GTT TCG ACT TTC TCT CTC TAG AGC T-3'), and a positive control rHP (5'-GAT CCA CTT CTT AGG GTA GAG AGA GAA AGT TTC GAC TTT CTC TCT CTA GAG CT-3').⁸⁷ The mutants were made by site-directed mutagenesis and have the sequences of U22C (5'-GAT CCT TTT TTA GGG TAG AGA GAG AAA GTT TCG ACT CTC TCT CTC TAG AGC T-3'), U25C (5'-GAT CCT TTT TTA GGG TAG AGA GAG AAA GTT TCG ACT TTC CCT CTC TAG AGC T-3'), and U29C (5'-GAT CCT TTT TTA GGG TAG AGA GAG AAA GTT TCG ACT TTC TCT CCC TAG AGC T-3').

The plasmids were linearized using PmlI restriction enzyme (NEB), followed by *in vitro* transcription to generate mRNA templates. 8.75 μ L nuclease treated Rabbit Reticulocyte Lysate mixture (Promega) was added into mRNA with a final volume of 12.5 μ L. The final concentration of mRNA is 0.1 μ M. The mixture was incubated at 30 $^{\circ}$ C for the *in vitro* translation reaction for 90 minutes. The *in vitro* translation was quenched by placing the tubes on ice for 20 minutes. The pH of the translation mixture is within 7.2-7.6.

The luminescence of the translation products was quantified by using the dual-luciferase reporter assay (Promega).^{87,90-93} The luminescence was measured using Tecan infinite M200 microplate reader on Nunclon™ flat-bottom 96-well black microplate with an integration time of 3 seconds at room temperature. The reading was taken immediately after the addition of 50 μ L of the respective substrates.⁸⁷ The *in vitro* translation reactions were conducted three times. The -1 frameshifting efficiency (-1 FS) was calculated using the equation, $-1 \text{ FS (\%)} = \frac{\text{FLuc(E)}/\text{RLuc(E)}}{\text{FLuc(C)}/\text{RLuc(C)}} \times 100\%$.⁹⁰ Here, FLuc (E) and RLuc (E) are the luminescence reading values of FLuc and RLuc, respectively, for the experimental constructs. FLuc (C) and RLuc (C) are the luminescence reading values of FLuc and RLuc, respectively, for the positive control construct of rHP.

Figure 2. Thermal denaturation experiments reveal limited pH dependency of thermal stability. (a) Representative normalized thermal melting curves (heating) monitored at 260 nm for rHP (red), U22C (cyan), U25C (magenta) and U29C (blue) at 200 mM NaCl, pH 7.3. The two thermal melting transitions of U22C are indicated with cyan arrows. (b) Melting (heating) temperatures (T_m) of rHP, U22C, U25C and U29C at 200 mM NaCl, pH 5.0, 5.5, 6.0, 6.5, 7.3, and 8.0. Errors of melting temperatures are reported as standard deviations. (c) Proposed one-step thermal unfolding and folding pathways for rHP, U25C and U29C. (d) Proposed two-step thermal unfolding and folding pathways for U22C. Due to the relatively large size of the hairpins, the thermal melting transitions are relatively broad and may involve more intermediate states. Hence, the thermodynamic parameters were not extracted from the thermal melting curves.

RESULTS AND DISCUSSION

Thermal unfolding

Hairpins U25C and U29C have a single A·C mismatch, respectively, at the middle and bottom part of the stem of the hairpin flanked by two G-C base pairs, whereas hairpin U22C has an A·C mismatch at the top part of the stem of the hairpin flanked by two A-U base pairs (**Figure 1b**). According to a nearest-neighbour model, at 1 M NaCl, pH 7, 37 °C (calculated using RNAstructure 5.3),^{3,94-96} a single A-U to A·C substitution induces a free energy destabilization effect for hairpins U22C, U25C, and U29C by 3.7, 4.8 and 4.8 kcal/mol, respectively.

The thermal melting data at 200 mM NaCl reveal that the heating and cooling curves are superimposable at pH 6.0, 6.5, 7.3 and 8.0 (**Figure S1**), indicating equilibrated (un)folding transitions. A single thermal unfolding transition was observed for hairpins rHP, U25C, and U29C (**Figures 1b and 2a,c**). Hairpin U22C, however, shows a two-step thermal unfolding from native hairpin structure to an intermediate, and then to single-stranded random coil (**Figure 2a,d**). By comparing the thermal melting curves of the four hairpins, we can conclude that the top part of the stem adjacent to the A11·C22 pair of hairpin U22C thermally melts first (with a $T_{m,1}$ value of ~53 °C), followed by the thermal melting of the remaining bottom stem (with a $T_{m,2}$ value of ~79 °C) (**Figures 1b and 2d**). Consistent with the prediction by the RNAstructure 5.3 program,^{3,94-96} we observed that, at 200 mM NaCl, pH 7.3, the T_m values of hairpins U22C, U25C, and U29C are lower than that of rHP (**Figure 2a,b and Table S1**). However, we observed position-dependent destabilization effect, with U25C being the least stable at pH 7.3, although U25C has the same nearest-neighbor combinations of base pairs as U29C.

Previous thermal unfolding studies for relatively short model RNAs and DNAs have revealed that pH dependent wobble A⁺·C pair formation enhances folding stability.^{9,16,19,52} We carried out thermal melting studies of the relatively large RNA hairpins with the pH ranging from 5.0

to 8.0 (**Figure 2b, Figure S1**). The thermal melting results reveal that pH doesn't influence the stability of rHP, with the T_m of rHP remaining at around 84 °C. Decreasing the pH from 8.0 to 5.0, however, results in an increase of the T_m values of U25C and U29C by 3.9 °C and 1.5 °C, respectively. Surprisingly, neither $T_{m,1}$ (53 °C) nor $T_{m,2}$ (79 °C) of U22C shows a significant change upon varying the pH.

Figure 3. Optical tweezers experiment setup and representative mechanical (un)folding force-extension curves (FECs) and proposed mechanical (un)folding pathways. (a) Schematic of experimental setup for mechanical (un)folding of RNA hairpins using Minitweezers (not to

scale). (b) Representative FECs. Force-loading and force-unloading curves are shown in colors and black, respectively. The curves are smoothed to 200 Hz. rHP has relatively higher unfolding forces than the three mutants. U22C has a relatively large hysteresis between unfolding and folding. (c,d) Proposed one-step mechanical (un)folding pathways. The terminal 4 base pairs of U29C do not form at pH 8.0.

The relatively broad thermal melting transitions of the relatively large hairpin constructs studied here may prevent the revelation of the effect of pH on structural stability. It's also probable that UV-absorbance-detected thermal melting studies are only informative for relatively short RNAs with T_m values close to physiologically relevant temperatures. In addition, the pK_a values of a wobble $A^+ \cdot C$ pair and other structures are often highly temperature dependent, which may complicate the analysis of the pH dependent thermal melting data.^{9,16,19,97} For example, due to the fact that the pK_a value of the A residue in a wobble $A^+ \cdot C$ pair decreases with increasing temperature,¹⁶ increasing temperature is expected to weaken the protonation dependent stabilization effect of a wobble $A^+ \cdot C$ pair. Interestingly, we observed hysteresis between thermal unfolding and folding transitions at pH 5.5 and 5.0 (**Figure S1**). We repeated the heating and cooling cycles, and observed superimposable heating and cooling curves, respectively, although the hysteresis exists between heating and cooling traces. Thus, we speculate that the purine rich part of the RNAs may form non-native multimeric structures stabilized by protonated $A \cdot A$ and $A^+ \cdot A$ pairs at relatively low pH^{20,21} before folding into native hairpin structures. We didn't extract the thermodynamic parameters from the thermal melting curves due to the reasons discussed above.

Mechanical (un)folding dynamics

We next employed single-molecule mechanical (un)folding experiments using high resolution optical tweezers to investigate at room temperature (**Figure 3a**) how a single A-U to A·C substitution and pH affect the stability and dynamics of the RNA hairpins. To facilitate the single-molecule mechanical pulling experiments, the hairpin constructs were extended on the 5' and 3' ends with DNA/RNA hybrid handles (**Figures 1b and 3a**). In a single-molecule force-ramp experiment, a varying force is applied to the terminal ends of RNA to facilitate the mechanical folding and unfolding of the RNA structures (**Figure 3**).

One-step mechanical (un)folding transitions (with unfolding indicated by an abrupt extension increase and force decrease) were observed for all hairpins at varied pH's (**Figure 3b,c**). The force-extension curves (FECs) of the force-ramp data suggest that rHP shows transitions near 17 pN with an end-to-end extension change ΔX around 12.5 nm (**Figure 3b**), which is consistent with the formation of a 32-mer hairpin (**Table S2**) as estimated by an extensible wormlike chain (EWLC) model (Equation 1).⁹⁸⁻¹⁰²

$$F = \frac{k_B T}{P} \left[\frac{1}{4 \left(1 - \frac{\Delta X + F}{L_c} \right)^2} - \frac{1}{4} + \frac{\Delta X}{L_c} - \frac{F}{K} \right] \quad (1)$$

Here k_B is Boltzmann's constant, T is the absolute temperature, P is persistent length, L_c is the contour length, K is stretch modulus, ΔX is the end-to-end distance and F is the stretching force. The L_c , P and K of single-stranded RNA are 0.59 nm per nucleotide, 1 nm, and 1500 pN, respectively.^{56,60,63,100}

To further characterize how the single mutations and pH affect mechanical (un)folding kinetics, we used a constant-force mode with the force maintained at a pre-set value by adjusting the pipette bead position (**Figures 3a, S2 and S3**).^{57,64} In a constant-force mode, we observed that at 16.7 pN, rHP has the folded state more populated, and the population distribution and (un)folding dynamics essentially show no change upon increasing the pH from 6.5 to 8.0 (**Figure 4a**), suggesting that varying pH does not affect the folding free energy landscape of rHP, consistent with the force-ramp results (**Figure 3b**). A relatively lower force (14.9 pN) is needed to obtain a similar population distribution at pH 6.5 for the three hairpin mutants with single mutations (**Figure 4b-d**), which clearly suggests that the single U-to-C mutations destabilize the hairpins. Upon varying the pH from 6.5 to 8.0, the population of the unfolded state (with a larger extension) increases for all three mutants (**Figure 4b-d**). Apparently, increasing pH lowers the mechanical stability (unfolding force) of the three mutants (U22C, U25C and U29C). The one-step mechanical unfolding pathway of hairpin U22C at 22 °C (**Figure 3c**) is in contrast to a two-step unfolding pathway observed in the thermal unfolding (temperature-ramp, 0.5 °C/min) experiment (**Figure 2a,d**). The relatively less stable top part of the stem of hairpin U22C thus thermally melts at a relatively low temperature (~53 °C, **Figure 2a,d**).

In addition, we used a passive mode to study the mechanical (un)folding kinetics, with the pipette bead kept at a fixed position, allowing the bead in the optical trap to move upon RNA (un)folding (**Figures 3a and S4**).⁶⁴ There is no electronic feedback in passive mode, which makes it suitable for measuring relatively fast kinetics.^{64,88} In the real-time (un)folding traces of passive mode, the high force and low force states correspond to a folded state and an unfolded state, respectively (**Figure S4**). The passive-mode and constant-force mode data give essentially the same kinetic parameters for various molecular tethers (**Figures S4-S6**).

The ΔX values can reveal if the RNAs form the designed native structures. The ΔX value (~ 12.5 nm) of rHP, U22C, and U25C does not vary with pH (**Figures 3b and 4a-c, Table 1**), which, based on EWLC model, corresponds to about 33 nucleotides, which is close to the 32 nucleotides present in all hairpin constructs. Interestingly, hairpin U29C unfolds at below 16 pN with a significantly shortened ΔX of ~ 10 nm at pH 8.0 (**Figures 3b and 4d, Table 1**). Furthermore, the ΔX of U29C decreases with increasing pH: ~ 12 nm at pH 5.0, ~ 11 nm at pH 6.5, and ~ 10 nm at pH 7.3 and 8.0 (**Table 1, Table S2**), suggesting that the bottom part of the stem of U29C is increasingly destabilized upon increasing pH. Even at pH 5.0, ΔX of U29C is still about 0.5 nm shorter than that expected for a 32-mer hairpin. Thus, at pH 7.3 and pH 8.0, there are 24 nucleotides folded for U29C hairpin, as calculated based on EWLC model (with the inter-nucleotide distance considered as 0.44 nm at 15 pN) (**Figures 1b and 3d**). The results suggest that substitution of the A4-U29 pair with A4-C29 pair in hairpin U29C causes the destabilization of the bottom part of the stem, resulting in the bottom base pairs unable to fully form in the presence of the mechanical stretching force applied on the terminal ends.^{103,104} Consistently, increasing pH results in an increasing number of base pairs unable to form in U29C hairpin, presumably due to the increased dynamics upon the loss of a hydrogen bond in the wobble A4-C29 pair.

We observed relatively fast unfolding and folding dynamics (designated as hopping) for rHP, U25C, and U29C (**Figures 3b and 4**). However, hairpin U22C shows significantly slower (un)folding dynamics, presumably due to the fact that the A11-U22 to A11-C22 mutation results in a decreased folding rate with the destabilization mutation near the hairpin loop. It

has been previously observed in thermal and mechanical (un)folding studies that destabilizing mutations of the base pairs near or closing the hairpin loop decrease the folding rate of nucleic acid hairpins.^{53-55,57,69,105,106} The highly dynamic unfolding and folding processes (hopping) as observed in the FECs (**Figure 3b**) make it hard to quantitatively measure the transition forces and the effect of pH.

Figure 4. Representative traces of mechanical (un)folding at a constant-force mode at pH 6.5 and 8.0. Only the extension curves are shown. The curves are smoothed to 200 Hz. The extension distributions are fit to two-peak Gaussian distributions (binned to 1 nm). A one-step mechanical unfolding transition is observed. The dashed cyan and green lines at the peaks of the Gaussian functions correspond to the unfolded states and folded states, respectively. (a) rHP at 16.7 pN. rHP folding and unfolding rates show no significant pH dependence. (b) U22C at 14.9 pN. U22C folding rate decreases with increasing pH. (c) U25C at 14.9 pN. With increasing pH, U25C folding rate decreases, whereas unfolding rate increases. (d) U29C at 14.9 pN. U29C shows a decreased extension change at pH 8.0. No significant difference in kinetics was observed between pH 6.5 and pH 8.0. However, unfolding rate significantly decreases at pH 5.0 (see **Figure 5d**).

Figure 5. Force-dependent unfolding (FU, open markers) and folding (UF, filled markers) rates at varied pH's obtained by constant-force mode. The critical forces (F_c) of the hairpins were obtained based on the crossing points of the force-dependent unfolding and refolding rates curves. The R^2 values of the linear fits range from 0.871 to 0.999. (a) rHP. No significant pH dependence is observed for rHP folding and unfolding rates. (b) U22C. pH dependence is observed for U22C folding but not unfolding. (c) U25C. pH dependence is observed for both unfolding and folding. (d) U29C. Unfolding rate is significantly slowed at pH 5.0.

The quantitative analysis of the pH- and force-dependent (un)folding kinetics was done using the constant-force and passive mode data. The extension versus time trajectories in a constant-force mode (**Figure 4**) are fit to a hidden Markov model¹⁰⁷ to extract the lifetimes of the unfolded and folded states at each fixed force (**Figure S3a**). A single exponential fit is then applied to extract the folding and unfolding rate constants (**Figure S3b**). To extract the

mechanical unfolding and folding transition state positions (**Figure 5 and Table S2**), the force-dependent rates are further fit to the Bell model (Equation 2).^{64,108}

$$\ln k(F) = \ln(k_0) + \frac{F\Delta X^\ddagger}{k_B T} \quad (2)$$

Here, k_0 is the rate coefficient at zero force, $\Delta X^\ddagger$ is the distance from unfolded or folded state to the transition state, k_B is Boltzmann constant, and T is absolute temperature. The force trajectories in passive mode (**Figure S4**) at each force were also fit to hidden Markov model¹⁰⁷ to extract the lifetimes of the mechanically unfolded and folded states. Overall, the constant-force mode data (**Table S2**) and passive mode data (**Table S3**) give essentially the same kinetic parameters.

The critical force F_c is defined as the force at which the unfolding rate equals to the folding rate. The critical forces of the hairpins were obtained based on the crossing points of the force-dependent unfolding and refolding rates curves (**Figure 5**). At all four pH's (5.0, 6.5, 7.3 and 8.0), the critical forces of rHP (16.7, 17.2, 16.5, and 16.8 pN) are higher than U22C (15.4, 15.2, 14.7, and 14.5 pN), U25C (16.4, 15.4, 14.7, and 14.4 pN), and U29C (16.2, 15.3, 15.0, and 15.1 pN) (**Figure 6a, Tables 1 and S2**). Clearly, a single A-U to A·C substitution destabilizes the mechanical stability of the hairpin.

The critical force of rHP remains at around 17 pN with a small change when pH is varied (**Figures 5a and 6a, Table 1**), suggesting that the mechanical stability of rHP is independent of pH. The critical forces of the three mutants, however, increase by over 1 pN when pH is lowered from 8.0 to 5.0 (**Figures 5b-d and 6a, Table 1**). At pH 8.0, the critical forces of U22C, U25C and U29C are significantly lower (by 2.3, 2.4 and 1.7 pN) than that of rHP. However, at pH 5.0, the critical forces of U25C and U29C are only slightly lower (by 0.3 and 0.5 pN, respectively) than that of rHP. Our data suggest that single-molecule mechanical (un)folding can reveal the stabilizing effect of a single proton binding at the N1 nitrogen atom of adenine in a wobble A·C pair to form a wobble A⁺·C pair with two hydrogen bonds (**Figure 1a**) in hairpins U22C, U25C and U29C.^{16,18,37,38}

Table 1. Parameters for mechanical (un)folding by constant-force experiments.^a

RNA molecule	pH	ΔX_c (nm)	# of folded nt ^c	F_c (pN)	W_{Fc} (kcal mol ⁻¹)	ΔG_o (kcal mol ⁻¹)
rHP	5.0	12.5 ± 1.4	32.8 ± 3.2	16.7 ± 0.1	30.0 ± 3.4	21.1 ± 3.3
	6.5	12.8 ± 1.1	33.4 ± 2.5	17.2 ± 0.1	31.5 ± 2.7	22.4 ± 2.7
	7.3	12.7 ± 1.1	33.3 ± 2.5	16.5 ± 0.1	30.2 ± 2.6	21.3 ± 2.6
	8.0	12.7 ± 1.1	33.3 ± 2.5	16.8 ± 0.1	30.7 ± 2.7	21.7 ± 2.7
U22C	5.0	12.6 ± 1.2	33.7 ± 2.8	15.4 ± 0.1	27.9 ± 2.7	19.5 ± 2.7
	6.5	12.4 ± 1.0	33.2 ± 2.3	15.2 ± 0.1	27.3 ± 2.2	19.0 ± 2.2
	7.3	12.5 ± 1.1	33.7 ± 2.6	14.7 ± 0.2	26.4 ± 2.3	18.3 ± 2.3
	8.0	12.6 ± 1.1	34.2 ± 2.6	14.5 ± 0.1	26.3 ± 2.3	18.3 ± 2.3
U25C	5.0	12.4 ± 1.0	32.7 ± 2.3	16.4 ± 0.2	29.3 ± 2.4	20.4 ± 2.4
	6.5	12.6 ± 1.1	33.7 ± 2.5	15.4 ± 0.1	27.9 ± 2.4	19.5 ± 2.4
	7.3	12.5 ± 1.0	33.8 ± 2.3	14.7 ± 0.1	26.5 ± 2.1	18.4 ± 2.1
	8.0	12.7 ± 1.0	34.5 ± 2.3	14.4 ± 0.1	26.1 ± 2.1	18.1 ± 2.1
U29C	5.0	12.2 ± 1.2	32.5 ± 2.7	16.2 ± 0.1	28.4 ± 2.8	19.7 ± 2.8
	6.5	11.1 ± 1.3	30.3 ± 3.0	15.3 ± 0.2	24.4 ± 2.9 ^b	16.1 ± 2.9
	7.3	10.2 ± 1.2	28.4 ± 2.8	15.0 ± 0.2	22.0 ± 2.6 ^b	13.8 ± 2.6
	8.0	9.7 ± 1.0	27.1 ± 2.3	15.1 ± 0.1	21.1 ± 2.2 ^b	12.8 ± 2.2

^a The critical force F_c is defined as the force at which the unfolding and folding rates are equal. W_{Fc} is the work for unfolding reaction at equilibrium at critical force ($W_{Fc} = N_A F_c \Delta X_c$). N_A is Avogadro constant. ΔG_o is the free energy for unfolding reaction at zero force at 22 °C ($\Delta G_o = W_{Fc} - W_{stretching}$). $W_{stretching}$ is the work for stretching single-stranded RNA from zero force to critical force at equilibrium, which can be calculated through the integration of the EWLC curve (Equation 1). All uncertainties are reported as standard errors.

^b The equilibrium unfolding work (W_{Fc}) decreases with increasing pH for all hairpins except for rHP. The W_{Fc} value for U29C decreases more significantly with increasing pH, mainly due to the decreasing ΔX_c value with the bottom base pairs not forming (see **Figure 3d**). The F_c values decrease with increasing pH for all hairpins except rHP.

^c The number of nucleotides folded are calculated based on the measured extension change (ΔX_c) values, with the diameter of RNA helix (2 nm) considered. The number of folded nucleotides of U29C decreases with increasing pH.

The critical force of U22C is about 1 pN lower than U25C and U29C at pH 5.0, which might be due to the fact that the A·C mismatch in U22C is located at a position adjacent to the hairpin loop and affects mechanical folding significantly. In addition, the A·C mismatch in U22C is flanked by two A-U pairs, whereas U25C and U29C have their A·C mismatch flanked by two G-C pairs. The nearest-neighbor flanking base pairs may affect the apparent pK_a differently.^{9,16,18,27,52} We expect that destabilizing mutations near the loop including the loop-closing base pair may generally destabilize a hairpin by decreasing mechanical folding rate, and the folding rate can be rescued if a potential protonation site exists to stabilize the base pair.

Detailed force-dependent kinetic studies reveal that proton binding affects the mechanical (un)folding kinetics of the A·C pair-containing hairpins in a position-dependent manner (**Figure 5**). Mechanical folding rate of hairpin U22C (with an A·C pair near the hairpin loop (A11·C22)) increases with decreasing pH, with the mechanical unfolding rate relatively less dependent on pH. In contrast, mechanical unfolding rate of hairpin U29C (with an A·C pair near the terminus of the stem (A4·C29)) decreases with decreasing pH, with the mechanical folding rate essentially unchanged. Hairpin U25C (with an A·C pair at the middle of the stem (A8·C25)) shows pH dependence for both mechanical unfolding and folding reactions. Thus, proton binding facilitates the rapid formation of an A⁺·C pair and enhances the apparent mechanical stability of an RNA stem-loop structure through the increased folding rate, or decreased unfolding rate, or both, depending on the location of the A·C pair in the hairpin.

To further understand the mechanical (un)folding mechanisms and the effects of protonation, we extracted the apparent two-state mechanical unfolding and folding transition state positions from the force-dependent (un)folding studies (Equation 2). The $\Delta X^\ddagger$ (or $\Delta n^\ddagger$) values correlate with the slopes of the rate versus force curves, and indicate the mechanical (un)folding transition state positions. It appears that the $\Delta X^\ddagger$ values or number of nucleotides disrupted and folded at the unfolding transition state ($\Delta n^{\ddagger, \text{FU}}$) and the folding transition state

($\Delta n^{\ddagger,UF}$) are about the same for rHP, U22C and U25C, and remain unchanged with varying pH (Figures 1b, 5 and 6b, Tables S2 and S3). At the apparent unfolding and folding transition state, hairpins rHP, U22C and U25C have their bottom 9 base pairs disrupted (Figure 1b). The rapid exchange of protonation and deprotonation of an A·C pair (on the μ s time)^{18,109,110} at the apparent two-state transition state allows us to observe the averaged effect of dynamic (de)protonation on mechanical (un)folding kinetics. The transition state positions revealed are consistent with the fact that U22C folding rate and U29C unfolding rate are most sensitive to pH, respectively. For U25C, the A⁺·C pair is located adjacent to the apparent transition state positions, resulting in both unfolding and folding rates of U25C being sensitive to proton binding.

Figure 6. Effects of pH on mechanical (un)folding. All errors are reported as standard errors. (a) pH dependence of critical forces. The data were obtained from constant-force mode (solid line) and passive mode (dashed line). Except for rHP, the critical forces decrease with increasing pH. (b) Number of nucleotides disrupted and folded at unfolding transition states

($\Delta n^{\ddagger, \text{FU}}$) and folding transition states ($\Delta n^{\ddagger, \text{UF}}$), respectively. The data were obtained by averaging the constant-force and passive mode data. The transition state positions show no significant pH dependence. (c) pH dependence of the total number of nucleotides in folded hairpins based on the measured ΔX values (see **Table 1**). U29C shows decreasing number of folded nucleotides with increasing pH. (d) pH dependence of mechanical unfolding Gibbs free energy at zero force at 22 °C (ΔG_0). Except for rHP, the ΔG_0 values decrease with increasing pH.

At pH 5.0, hairpin U29C has the bottom part of the stem nearly fully formed (**Figures 1b and 6c**), and the force-dependent kinetic studies (**Figure 5d**) suggest that the apparent unfolding and folding transition states (Equation 2) of U29C are the same as rHP. As discussed above, the bottom base pairs of hairpin U29C are not fully formed with an increased pH. However, the slopes of the rate versus force curves for hairpin U29C at pH 8.0 are largely the same as those at pH 5.0 (**Figure 5d**). We can conclude that the apparent folding transition state position of hairpin U29C is the same as the other three hairpins (around the A10-U23 pair, see **Figure 1b**), and is independent of pH. However, the apparent unfolding transition state position of hairpin U29C instead shifts up by two base pairs at a relatively high pH (7.3 and 8.0), i.e., the bottom 11 base pairs are disrupted at the unfolding transition state (**Figure 1b**). Consistently, unfolding rate of U29C changes significantly upon varying pH from 5.0 to 6.5, but not as much from pH 6.5 to 8.0 (**Figure 5d**).

The Gibbs free energy change for unzipping a hairpin at zero force ΔG_0 is obtained by subtracting the work of the mechanically unfolding hairpin by the work of stretching the single-stranded RNA from zero force to the critical force (**Table 1**)^{13,60}. Similar to the trend observed for critical forces, ΔG_0 of rHP is the highest at all pH's and remains at ~22 kcal·mol⁻¹, confirming that a single A-U to A·C replacement can decrease the thermodynamic stability of the hairpin. The ΔG_0 values of U22C, U25C, and U29C decrease gradually with pH increasing from 5.0 to 8.0 (**Figure 6d**). It is of note that, compared to U22C and U25C,

U29C shows a more significant drop in ΔG_o , which is mainly due to the fact that several bottom base pairs in U29C fail to form when pH is above 5.0.

Minus-one ribosomal frameshifting efficiency positively correlates with critical force and folding rate at around 15 pN

Our mechanical (un)folding results show that the mechanical forces needed for unfolding the studied RNA hairpin structures range from 14-18 pN, which is in the same range of the force exerted by translating ribosomes (12-20 pN).^{72,73} Thus, mRNA structures could affect the ribosome translation by serving as mechanical and kinetic barriers.¹¹¹ Hence, it is important to unravel the potential correlations between mechanical (un)folding parameters of the studied RNA hairpins and ribosomal translation. We carried out *in vitro* translation assay using a dual-luciferase reporter system to characterize how A-U to A-C substitutions in an mRNA containing the 32-mer hairpins (**Figure 7a**) may affect -1 ribosomal frameshifting efficiency.⁸⁷ We observed -1 frameshifting efficiencies of $2.2 \pm 0.2\%$, $1.8 \pm 0.1\%$, $1.2 \pm 0.1\%$, and $1.0 \pm 0.1\%$, respectively, for rHP, U29C, U25C, and U22C (**Figure 7b,c**). The frameshifting efficiency for rHP is consistent with our previously reported value.⁸⁷ We obtained the frameshifting efficiencies with relatively small errors by rigorously controlling the timing of the luminescence measurement.⁸⁷

It is interesting to note that U29C and U22C show the highest and lowest frameshifting efficiencies among the three mutated hairpins. The data suggest that, for the hairpins studied, the local stability of the top part of the stem is more important than the bottom part for stimulating frameshifting. It is known that a 5-8 nucleotide single-stranded spacer between the slippery site and downstream RNA structure is optimal for stimulating frameshifting.^{74,79} Thus, with a 3-nt single-stranded spacer (**Figure 7a**), the bottom 2-5 base pairs of the hairpins may be pre-melted by ribosome at the slippery site. The pre-melting of the RNA structure could be due to the ribosome biasing the thermal fluctuations towards the unfolded state or the mechanical force generated during ribosome translocation.¹¹¹ Thus, we propose

that the bottom four base pairs of the hairpins studied here are pre-melted at the mRNA entry site when ribosome is initially paused at the slippery site, followed by consequent unzipping of the rest of the hairpin structure (**Figure 7a,d**). Hence, it is reasonable that U29C shows a minimal reduction in frameshifting efficiency compared to rHP. Consistently, our single-molecule mechanical pulling data suggest that, on average, the bottom 2-4 base pairs of U29C do not form under mechanical stretching force at pH 7.3-8.0. We observed a correlation between critical force (which is determined by both unfolding and folding rates, see **Figure 5**) and frameshifting efficiency (**Figure 7b**). Thus, for the hairpins studied, mechanical (un)folding may mimic the mRNA structure (un)folding in the presence of translating ribosomes.

U22C has a very similar frameshifting efficiency compared to U25C, which seems to be counter-intuitive, because base pairs closer to mRNA entry site of ribosome at the slippery site is expected to have a more significant effect in affecting frameshifting efficiency.^{79,80} It is likely that, in addition to local stem stability, folding rate may also affect frameshifting significantly.⁸⁵ Timely folding of the downstream mRNA structure may be critical for pausing a translating ribosome and stimulating frameshifting in consideration of the fact that multiple ribosomes translate one mRNA. U22C shows a significantly lower folding rate compared to U25C, which may result in a similar frameshifting efficiency for U22C and U25C, even though the mismatch for U22C is located further away from the slippery site. We indeed observed a correlation between frameshifting efficiency and folding rate at 15 pN (**Figure 7c**), with the force well within the range that can be exerted by a translating ribosome.^{72,73} No strong correlation was observed between frameshifting efficiency and folding rate at other forces (**Figure S7**). It is likely that the magnitude of such steric hindrance-induced effect is dependent on the density of ribosomes on mRNAs. It is possible that for a relatively large mRNA in the presence of multiple translating ribosomes,¹¹² the folding of a hairpin structure may experience mechanical resistance in a relatively crowded environment of cell lysate and steric hindrance of translating ribosomes at and adjacent to the hairpin sequence (**Figure 7d**). We hypothesize that steric and crowding effects may be mimicked by the stretching force exerted in optical tweezers experiments.

For the hairpins studied, surprisingly, no apparent correlation is observed between frameshifting efficiency and unfolding rate (**Figure S8**). The results further suggest that, folding rate may also affect frameshifting for the hairpins studied. In addition, the bottom stem of U29C is not fully formed at $\text{pH} > 5.0$, as revealed by our pulling experiments. Taken together, our data suggest that mechanical (un)folding of RNA hairpins may mimic how RNAs unfold and fold in the presence of translating ribosomes.

The thermal melting pathway of U22C is not consistent with the directional mRNA unwinding process of a ribosome. Thus, thermal melting data may not be meaningfully compared with the frameshifting data (**Figure S8a**). Taken together, our results suggest that, for the hairpins studied, incorporating an A·C mismatch may reduce the mechanical stability of the stem and decrease the folding rate, hence attenuating -1 ribosomal frameshifting efficiency.

Figure 7. Comparison of frameshifting efficiency at 30 °C and mechanical (un)folding parameters at 22 °C. (a) Experimental constructs for *in vitro* frameshifting assay. The slippery sequence (U UUU UUA) is shown in red and underlined. Minus-one ribosomal frameshifting results in the slippery sequence to be decoded as UUU UUU A instead of U UUU UUA, resulting in the expression of downstream FLuc at -1 frame in addition to RLuc at normal zero frame. The single-stranded spacer (GGG, brown) is located between the slippery sequence and the hairpin structure (blue). (b) Frameshifting efficiency increases with increasing critical force. The critical forces shown were averaged values based on the constant-force mode and passive mode data at pH 7.3. (c) Frameshifting efficiency increases with increasing folding rate at 15 pN. No clear correlation is observed at forces far away from 15 pN (see **Figure S7**). Errors for the critical forces are standard errors. Errors for

frameshifting efficiency values are standard deviations based on three independent measurements. The mRNAs are at 0.1 μM with the translation mixture at pH 7.2-7.6. (d) Schematics of a translating ribosome positioned at various sites of an mRNA. Multiple ribosomes may simultaneously translate one mRNA. (Top) Ribosome positioned upstream of the slippery site, with the mRNA hairpin structure unaffected. (Middle) Ribosome positioned at the slippery site, with the bottom four base pairs of the mRNA hairpin disrupted. (Bottom) Ribosome positioned downstream of the slippery site at -1 frame, with mRNA hairpin completely disrupted.

CONCLUSION

In summary, compared to the traditional ensemble thermal melting method, single-molecule mechanical (un)folding studies can reveal the detailed real-time folding dynamics at near physiological temperature and facilitate a deeper understanding of how a single A-U to A·C mutation and single proton binding may affect the stabilities, (un)folding dynamics, and transition states of RNA in a position-dependent manner. For example, although our ensemble thermal melting studies show that a single A-U to A·C replacement lowers the thermal stability, the detailed dynamics and kinetic parameters and the effects of protonation, however, may not be easily obtained by pH-dependent thermal melting studies likely due to the facts that (i) relatively large hairpins may show non-two-state thermal melting transitions, (ii) the pK_a values for an A·C pair is temperature dependent, and (iii) non-native structures may form during thermal melting. Our single-molecule mechanical (un)folding studies at a constant temperature (room temperature) clearly suggest that varying proton concentration from 10 nM (pH 8.0) to 10 μM (pH 5.0) can specifically enhance the formation of a wobble A⁺·C pair and in turn the mechanical stability of an A⁺·C pair-containing hairpin.

The mechanical unfolding forces for U22C, U25C, and U29C increase gradually with decreasing pH (**Figure 6a**), consistent with the fact that the protonation of the A·C mismatch is relatively fast. The exchange of protonation and deprotonation of a wobble A·C pair is on

the μs time scale.^{18,109,110} Thus, in our optical tweezers experiments with a time resolution of ms, we observed an averaged effect of dynamic (de)protonation on the stability of the apparent two-state transition state and mechanical (un)folding kinetics. The proton binding effect is similar to the salt concentration dependence as observed before.^{69,113,114} However, proton binding is a site-specific binding and is sequence and structure specific as observed for some small molecules, oligonucleotides, and proteins.^{56,115-120} It may be expected that, with more extensive studies in an expanded pH range, the apparent $\text{p}K_{\text{a}}$ values may be extracted¹⁹ from the single-molecule mechanical unfolding studies.

Our results have important implications in how a wobble A·C pair and other structures may affect RNA (un)folding dynamics and function at near physiological conditions.^{29,31,32} For example, compared to RNA thermal unfolding, RNA unfolding by mechanical stretching force may better mimic RNA (un)folding dynamics in the presence of RNA helicases (such as ribosome). Our *in vitro* translation data for the mRNAs containing the hairpins suggest that mechanical unfolding force at equilibrium (critical force) and folding rate at around 15 pN of the hairpins positively correlate with -1 ribosomal frameshifting efficiency. For the mRNA hairpins studied, mechanical unfolding rate shows no correlation with frameshifting efficiency (**Figure S8**), probably because U29C hairpin is partially unfolded during pulling experiments. Indeed, with a 3-nt single-stranded spacer between the slippery site and downstream hairpin (GGG, **Figure 7a**), two to five base pairs of the bottom part of the stem of the hairpins are predicted to be melted by ribosome at the slippery site (**Figure 7d**). It is expected that the length of the single-stranded spacer is critical in determining the correlation between mechanical unfolding parameters and frameshifting efficiency. For example, the observed correlations (**Figure 7b,c**) are not linear correlations, which might be specific to the studied hairpins with a relatively short 3-nt single-stranded spacer.

U22C and U25C have a similar frameshifting efficiency ($\sim 1\%$), indicating that both unfolding and folding rates of an mRNA hairpin may affect frameshifting. It is likely that for

a relatively large mRNA in the presence of multiple translating ribosomes, the folding of a hairpin structure slows down due to the steric hindrance of translating ribosomes¹¹² at and adjacent to the hairpin sequence (see **Figure 7d**) and mechanical resistance in a relatively crowded environment of cell lysate. Thus, RNA (un)folding under mechanical stretching force provides unique insights into how RNA structural stability and dynamics involving A·C pairs and other non-canonical pairs¹²¹ may regulate RNA-acting molecular motors such as ribosome.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. Detailed single-molecule data analysis protocols. Thermal melting temperatures (Table S1), Parameters obtained from constant-force (Table S2) and passive mode (Table S3) experiments, normalized thermal melting curves (Figure S1), Representative constant-force mode traces (Figure S2), representative constant-force mode trace and fitting curves for the extraction of (un)folding rates (Figure S3), representative passive mode traces (Figure S4), mechanical (un)folding rates obtained by passive mode (Figure S5), comparison of (un)folding rates obtained from different single-molecule tethers (Figure S6), comparison between frameshifting efficiency and mechanical folding rate (Figure S7), and comparison between frameshifting efficiency and thermal melting temperatures and mechanical unfolding rate (Figure S8).

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Notes

The authors declare no competing financial interest.

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TOC figure

figure 1

190x140mm (300 x 300 DPI)

Fig 2

190x170mm (300 x 300 DPI)

Fig 3

190x209mm (300 x 300 DPI)

figure 4

190x142mm (300 x 300 DPI)

figure 5

190x140mm (300 x 300 DPI)

Fig 6

190x139mm (300 x 300 DPI)

Fig 7

190x177mm (300 x 300 DPI)

TOC figure

88x44mm (300 x 300 DPI)