Structural dynamics of single SARS-CoV-2 pseudoknot molecules reveal topologically distinct conformers

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The RNA pseudoknot that stimulates programmed ribosomal frameshifting in SARS-CoV-2 is a possible drug target. To understand how it responds to mechanical tension applied by ribosomes, thought to play a key role during frameshifting, we probe its structural dynamics using optical tweezers. We find that it forms multiple structures: two pseudoknotted conformers with different stability and barriers, and alternative stem-loop structures. The pseudoknotted conformers have distinct topologies, one threading the 5’ end through a 3-helix junction to create a knot-like fold, the other with unthreaded 5’ end, consistent with structures observed via cryo-EM and simulations. Refolding of the pseudoknotted conformers starts with stem 1, followed by stem 3 and lastly stem 2; Mg2+ ions are not required, but increase pseudoknot mechanical rigidity and favor formation of the knot-like conformer. These results resolve the SARS-CoV-2 frameshift signal folding mechanism and highlight its conformational heterogeneity, with important implications for structure-based drug-discovery efforts.
like most coronaviruses, the Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2) causing the COVID-19 pandemic makes use of −1 programmed ribosomal frameshifting (−1 PRF) to express proteins that are essential for viral replication. In −1 PRF, a shift in the reading frame of the ribosome at a specific location in the RNA message is stimulated by a structure in the mRNA located 5–7 nt downstream of the slippery sequence where the reading frameshift occurs, thereby generating alternate gene products. Previous work on viruses slippery sequence where the reading frameshift occurs, thereby generating alternate gene products. Previous work on viruses

Fig. 1 Three-stemmed architecture of SARS-CoV-2 frameshift-stimulatory pseudoknot. a Secondary structure from Ref. 1, with stems and loops color-coded (S1: stem 1, cyan; S2: stem 2, orange; S3: stem 3, purple; L1/2/3: loop 1/2/3, gray). Spacer region linking to slippery sequence shown in red. b 3D structures of 5’-threaded (left) and -unthreaded (right) conformers from Ref. 18. c Cryo-EM structure from Ref. 15 (PDB ID 7O7Z).

Results

To probe the conformations formed by the SARS-CoV-2 pseudoknot, their folding pathways, and the dynamics under tension, we annealed a single RNA molecule containing the sequence of the pseudoknot flanked by handle regions to RNA handles that were attached to beads held in optical traps (Fig. 2a). We then moved the traps apart to ramp up the force and unfold the RNA, and brought them back together to ramp down the force and refold the RNA. Force-extension curves (FECs) measured during unfolding in near-physiological ionic conditions (130 mM K+, 4 mM Mg2+) showed one or more characteristic transitions in which the extension abruptly increased and force simultaneously decreased when part or all of the structure unfolded cooperatively (Fig. 2b). Unfolding events were observed over a range of forces from ~5 to 50 pN; similar transitions were seen in refolding FECs, but at forces below ~15 pN (Fig. 2c).

Examining the unfolding FECs in more detail, we found two qualitatively different behaviors distinguished by different length changes. Measuring the amount of RNA unfolded by fitting the FECs before and after the transition to worm-like chain (WLC) polymer elasticity models (Eq. (1), Methods) for the handles and unfolded RNA (Fig. 2b, dashed lines), we found that ~80% of FECs (Fig. 2b, black and magenta) showed a contour length change of ΔLc = 35.6 ± 0.4 nm for complete unfolding (Supplementary Table 1). This result was consistent with the value expected for full unfolding of the pseudoknot, 34.7–36.5 nm, based on cryo-EM reconstructions10,15 and computational modeling of likely structures18. Sometimes these curves contained an intermediate state, I1 (Fig. 2b, magenta), which unfolded with a length corresponding to stem 1 (ΔLc = 17.5 ± 0.4 nm), but most of the time they showed a single cooperative unfolding transition (Fig. 2b, black). The remaining ~20% of the FECs (Fig. 2b, green) unfolded with a smaller total length change of ΔLc = 25 ± 1 nm, indicating an alternate structure that was incompletely folded (denoted as Alt), at forces of ~10–20 pN characteristic of hairpins33 (Supplementary Fig. 1). Such alternative structures have been observed previously for many frameshift signals22,34. This second class of FECs also often contained at least one unfolding intermediate.

There is a characteristic shape expected for the distribution of unfolding forces, p(F), for unfolding across a single barrier35.
hence $p(F_u)$ can reveal the presence of distinct initial states during the unfolding.\textsuperscript{36,37} For the population of FECs with full-length unfolding, two peaks were seen in $p(F_u)$: a minor peak near 16 pN and a larger peak near 30 pN (Fig. 2d, black). The double peak indicates the presence of at least two distinct initial conformers, which despite sharing the same total length change nevertheless unfold over different barriers, leading to different shapes for their unfolding force distributions. Such behavior has been seen previously in ligand-bound riboswitches\textsuperscript{38} and proteins\textsuperscript{36}. By fitting $p(F_u)$ to a kinetic model for barrier crossing (Eq. 2, Methods), the shape of the energy barrier can be characterized through its height ($\Delta G^\ddagger$) and distance from the folded state ($\Delta x^\ddagger$), reporting on the nature of the interactions that hold the structure together\textsuperscript{32}. We found that $p(F_u)$ fit better to the distribution expected for two initial states (Fig. 2d, red; fit parameters listed in Supplementary Table 2) than to the distribution expected for a single initial state (Fig. 2d, blue), as assessed by the Akaika information criterion (AIC) (see Methods)\textsuperscript{39}. The results for $\Delta G^\ddagger$ were similar within error for the two initial states, respectively, 31 ± 4 kJ/mol for the higher-force state (denoted N) and 33 ± 5 kJ/mol for the lower-force state (denoted N'), but $\Delta x^\ddagger$ was notably smaller for N: 0.7 ± 0.1 nm, compared to 2.1 ± 0.3 nm for N', implying a more rigid structure for N. In both cases, however, the value for $\Delta x^\ddagger$ was consistent with the range characteristic of pseudoknots\textsuperscript{22,40} and other structures containing tertiary contacts\textsuperscript{41–43}, but too short for structures consisting only of stem-loops\textsuperscript{32}. The great majority of the unfolding FECs showing full-length ($\Delta L_c$ started in state N (91 ± 2%), with only a small minority (9 ± 2%) starting in state N'.

![Image](image_url)
Fig. 3 Occlusion of 5′ end suppresses folding of threaded conformer. a Unfolding FECs measured with 1-nt spacer between duplex handle and pseudoknot show same length changes as in Fig. 2. Dashed lines show WLC fits. Inset: schematic showing location of handle duplex. b Unfolding force distribution with 1-nt spacer between handle and pseudoknot (N = 342 FECs from 8 molecules) shows more prominent low-force peak and less prominent high-force peak. c Re-measuring unfolding with handle extending 2 nt into stem 1 of pseudoknot (inset: schematic showing location of handle duplex) yields unfolding force distribution (N = 220 FECs from 3 molecules) with even more prominent low-force peak and even less prominent high-force peak. d Fraction of FECs showing higher-order pseudoknot unfolding (red) decreases systematically as duplex handle is extended towards stem 1 of pseudoknot (or invades it), with compensating increase in fraction showing lower-order pseudoknot unfolding (orange), whereas fraction showing alternative stem-loop unfolding (brown) remains constant. Error bars on occupancies of pseudoknotted (sum of N and N′) and alternate conformers (Alt) represent standard error of proportion from numbers of FECs showing each conformation type; error bars on fraction of pseudoknotted conformers occupying N/N′ represent fitting errors from analysis of unfolding force distributions with two-population landscape model (errors correlated for N and N′, as shown). N = 1825 FECs from 10 molecules for 6-nt spacer, 491 FECs from 8 molecules for 1-nt spacer, and 270 FECs from 3 molecules for −2-nt spacer.

to refold through an additional intermediate (Fig. 2c, blue). The cumulative length changes from the unfolded state for these subsequent transitions were ΔLc = 29.2 ± 0.6 nm and 35.3 ± 0.6 nm, consistent with expectations respectively for folding stem 3 in addition to stem 1 (29.8 nm), and then for folding stem 2 as well to form the complete pseudoknot (matching the value seen for complete unfolding). Stem 2 thus folded last, after stem 3. Intriguingly, this order is precisely what is needed to form a 5′-threaded fold topology: stem 2 must form last, after the 5′ end is threaded across the junction between stems 1 and 3. To confirm the identification of these structures in the intermediates, we repeated the measurements using anti-sense oligos to block the formation either of stem 1 (oligo 1) or stem 2 and part of stem 3 (oligo 2), as shown in Fig. 4a. The initial refolding transitions with oligo 2 present (Fig. 4b) showed effectively the same p(Fc) (Fig. 4c, red) as without the oligo (Fig. 4c, black), and close to the same ΔLc, too, albeit elongated by an extra ~2 base-pairs formed with the part of stem 3 liberated by oligo 2 (Supplementary Table 1 and Supplementary Fig. 4e), confirming that stem 1 was first to refold. FECs with oligo 1 (Fig. 4d), on the other hand, showed refolding at notably lower force than stem 1 (Fig. 4c, blue); unfolding proceeded via an intermediate with a length corresponding to the lower half of stem 3, ΔLc = 8 ± 1 nm (Supplementary Table 1 and Supplementary Fig. 4d).

We also tested the importance of Mg2⁺ for the folding and stability of the pseudoknot by re-measuring FECs in the absence of Mg2⁺ (Fig. 4e). We found that almost all (97%) of the curves showed the length change for pseudoknot unfolding, although ΔLc was ~1 nm shorter than previously (Supplementary Table 1), indicating that the absence of Mg2⁺ disfavored Alt. Two populations were still present in p(Fc) (Fig. 4f), but the high-force population (N) was greatly reduced from what was observed with Mg2⁺ present (Fig. 2d), down to only 40 ± 8% of the FECs showing full-length unfolding, and it peaked at lower forces. Mg2⁺ was therefore not required for folding of the pseudoknot, but it played a key role in promoting the formation of the higher-force population attributed to the ring-knot fold topology. Fitting p(Fc) to characterize changes in the landscape (Fig. 4f, red), we found that ΔGc was little changed, but Δx was significantly higher, rising to 4.0 ± 0.6 nm for N′ and 2.8 ± 0.7 nm for N (Supplementary Table 2). The pseudoknots were thus much less rigid without Mg2⁺.

Finally, we examined the thermodynamic stability of N and N′ by using the Jarzynski equality44 to estimate the free-energy change relative to the unfolded state based on the non-equilibrium work done during unfolding15, while accounting for non-equilibrium populations of N and N′. The stability of the threaded conformer N in the presence of Mg2⁺ was estimated as ΔGN = 61 ± 7 kBT. This value was nominally somewhat higher than the stability of the unthreaded conformer, estimated as ΔGN = 55 ± 6 kBT, but similar within the error, which was relatively large because the unfolding was not near equilibrium. Repeating the analysis for the FECs measured without Mg2⁺, we found stabilities of 55 ± 2 kBT and 53 ± 2 kBT, respectively, for N and N′, suggesting that the threading does not significantly change the thermodynamic stability of the pseudoknot, even though it does change the mechanical stability.

Discussion

These results confirm the suggestion from simulations and cryo-EM imaging that the SARS-CoV-2 frameshifting signal can form a variety of different structures. The state N, which was by far the most common conformation under physiological-like conditions (handle far from stimulatory structure, with Mg2⁺ but without oligos), unfolded through the full length of the pseudoknot at moderately high force. This conformation was suppressed significantly by occlusion of the 5′ end by the duplex handle, precisely as would be expected for a 5′-end threaded structure such as those seen in cryo-EM images on and off the ribosome10,15 or predicted from simulations18. In contrast, the conformation unfolding at lower force, N′, while occurring ten-fold less frequently than N under normal conditions, increasingly replaced N as occlusion of the 5′ end suppressed the occupancy of N, as would be expected for a conformation in which the 5′ end remains unthreaded. Unthreaded conformers have been predicted computationally18 but not yet characterized structurally in experiments, although some individual cryo-EM images show the straight morphology expected for unthreaded conformers, in contrast to the bent shape of threaded conformers10.

The picture of the pseudoknot folding and unfolding that emerges from this work is illustrated in Fig. 5. Stem 1 always folds first, followed by sequential folding of stem 3 and then stem 2. The orientation of the 5′ end at the moment of stem 2 formation leads to two distinct fold topologies that cannot interconvert: 5′-
Fig. 4 Effects of anti-sense oligomers and Mg$^{2+}$ ions. a Anti-sense oligo 1 blocks formation of stem 1, oligo 2 blocks formation of stem 2. Participation of bases in secondary structure indicated by the same colors and labels as in Fig. 1a. b FECs with oligo 2 present unfold (black) and refold (red) through an extended stem 1 (I$_1$). Dashed lined: WLC fits (purple: I$_{b}$, cyan: I$_{f}$, gray: U). Inset: cartoon showing effect of oligo binding. c Refolding force distribution with oligo 2 present (red, N = 247 FECs from 3 molecules) is same as without oligos (black, N = 416 FECs from 10 molecules), but higher than with oligo 1 present (blue, N = 116 FECs from 2 molecules). d FECs with oligo 1 present (black: unfolding, red: refolding) show folding of stems 2 and 3 but not stem 1, through an intermediate (I$_{f}$) consistent with the lower part of stem 3. Dashed lines: WLC fits (brown: I$_{f}$, green: I$_{b}$). Inset: cartoon showing effect of oligo binding. e FECs for construct with 6-nit spacer in absence of Mg$^{2+}$ show unfolding (black) and refolding (red) of pseudoknotted structures (N') similar to FECs with Mg$^{2+}$, through same intermediate (I$_{f}$ in unfolding curves, I$_{b}$ in refolding curves), but with slightly shorter contour length changes. Curves offset for clarity. Dashed lined: WLC fits (navy: N', cyan: I$_{f}$/I$_{b}$). f Unfolding force distribution (N = 290 FECs from 2 molecules) shows two peaks, both at lower force than with Mg$^{2+}$. Red: two-population fit to landscape model.

Fig. 5 Pseudoknot folding and unfolding pathways. Schematic of pathways for folding (red) and unfolding (black) the pseudoknotted conformers. Stem 1 forms first, then stem 3, with a bifurcation leading to 5'-threaded (top) or -unthreaded (bottom) conformers depending on orientation of 5' end just before stem 2 forms. RNA segments are colored corresponding to their participation in native secondary structure elements as in Fig. 1a.

threaded or -unthreaded. These two topologies give rise to distinct unfolding behaviors: higher forces for the threaded fold, lower forces for the unthreaded fold. The partitioning of the folding at the point when stem 2 forms—depending on whether or not the 5' end is lying across the stem 1/stem 3 junction, as required for threading—ensures the presence of both threaded and unthreaded conformers, with the minority unthreaded state populated at some finite level, similar to what was seen in the folding of the Zika exoribonuclease-resistant RNA (xRNA)\textsuperscript{21}. This folding mechanism is dependent on stem 2 folding last; as it happens, stem 2 is also predicted by mfold\textsuperscript{17} to be the least stable thermodynamically, whereas stem 1 is expected to be the most stable, so that the folding is ordered by the relative stabilities of the stems as seen previously for two-stem pseudoknots\textsuperscript{34,48}. Intriguingly, this order is also the same one in which the stems would refold co-translationally as the ribosome leaves the frameshift signal: stem 1 is at the 5' end and would be expected to refold first, whereas stem 2 is at the 3' end and would not be able to refold until after the whole pseudoknot emerged from the ribosome. Notably absent from this picture of the folding, however, is a third fold that was predicted computationally with the 3' threaded through the stem 2/stem 3 junction\textsuperscript{18}, which makes sense given that this fold requires stem 1 to form last instead of first.
The energy landscape parameters for unfolding states N and N' further support this picture, in particular $\Delta x$, which reports on the mechanical rigidity of the RNA structure. Smaller $\Delta x$ implies a rigid structure that is less sensitive to tension and hence more likely to rupture in a brittle manner at high force, whereas larger $\Delta x$ implies a compliant structure that is more sensitive to tension and ruptures in a lower, narrower range of forces. State N was much more mechanically rigid than N', with $\Delta x$ roughly three times smaller, which makes sense in terms of the mechanical effects of threading: threading of the 5'-end should rigidify the fold via interactions between the 5'-end and the helical junction in the pseudoknot that constrain the motion of the terminus in unfolding. However, comparing NMR results to computational modeling suggests that Mg$^{2+}$ ions or determined how Mg$^{2+}$ affects the pseudoknot structure. Even though the observation of state N in the absence of Mg$^{2+}$ shows that Mg$^{2+}$ is not absolutely required for the pseudoknot folding, consistent with results from NMR, Mg$^{2+}$ clearly plays an important role. Given that $\Delta x$ increased significantly for both N and N' in the absence of Mg$^{2+}$, the latter must be essential for stabilizing tertiary contacts that rigidify both the threaded and unthreaded folds. Structural studies have not yet resolved coordinated Mg$^{2+}$ ions or determined how Mg$^{2+}$ affects the pseudoknot structure. However, comparing NMR results to computational modeling suggests that Mg$^{2+}$-mediated tertiary interactions may be especially important in the stem 2/loop 1 region, possibly explaining why both N and N' are more compliant in the absence of Mg$^{2+}$. Mg$^{2+}$ must also be important in stabilizing threading of the 5'-end into the stem 1/stem 3 junction, since removing Mg$^{2+}$ greatly reduced the incidence of the threaded conformer. Presumably, Mg$^{2+}$ binds near the stem 1/stem 3 junction to help coordinate the threading, consistent with suggestions from simulations showing Mg$^{2+}$-mediated interactions between the 5'-end and stem 1 that make the three-helix junction more compact. Such contacts with the threaded end in N might explain why the rigidification is twice as large for N as it is for N' (four-fold decrease in $\Delta x$ instead of two-fold).

Comparing the SARS-CoV-2 pseudoknot to the Zika virus xrRNA, the only other RNA forming a similar ring-knot whose folding has been studied, reveals some interesting differences. The ring-knot in the Zika virus xrRNA unfolds at a much higher force, above 60 pN, acting as a mechanical road-block to digestion of the viral RNA by host exoribonucleases. Such mechanical resistance would be functionally counterproductive for the SARS-CoV-2 pseudoknot, however, because although the latter acts to induce a frameshift, it nevertheless must not prevent ribosomal translocation. Concurrently, these two RNAs differ in the importance of Mg$^{2+}$ for threading of the 5'-end before closure of the pseudoknot: the absence of Mg$^{2+}$ abolishes threading entirely for the Zika virus xrRNA, leading to the formation of different structures, but it has a much less dramatic effect on the SARS-CoV-2 pseudoknot, partially inhibiting rather than abolishing the threading so as to rebalance the proportions of N and N'. We speculate that the reduced role of Mg$^{2+}$ in 5'-threading in the SARS-CoV-2 pseudoknot may help to reduce the mechanical stability of the ring-knot sufficiently to allow the ribosome to unfold it during −1 PRF.

Turning to the non-pseudoknotted conformer, Alt, the FECs provide several clues to its identity. Its low unfolding force and the relatively large distance to the barrier found from fitting the force distribution (Supplementary Fig. 1, red), $\Delta x^2 = 4.5 \pm 0.8$ nm, indicate that it involves secondary structure only. Moreover, the length change reveals that 46 ± 2 nt are folded in Alt, and the fact that Alt does not rapidly convert into N or N' implies that it involves stems that differ from those in the pseudoknotted conformers. The most likely structure consistent with these results is the hairpin with multiple bulges shown in Supplementary Fig. 4c. Because Alt is much less stable than N/N', it would be expected eventually to convert into a pseudoknot, given enough time. Indeed, analyzing only the first FEC measured for each molecule (for which the RNA had much more time to find the minimum-energy state than during repeated unfolding/refolding cycles) supports this picture: the fraction of FECs starting in Alt was reduced significantly, by roughly a factor of 3, to 6 ± 4%. Moreover, this conversion was occasionally seen directly, in rare examples where the RNA folded into Alt but converted to N/N' during the subsequent unfolding FEC (Supplementary Fig. 5). Alt was almost eliminated in the absence of Mg$^{2+}$, suggesting that Mg$^{2+}$ stabilizes it and helps to trap the RNA in Alt kinetically.

The significant heterogeneity seen here for the SARS-CoV-2 frameshift signal is entirely consistent with the direct correlation between conformational heterogeneity and −1 PRF efficiency found in recent work, given the relatively high level of −1 PRF in SARS-CoV-2 observed in functional assays, underscoring the functional relevance of understanding the force-dependent conformational dynamics. We note that the SMFS assay mimics several physiologically important features of −1 PRF: the stimulatory structure is indeed under tension applied directly by the ribosome, this tension is ramped up and down as the ribosome attempts to unfold the RNA during −1 PRF, and the stimulatory structure undergoes repeated unfolding/refolding cycles as multiple ribosomes translocate through it, sometimes in rapid bursts. However, SMFS does not perfectly recapitulate the circumstances in the cell. For example, the ribosome only applies force to the 5'-end of the RNA (not both ends as in the tweezers), and the force profile over time is more complex, including periods of sustained tension on the RNA while the ribosome is paused at the frameshift site, in addition to ramps up and down. Specific contacts between the SARS-CoV-2 pseudoknot and the ribosome are also proposed to play a role in −1 PRF. As a result, the forces needed to unfold the RNA in SMFS may differ from those involved in unfolding it in the cell. Although the duplex handles in the SMFS assay are not present in the cell, the fact that no change is seen when moving the handle from 6 to 12 nt away from the pseudoknot suggests that a 6-nt spacer is sufficient for the handle to have little to no effect on the folding.

Finally, we note that the existence of distinct fold topologies has important implications for structure-based drug-discovery efforts targeting the SARS-CoV-2 frameshift signal, because the structure of the junction between the helices in the pseudoknot, which is the locus of the most likely binding pockets for small molecules, is strongly affected by whether the 5'-end is threaded or not. Combining this observation with the previous result showing that −1 PRF efficiency varies linearly with the conformational heterogeneity as measured by the Shannon entropy, suggests a strategy for developing small-molecule modulators of −1 PRF in SARS-CoV-2: ligands that stabilize the 5'-threaded conformer (thereby decreasing the heterogeneity) should be sought for inhibiting −1 PRF, whereas ligands that stabilize the unthreaded conformer (thereby increasing the heterogeneity) should be sought for enhancing −1 PRF. Future work characterizing the interactions of the SARS-CoV-2 frameshift signal with ligands that have been found to modulate −1 PRF should help clarify the molecular mechanisms of action.
Methods

Sample preparation. Samples consisting of a single RNA strand linked at each end to double-stranded handles were prepared in two ways. (1) An RNA strand containing the SARS-CoV-2 pseudoknot and spacer sequences (Fig. 1a and Supplementary Table 4) flanked by long handle sequences was annealed to single-stranded (ss) DNA complementary to the handles sequences. The DNA fragment containing the SARS-CoV-2 pseudoknot and spacer sequences (Fig. 1a and Supplementary Fig. 1) was then annealed to the DNA handles, completing the construct. The sequences of the primers used in the two construct designs are listed in Supplementary Table 5.

Sample preparation. Samples consisting of 40 U/mL glucose oxidase, 185 U/mL catalase, and a scavenging system consisting of 40 U/mL glucose oxidase, 185 U/mL catalase, and 50 mM MOPS pH 7.5, 130 mM KCl, 4 mM MgCl2, and 200 mM U/mL RNase inhibitor (SUPERase In, Ambion). An oxygen scavenging system consisting of 40 U/mL glucose oxidase, 185 U/mL catalase, and 250 mM D-galactose was also included in the buffer. The diluted dumbbells were then annealed to the RNA, bringing them back together at the same speed to ramp the force back down to ~0 pN, waiting 5–10 s to allow refolding, and then repeating the cycle. Trap stiffnesses were 0.45–0.62 pN/nm. Data were sampled at 20 kHz and filtered online at the Nyquist frequency. Measurements with anti-sense oligos added oligo 1 or oligo 2 to the measuring buffer at a final concentration of 10 μM. For measurements in the absence of Mg2+, MgCl2 was removed from the measuring buffer and EDTA was added to a final concentration of 1 mM.

The thermodynamic stabilities of N and N' were determined by calculating distributions of work done during unfolding, found by integrating the fitted unfolding FECs from the extension corresponding to F = 2 pN up to the point where the unfolded state was reached, while subtracting the work done to stretch out the unfolded RNA (found from integrating the WLC for the unfolded state between the same two end-points). Because N and N' have very similar ΔLp and their unfolding force distributions overlap significantly, it was not possible to assign definitively the initial state of any given FEC to N or N', as needed to build the work distribution for each state. Instead, we used a probabilistic approach, determining the relative likelihood that a given FEC unfolded from N or N' based on its unfolding force, using the fits of the unfolding force distributions to Eq. 2: the likelihood that a curve with unfolding force F0 started in state N (or N') was given by the probability density function P(W; F0) = exp[−B(W−W0)]; We used this likelihood function to assign each curve to N or N' while sampling a number of curves equal to the total number measured, with replacement, thereby generating the unfolding work distribution for each state for this sampling. We then calculated the free energy for unfolding from the Jarzynski equality \[ ΔG = k_B T \ln \left( \frac{\exp[-B(W)]}{\exp[-B(W0)]} \right) \]

where \( B = k_B T \phi \), \( k_B \) is the Boltzmann constant, and \( T \) is the temperature. Using this approach for the forces in the Jarzynski estimate using the unbiased sum of the free energy values to calculate the dissipated work. We also corrected for the non-equilibrium populations of N and N, adding an additional energy \( -k_B T \ln (\phi) \), where \( \phi \) is the fraction of refolding curves that end in the state (N or N') whose stability is being calculated. This procedure was then repeated 5000 times while resampling the curves and recalculating their assignments to N or N' yielding the average values and standard deviation for ΔG reported for N and N'.

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References

1. Kelly, J. A. et al. Structural and functional conservation of the programmed −1 ribosomal frameshift signal of SARS coronavirus 2 (SARS-CoV-2). J. Biol. Chem. 295, 10741–10748 (2020).
2. Brierley, L., Gilbert, R. J. C. & Pennell, S. Pseudoknot-dependent programmed −1 ribosomal frameshifting: structures, mechanisms and models. Recoding: Expansion Decoding Rules Enriches Gene Exp. 24, 149–174 (2009).
3. Akams, J. F., Loughran, G., Bhatt, P. R., Firth, V. E. & Baranov, P. V. Ribosomal frameshifting and transcriptional slippage: from genetic steganography and crytptography to adventitious use. Nucleic Acids Res. 44, 7007–7017 (2016).
4. Dulude, D., Berchiche, Y. A., Gandron, K., Braker-Gingras, L. & Heveker, N. Deciphering the frameshift efficiency translates into an equivalent reduction of the replication of the human immunodeficiency virus type 1. Virology 345, 127–136 (2006).
5. Plant, E. P., Rakauskaite, R., Taylor, D. R. & Dinman, J. D. Achieving a golden mean: mechanisms by which coronaviruses ensure synthesis of the correct stoichiometric ratios of viral proteins. J. Virol. 84, 4330–4340 (2010).
6. Plant, E. P., Sims, A. C., Baric, R. S., Dinman, J. D. & Taylor, D. R. Altering SARS Coronavirus frameshift efficiency affects genomic and subgenomic RNA production. Viruses 5, 279–294 (2013).
7. Belew, A. T. & Dinman, J. D. Cell cycle control (and more) by programmed -1 ribosomal frameshifting: implications for disease and therapeutics. Cell Cycle 14, 172–178 (2015).

8. Park, S.-J., Kim, Y.-G. & Park, H.-J. Identification of RNA pseudoknot-binding ligand that inhibits the −1 ribosomal frameshifting of SARS-CoV-2 RNA genome. bioRxiv 2020.07.18.209270. https://doi.org/10.1101/2020.07.18.209270 (2020).

9. Neupane, K. et al. Anti-frameshifting ligand active against SARS Coronavirus-2 is resistant to natural mutations of the frameshift-stimulatory pseudoknot. J. Mol. Biol. 432, 5843–5847 (2020).

10. Zhang, K. et al. Cryo-electron microscopy and exploratory antisense targeting of the 28-kDa frameshift stimulation element from the SARS-CoV-2 RNA genome. bioRxiv 2020.07.18.209270. https://doi.org/10.1101/2020.07.18.209270 (2020).

11. Neupane, K. et al. Anti-frameshifting ligand active against SARS Coronavirus-2 is resistant to natural mutations of the frameshift-stimulatory pseudoknot. J. Mol. Biol. 432, 5843–5847 (2020).

12. Chen, Y. et al. A drug screening toolkit based on the −1 ribosomal frameshifting of SARS-CoV-2. Heliyon 6, e04793 (2020).

13. Sun, Y., Abriola, L., Sunovtseva, Y. V., Lindenbach, B. D. & Guo, J. U. Restriction of SARS-CoV-2 replication by targeting programmed −1 ribosomal frameshifting, Proc. Natl Acad. Sci. U.S.A. 118, 2023051118 (2021).

14. Kelly, J. A., Woodside, M. T. & Dinman, J. D. Programmed −1 ribosomal frameshifting in coronaviruses: a therapeutic target. Virology 554, 73–82 (2022).

15. Bhatt, P. R. et al. Structural basis of ribosomal frameshifting during translation of the SARS-CoV-2 RNA genome. Science 372, 1306–1313 (2021).

16. Wacker, A. et al. Secondary structure determination of conserved SARS-CoV-2 RNA elements by NMR spectroscopy. Nucleic Acids Res. 48, 12415–12435 (2020).

17. Plant, E. P. & Dinman, J. D. The role of programmed−1 ribosomal frameshifting in coronavirus propagation. Front. Biosci. 13, 4873–4881 (2008).

18. Omar, S. I. et al. Modeling the structure of the frameshift-stimulatory pseudoknot in SARS-CoV-2 reveals multiple possible conformers. PLoS Comput. Biol. 17, e1008665 (2021).

19. Akiyama, B. M. et al. Zika virus produces noncoding RNAs using a multi-pseudoknot structure that confounds a cellular exonuclease. Science 354, 1148–1152 (2016).

20. Steckelberg, A. L. et al. A folded viral noncoding RNA blocks host cell exoribonucleases through a conformationally dynamic RNA structure. Proc. Natl Acad. Sci. U.S.A. 115, 6404–6409 (2018).

21. Zhao, M. & Woodside, M. T. Mechanical strength of RNA knot in Zika virus protects against cellular defenses. Nat. Chem. Biol. https://doi.org/10.1038/s41589-021-00829-2 (2021). in press.

22. Ritchie, D. B., Foster, D. A. N. & Woodside, M. T. Programmed −1 frameshifting efficiency correlates with RNA pseudoknot conformational plasticity, not resistance to mechanical unfolding. Proc. Natl Acad. Sci. U.S.A. 109, 16167–16172 (2012).

23. de Messieres, M. et al. Single-molecule measurements of the CCR5 mRNA unfolding pathways. Biophys. J. 106, 244–252 (2014).

24. Ritchie, D. B. et al. Conformational dynamics of the frameshift stimulatory structure in HIV-1 RNA. 23, 1357–1384 (2017).

25. Halma, M. T. J., Ritchie, D. B., Cappellano, T. R., Neupane, K. & Woodside, M. T. Complex dynamics under tension in a high-efficiency frameshift stimulatory structure. Proc. Natl Acad. Sci. U.S.A. 116, 19500–19505 (2019).

26. Ritchie, D. B., Soong, J., Sikkema, W. K. A. & Woodside, M. T. Anti-frameshifting ligand reduces the conformational plasticity of the SARS virus pseudoknot. J. Am. Chem. Soc. 136, 2196–2199 (2014).

27. Halma, M. T. J., Ritchie, D. B. & Woodside, M. T. Conformational shannon entropy of mRNA structures from force spectroscopy measurements predicts the efficiency of −1 programmed ribosomal frameshift stimulation. Phys. Rev. Lett. 126, 038102 (2021).

28. Qu, X. et al. The ribosome uses two active mechanisms to unwind messenger RNA during translation. Nature 475, 118–121 (2011).

29. Liu, T. et al. Direct measurement of the mechanical work during translocation by the ribosome. eLife 3, e03406 (2014).

30. Yan, S., Wen, J.-D., Bustamante, C. & Tinoco, I. Ribosome excursions during mRNA translation mediate broad branching of frameshift pathways. Cell 160, 870–881 (2015).

31. Ritchie, D. B. & Woodside, M. T. Probing the structural dynamics of proteins and nucleic acids with optical tweezers. Curr. Opin. Struct. Biol. 34, 43–51 (2015).

32. Woodside, M. T. & Block, S. M. Reconstructing folding energy landscapes by single-molecule force spectroscopy. Annu. Rev. Biophys. 43, 19–39 (2014).

33. Woodside, M. T. et al. Nanomechanical measurements of the sequence-dependent folding landscapes of single nucleic acid hairpins. Proc. Natl Acad. Sci. U.S.A. 103, 6190–6195 (2006).
