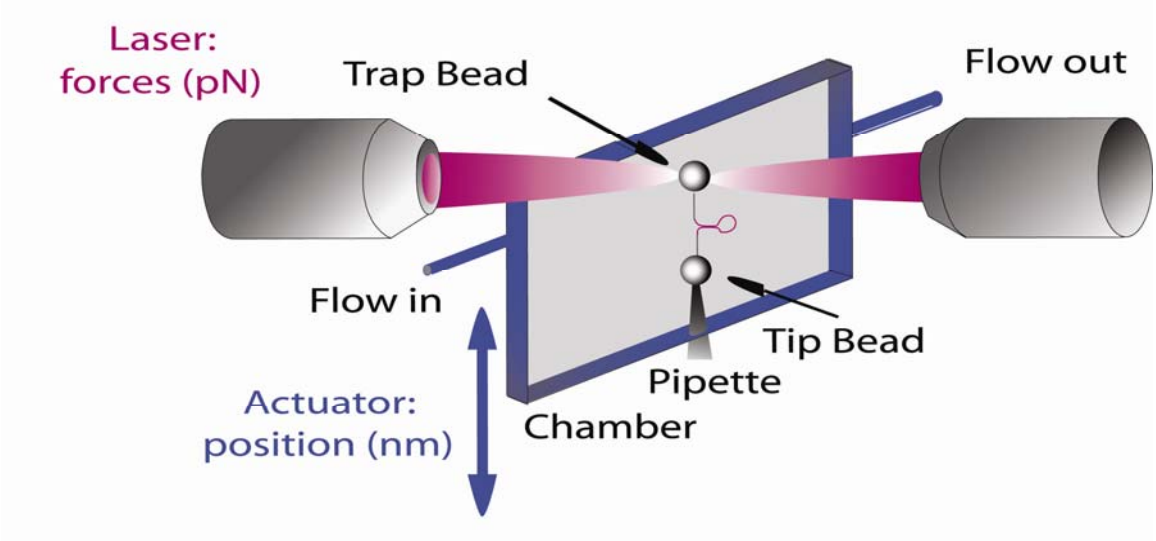
Supplementary Materials

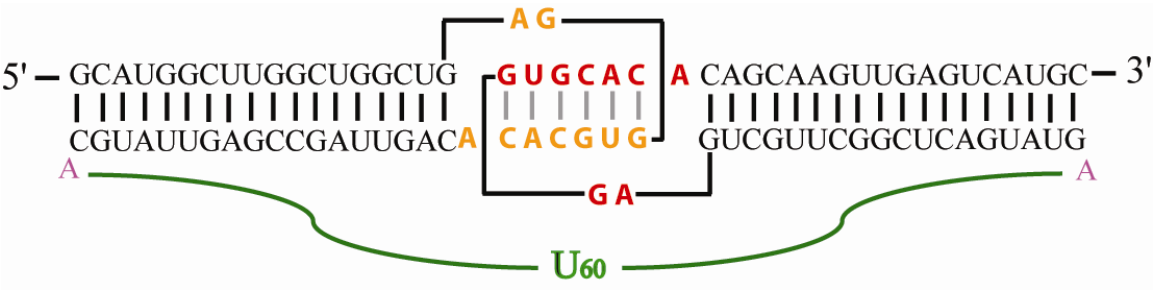
Figure S1 Setup of an optical tweezers' experiment.



A dual-beam laser trap was set up in the middle of a flow chamber.[1] The RNA molecule was tethered between the two micro-size beads. One bead was held by an optical trap and the other by a micropipette mounted on a piezoelectric stage. The piezoelectric flexure stage moves the pipette and stretches the RNA. Force is measured by the spring constant of the trap and distance of the trapped bead from the center of the trap. Change in the extension of the molecule was measured by relative movements of the trapped bead and the piezoelectric flexure stage. Force and extension of the molecule were recorded at a rate of 100 Hz.

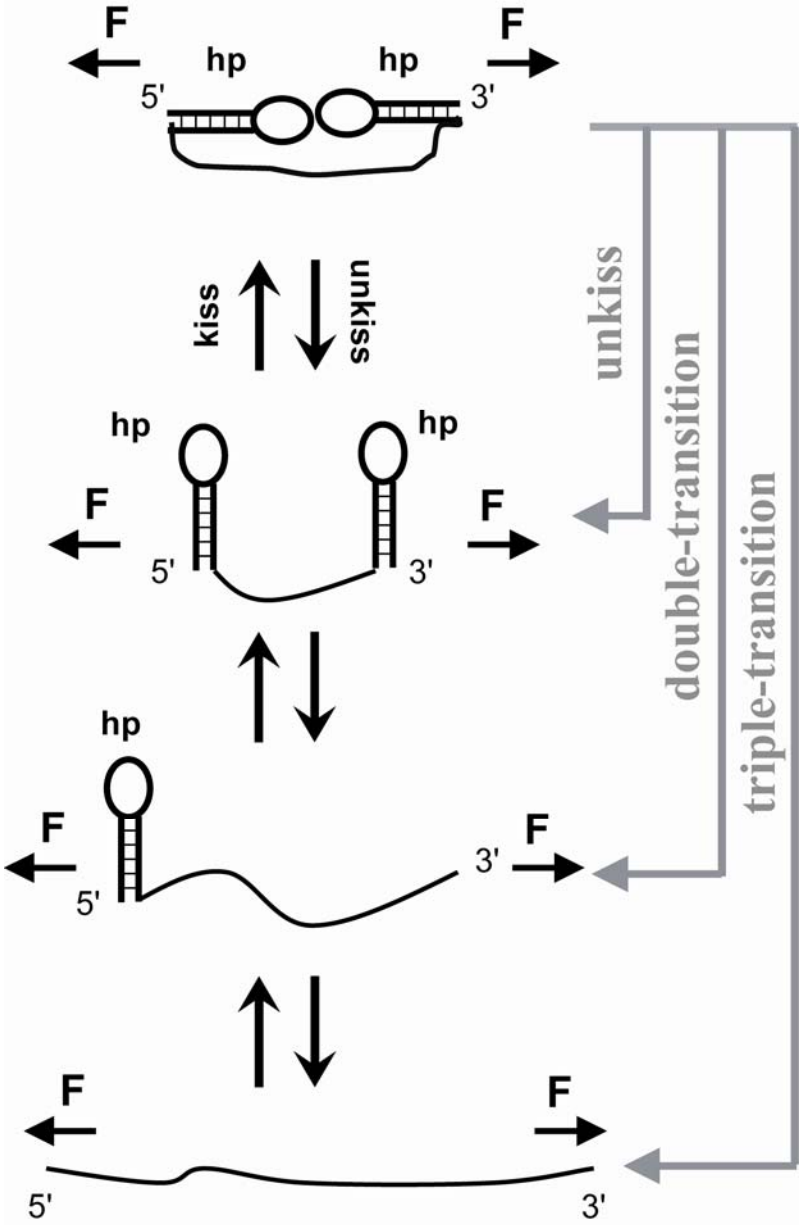
[1] Smith, S. B.; Cui, Y.; Bustamante, C., *Methods Enzymol.* **2003,** 361, 134-62.

Figure S2 Detailed structure of the Mal type kissing complex.



The loop sequences are colored in orange and red. A few G•U mismatches are incorporated so that the two hairpins have slightly different stability (0.5 kcal/mol by the RNA secondary structure prediction program Mfold). The Lai type kissing complex has the same structure except different loop sequence.

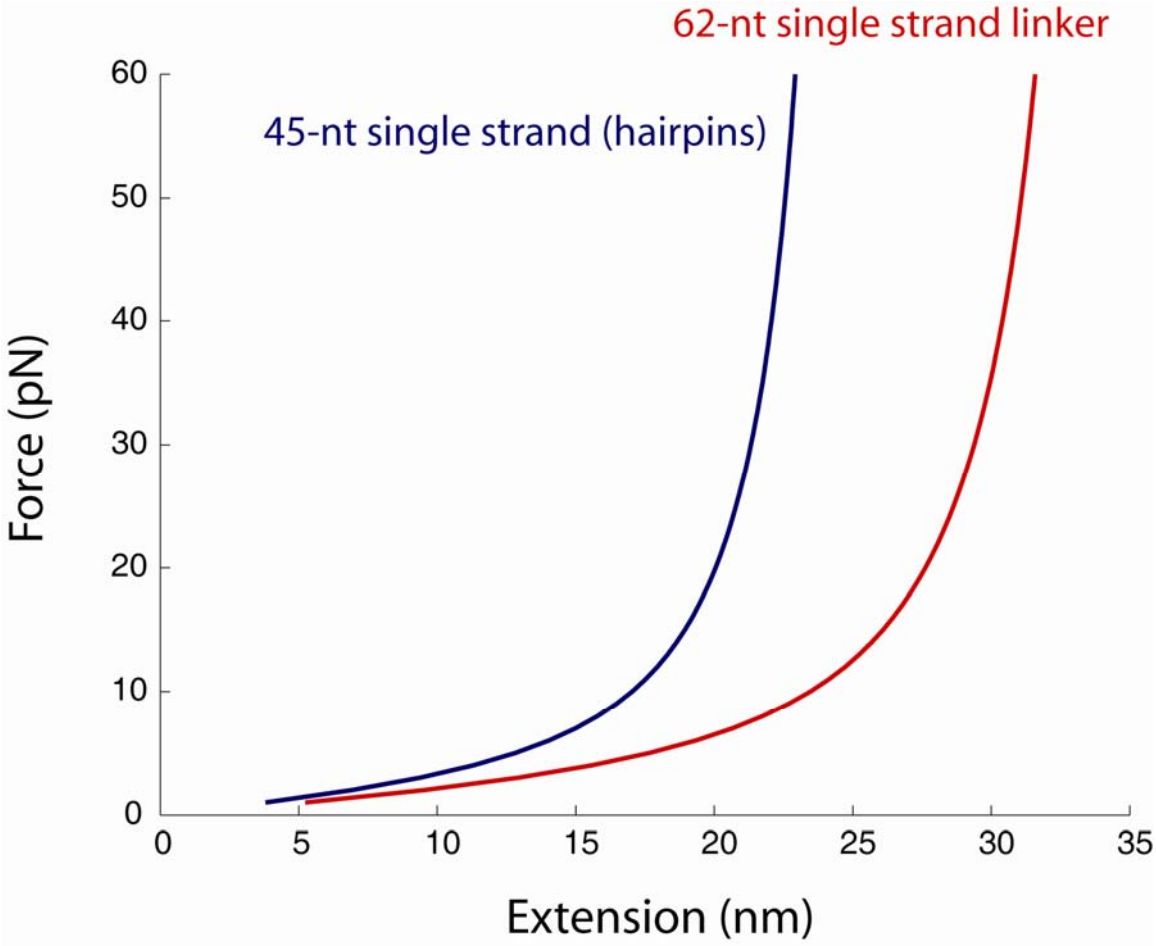
Figure S3 Unfolding and refolding pathways of the kissing complex.



The unfolding and refolding pathways of the DIS kissing complexes are similar to those of the minimal kissing complex. [2] The first unfolding transition is always disruption of the kissing interaction, followed by unfolding of the hairpins. The extension of the molecule increases as unfolding progresses. Double- and triple-transitions follow the same unfolding pathways but there is no apparent delay between sequential steps. On refolding, the two hairpins fold first; and the kissing occurs at lower force.

[2] Li, P. T. X., C. Bustamante, and I. Tinoco, Jr. (2006) Proc. Natl. Acad. Sci. U. S. A. 103:15847-15852.

Figure S4 Estimation of ΔX as a function of force.



Based on the structure of the kissing complexes (Figure S2), we estimated ΔX for unfolding and refolding transitions as a function of force. In the RNA structures, the entire double stranded region, including the kissing base pairs, has 42 base pairs, which is smaller than the persistence length of the double stranded DNA (50 base pairs). Therefore, we assumed that the double stranded segments in the structures are rigid and not extendable with a helical rise of 0.3 nm per base pair. Elasticity of single strands is described by a worm-like-chain interpolation formula: [3]

$$F = \frac{k_B T}{P}\left[\frac{1}{4(1-X/L)^2} + \frac{X}{L} - \frac{1}{4}\right]$$

in which F is the force; X is the extension, L is the contour length, P is the persistence length, T is the temperature, F is the force, and k_B is Boltzmann constant. We chose a persistence length of 1 nm and a contour length of 0.59 nm per nucleotide for single strand RNA. Figure S4 shows computed force-ΔX relationship of two single strands. The 62-nt single strand corresponds to the polyU linker (with two adenosines) and the 42-nt one resembles the unfolded form of the two hairpins. We also assumed that the helical axis of a hairpin is 2 nm.

[3] Bustamante, C., Marko, J., Siggia, E., and Smith, S. (1994) *Science* **265**, 1599-1600.

The end-to-end distance of the kissing hairpins, X_{kiss}, is

$$X_{kiss} = 42bp \times 0.30nm/bp = 12.6nm$$ (18 bp per hairpin + 6 kissing bp)

ΔX_{unkiss} for breaking the kissing interaction is

$$\Delta X_{unkiss} = X_{linker} - X_{kiss} + 4nm = X_{62-nt} - X_{kiss} + 4nm$$

where X_{linker} is the length of the single-stranded linker (red curve).

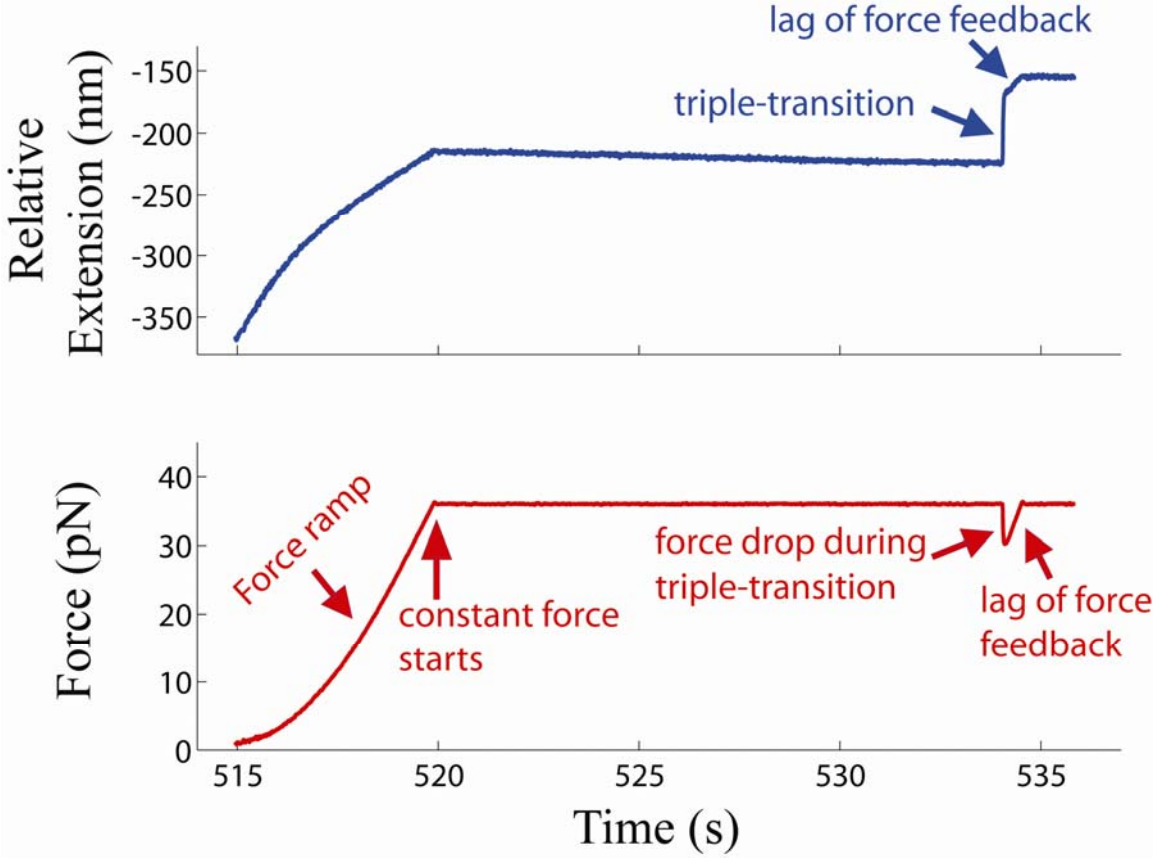
In the double-transition, one hairpin breaks right after the unkiss, so

$$\Delta X_{double} = \Delta X_{unkiss} + \Delta X_{hp} = \Delta X_{unkiss} + \Delta X_{45-nt} - 2nm$$

And in the triple-transition

$$\Delta X_{triple} = \Delta X_{unkiss} + 2\Delta X_{hp} = \Delta X_{unkiss} + 2\Delta X_{45-nt} - 4nm$$

Figure S5 Long lifetime of the kissing structures at high force.

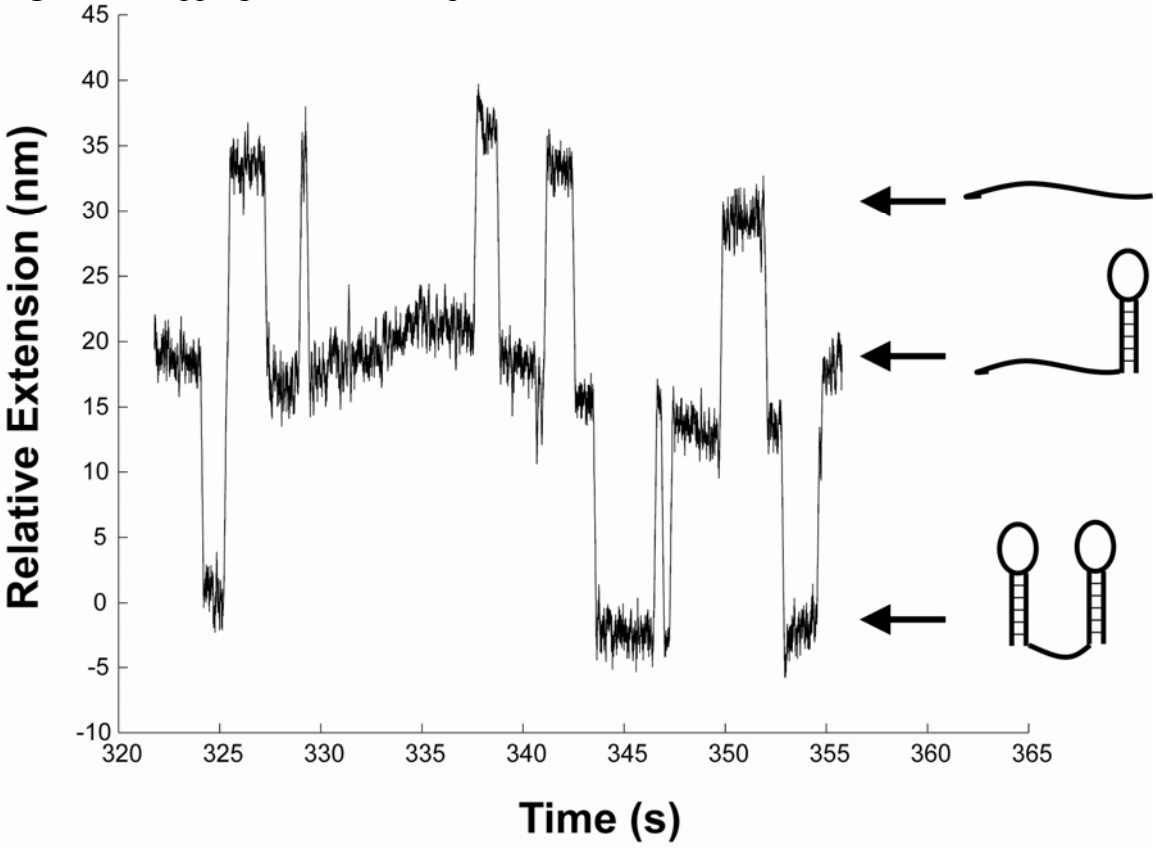


The kissing structures show a wide rip force distribution and also high rip forces indicating slow unkiss kinetics at relatively high forces. To test this hypothesis, we did a force-jump experiment[4] to directly measure the lifetimes of the kissing structures at high forces. Because the DIS kissing complexes are stable, it usually require a >30 pN jump to break the kissing interaction in less than a minute. Such a large force jump occurs within 100 ms usually breaks tethering of the molecule between the beads. We then modified the procedure to raise the force with a loading rate ~5-10 pN/s. Because of the long duration of force ramp, observed lifetimes of the kissing structures at a constant force do not reflect the unkiss kinetics directly. Nevertheless, it is clear from the figure that the Mal type kissing structure lasted for more than 20 seconds at 36 pN, which is consistent with our hypothesis.

During the triple-transition, force decreased by over 5 pN and it took almost a second for the instrument to return to the set force. This lag is largely caused by the fact that the PID (proportional-integral-differential) setting of the feedback has been optimized to dampen force fluctuations within a ±0.5 pN range. The feedback did not handle large force changes as efficiently as small force fluctuation. However, since the lag of force feedback occurred after the unfolding transition, it had no effect on the measured lifetimes of the RNA structures.

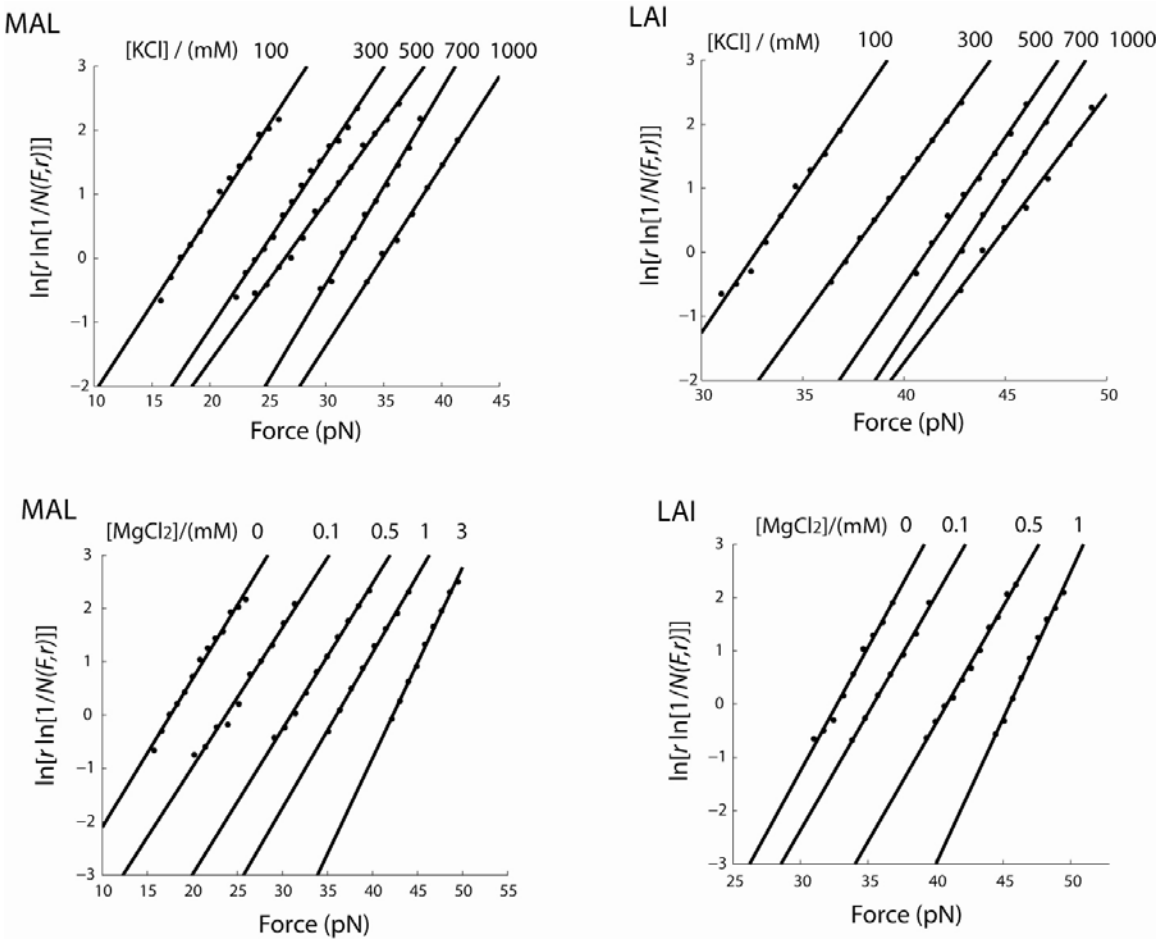
[4] Li, P. T. X., D. Collin, S. B. Smith, C. Bustamante, and I. Tinoco, Jr. (2006) Biophys. J. 90:250-260.

Figure S6 Hopping of the two hairpins.



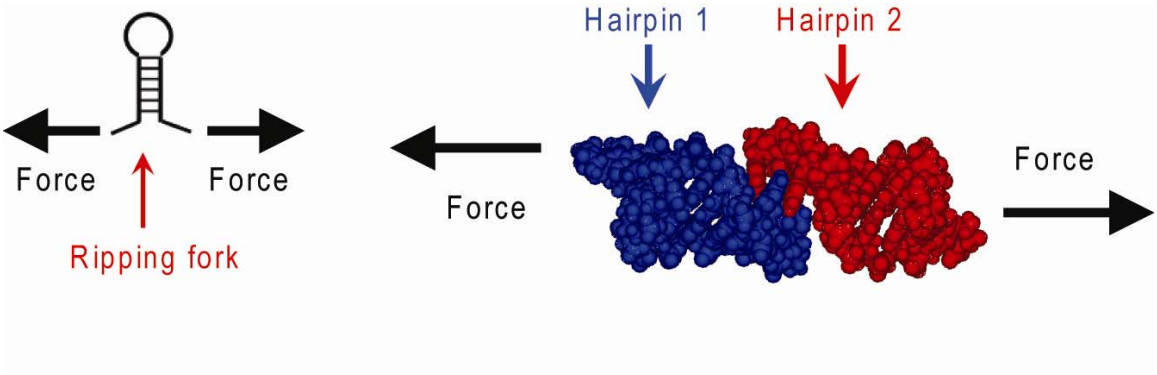
The two hairpins have same length and similar sequence (Figure S2). Their ΔGs are predicted to be different only by 0.5 kcal/mol. The hairpins hop between 16 pN and 18 pN. The figure shows a trace at 17 pN that the unkissed RNA existed in three different structures in order of decreasing extension: single strand, one hairpin and two-hairpin. Above 18 pN, the dominant form of the unkissed RNA is a single strand.

Figure S7 Evans and Ritchie analyses of unkiss force distributions



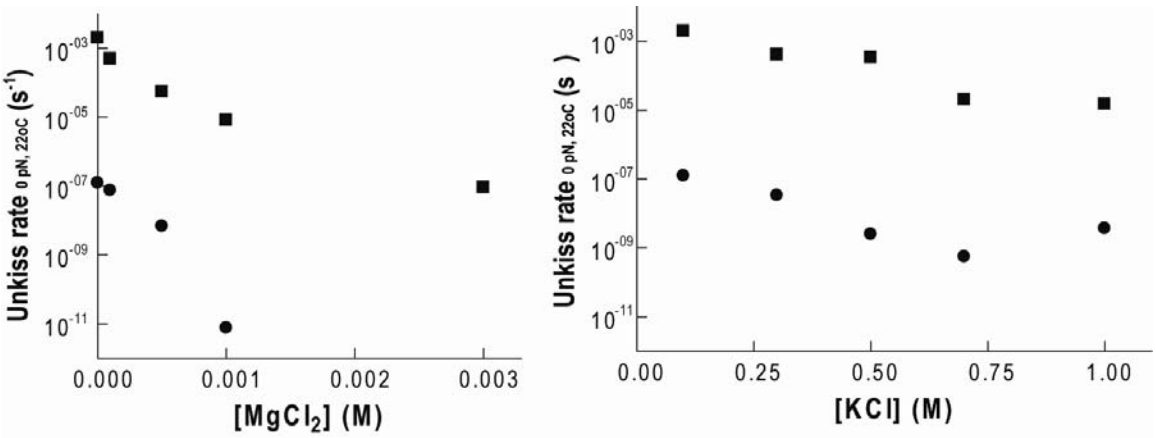
Distributions of unkiss forces shown in Figure 4 and 5 were transformed to *ln[r ln[1/N(F,r)]]* vs. *F* relations. Solid lines were fit to Eq. 1. Slopes of the fitted lines equal to $\Delta X^{\ddagger}/ k_B T$; y-intercepts equal to $k_0 - ln(\Delta X^{\ddagger}/ k_B T)$. Values of k_0 and $\Delta X^{\ddagger}/ k_B T$ were listed in Tables 1 and 2.

Figure S8 Direction of applied force in unfolding tertiary and secondary structures.



The hairpin is unfolded by unzipping (left) and the two GC kissing base pair is under shearing force (right). As ripping fork moves up in the hairpin stem, one base pair breaks at a time. In contrary, the two kissing base pairs are both broken at the unfolding transition state. The direction of applied force relative to a structure has impact on the mechanical rupture of the structure.

Figure S9 Unkiss rate constants extrapolated to zero force.



Unkiss rate of Mal (■) and Lai (●) kissing complexes are extrapolated to zero force using Eq. 1.