Supplementary Note

DNA Duplex Design and Construction

Double-stranded DNA was amplified from a M13mp18 plasmid (Bayou Biolabs) by Autosticky PCR[1], which in our hands gave higher yields than asymmetric PCR[2]. This approach generates the necessary 15-bp overhang (5′—*TTGAAATACCGACCG*—3′) by using a primer with an internal abasic site that prevents read-through by the polymerase. All linkages and fluorophores were incorporated via conjugation to the PCR primers (QIAGEN Operon).

To assemble the bead:DNA complexes, the long dsDNA with a 15-bp single-stranded overhang was incubated with a 5-fold excess of the complimentary 15-bp digoxygenin-labeled oligonucleotide. Annealing the strands for 5 min at 60°C, followed by a slow cooling at 1°C/min to 10°C, ensured faithful base-pairing of the two single-stranded components. For experiments requiring dyes on the 15-mer, we used oligonucleotides with TAMRA conjugated to the 3′-end. To confirm the specificity of the attachment of DNA to the glass surface, we prepared complexes lacking digoxygenin to verify that no bead tethers formed. Avidin-coated polystyrene beads (500˚nm diameter) were prepared as described previously[3]. The molar concentration of beads used was 4-fold that of the dsDNA to ensure that most beads would be tethered to the coverglass by a single DNA molecule.

Complexes were bound to the surface of a coverglass in a ~20 µL flowcell, constructed by using two strips of double-sticky tape to form a channel between the microscope slide and a #1_ thickness coverglass (Corning). Slides and coverglasses were cleaned by sonication in 5M ethanolic KOH, rinsed in ethanol followed by water, then oven-dried. The cell was incubated for 40 min with 20 µg/ml anti-digoxygenin polyclonal antibody (Roche Molecular Biochemicals) dissolved in PBS, rinsed with 0.1˚mg/ml BSA solution, incubated with 3.0 mg/ml BSA solution for 20 min, then incubated with bead:DNA complexes for 20 min. After incubation with the complexes, the flow cells were rinsed with 0.1 mg/ml BSA solution, then rinsed again with 3.0˚mg/ml BSA solution containing 0.1% β-mercaptoethanol and de-gassed with a light

vacuum. All BSA solutions were prepared in 100 mM Na-phosphate buffer pH 7.5 containing 0.1% Tween to prevent beads from clumping.

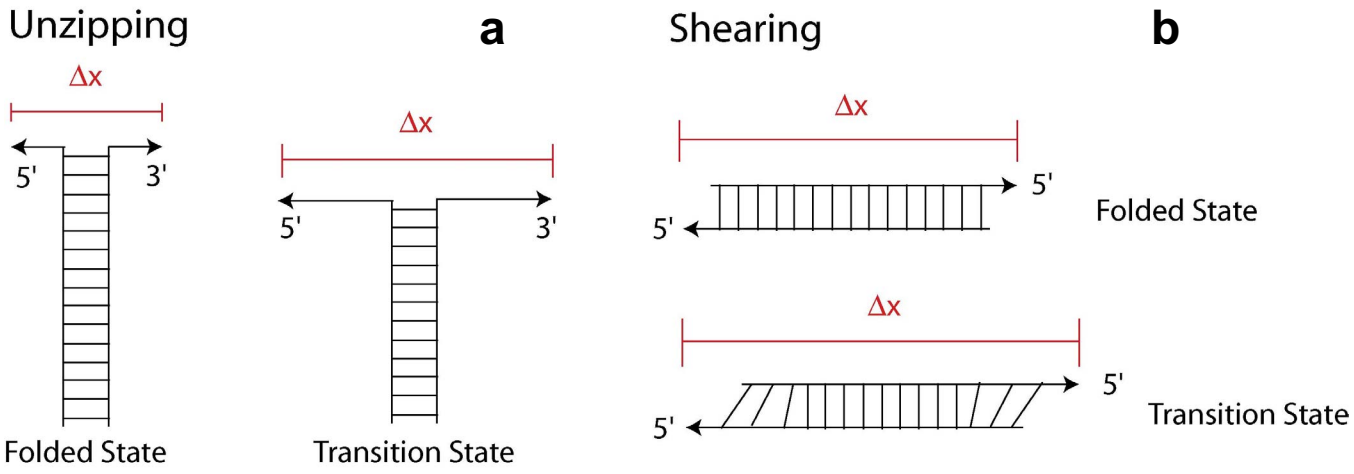
Determination of Zero-Load Parameters from Rupture Force Distributions

To determine the distance to the transition state and the thermal off rate in the absence of load, we directly fit the rupture force distribution to the theoretical rupture probability density function. Recent evidence suggests that this supplies a more accurate means of estimating these two parameters than the traditional approach of analyzing the dependence of rupture force on pulling rate[4]. Here, we apply the single energy-barrier formalism developed by Evans and Ritchie[5] and fit the rupture distributions to the following probability density[4,5]:

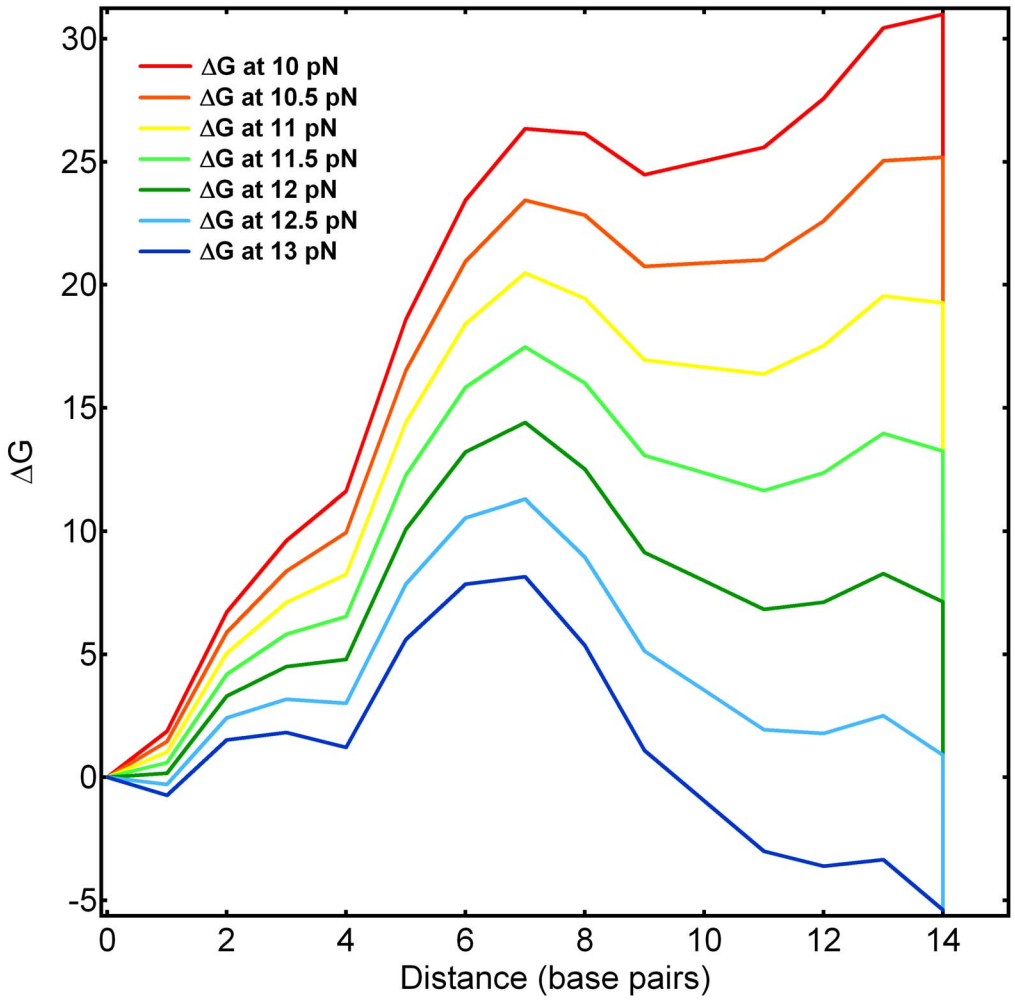
$$p(F) = \frac{v_0}{\left[\partial F / dt\right]} \exp\left[\frac{Fx}{k_B T}\right] \exp\left\{\frac{v_0 k_B T}{\left[\partial F / \partial t\right] x}\left[1 - \exp\left(\frac{Fx}{k_B T}\right)\right]\right\},$$

where v_0 is the thermal off-rate in the absence of load, $k_B T$ is Boltzmann s constant times the temperature, $\partial F/\partial t$ is the (constant) loading rate, x is the distance to the transition state, and F represents the x-component of the rupture force; v_0 and x were used as free parameters in the fit.

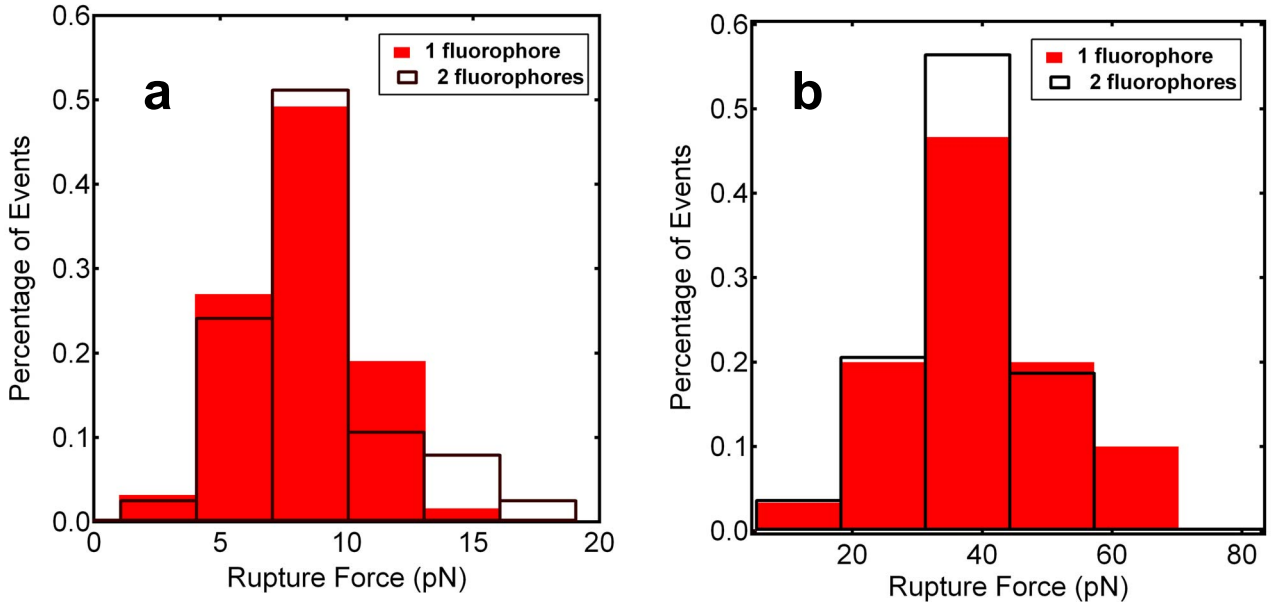
Supplementary Figure 1 Physical interpretation of distance to the transition state for unzipping and shearing forces. **(a)** Diagrams showing 5′-to-3′-end distances for folded and transition states during unzipping. Distance to the transition state is the difference between the 5′-to-3′-end distances in each case. **(b)** Diagrams showing 5′-to-5′-end distances for folded and transition states during shearing. Distance to the transition state is the difference between the 5′-to-5′-end distances in each case.



Supplementary Figure 2 Computed free energy surfaces of the 15-mer duplex DNA unzipping under varying external loads. Free energies were predicted according to polymer, dumbbell, and oligonucleotide nearest-neighbor thermodynamics[6], with the energy landscapes tilted by different slopes corresponding to the applied force (legend). The shape of the energy landscape suggests that the entire complex should unzip after ~7˚bp have been separated, in contrast with the 2-3˚bp predicted by directly fitting the rupture force distribution. A similar discrepancy has also been reported in studies of folding and unfolding RNA hairpins[7].



Supplementary Figure 3 Data showing rupture force distributions for unzipping and shearing apart complexes terminally labeled with either one and two fluorophores. **(a)** Unzipping force histograms. The average rupture force was 10.0˚± 0.3˚pN (s.e.m.) for singly-labeled complexes (n˚= 63) and 10.6˚± 0.5˚pN (s.e.m.) for doubly-labeled complexes (n˚= 37). **(b)** Shearing force histograms. The average rupture forces were 38˚± 2˚pN (s.e.m.) for singly-labeled complexes (n˚= 30) and 37˚± 1˚pN (s.e.m.) for doubly-labeled complexes (n˚= 53). Histograms of the rupture forces for singly and doubly labeled complexes were statistically indistinguishable for each rupture mode.



References for Supplementary Information

1. Gal, J., Schnell, R., Szekers, S. & Kalman, M. Directional cloning of native PCR products with preformed sticky ends (Autosticky PCR*). *Molecular. Gen. Genet.* **260**, 569-573 (1999).
2. Ausubel, F.M. *et al. Short protocols in molecular biology* (John Wiley and Sons, Inc., 2002).
3. Neuman, K.C., Abbondanzieri, E.A., Landick, R., Gelles, J. & Block, S.M. Ubiquitous transcriptional pausing is independent of RNA polymerase backtracking. *Cell* **115**, 437-447 (2003).
4. Friedsam, C., Wehle, A.K., Kuhner, F., and H.E. Gaub. Dynamic single-molecule force spectroscopy: bond rupture analysis with variable spacer length. *J. Phys. - Condens. Mat.* **15**, S1709-S1723 (2003).
5. Evans, E. & Ritchie, K. Dynamic strength of molecular adhesion bonds. *Biophys. J.* **72**, 1541-1555 (1997).
6. SantaLucia, J. J. A unified view of polymer, dumbbell, and oligonucleotide DNA nearest-neighbor thermodynamics. *Proc. Natl. Acad. Sci. U S A* **95**, 1460-1465 (1998).
7. Liphardt, J., Onoa, B., Smith, S.B., Tinoco, I. Jr., and C. Bustamante. Reversible unfolding of single RNA molecules by mechanical force. *Science* **292**, 733-737 (2001).