Submicrometer elasticity of double-stranded DNA revealed by precision force-extension measurements with magnetic tweezers

Min Ju Shon†‡, Sang-Hyun Rah†, Tae-Young Yoon1,2*

Submicrometer elasticity of double-stranded DNA (dsDNA) governs nanoscale bending of DNA segments and their interactions with proteins. Single-molecule force spectroscopy, including magnetic tweezers (MTs), is an important tool for studying DNA mechanics. However, its application to short DNAs under 1 μm is limited. We developed an MT-based method for precise force-extension measurements in the 100-nm regime that enables in situ correction of the error in DNA extension measurement, and normalizes the force variability across beads by exploiting DNA hairpins. The method reduces the lower limit of tractable dsDNA length down to 198 base pairs (bp) (67 nm), an order-of-magnitude improvement compared to conventional tweezing experiments. Applying this method and the finite worm-like chain model we observed an essentially constant persistence length across the chain lengths studied (198 bp to 10 kbp), which steeply depended on GC content and methylation. This finding suggests a potential sequence-dependent mechanism for short-DNA elasticity.

INTRODUCTION

Bending of short double-stranded DNA (dsDNA) represents a critical step in many processes within cells including regulation of transcription, chromatin remodeling, and packaging of genome (1). Such bending depends on the intrinsic elasticity of DNA as well as additional modulation by DNA binding proteins. The lengths of the dsDNA segments under elastic deformations can be as short as their well-accepted persistence length, ~50 nm or ~150 base pairs (bp) (2, 3), thereby incurring a substantial energy cost in bending. Thus, it has been questioned how such an energetically demanding process occurs and how much the native, protein-independent flexibility of DNA contributes to the steep curves of dsDNA observed in cells. Consequently, the nanomechanical properties of short dsDNA have been the subject of extensive theoretical and experimental investigations (1).

Traditionally, elasticity of DNA has been studied by diverse approaches including cyclization assays, optical methods, and single-molecule techniques [reviewed in (1, 2)]. Clever designs that can enforce high curvatures in DNA have also been devised (4–6). Virtually, all of the results to date support the classical worm-like chain (WLC) model for long dsDNA molecules, which treats a chain of DNA as an elastic rod with a harmonic bending potential. In contrast, many of the previous studies have observed that the elastic response of a short DNA molecule deviates from the WLC prediction when its contour length is below 1 μm (7–12). The complications with short dsDNAs are both theoretical and experimental: (i) The WLC model presuming a sufficiently long chain breaks down because of the finite chain length (9). (ii) The mechanical measurements on DNA become increasingly challenging as the molecular extension reaches the nanoscale length regime.

Direct manipulation and observation of DNA became possible by single-molecule force spectroscopy (SMFS) techniques such as magnetic tweezers (MTs), optical tweezers, and atomic force microscopy (3, 13, 14). Application of force in SMFS setups stretches DNA and provides a wealth of information inaccessible from spontaneous bending. For example, profiling force-extension curves (FECs) with SMFS is one of the most direct methods to probe the elasticity of nucleic acids. In this method, a stretching force is applied to the ends of a molecule via a microsphere, or “bead,” tethered to the molecule. The resulting force-induced extension of the molecule is recorded from the changes in bead position. The FECs thus generated allow straightforward tests of various polymer models (14).

Despite the tremendous contributions of SMFS to the understanding of nucleic acid elasticity, different SMFS methods have their own limitations in their applications to submicrometer DNAs. First, atomic force microscopes use large and stiff cantilevers, not well suited to the nanometer- and piconewton-regime force-extension measurements on short DNAs. Optical tweezers provide a high sensitivity and spatiotemporal resolution, but their standard horizontal-pulling geometry suffers from the steric hindrance between a pulling bead and the glass surface, especially when using short tethers. In addition, the orientation of the tether changes considerably during the horizontal pulling, requiring substantial geometric corrections in calculating tether extension. While dual-trap (15, 16) or axial optical tweezers (10) are largely free from these problems associated with pulling configuration, the measurement becomes particularly challenging as the length of the pulled DNA approaches the optical diffraction limit due to the difficulties in maintaining constant force and avoiding optical interference between trapping lasers.

MTs offer several advantages in monitoring the force-extension relation of short DNA molecules, such as a wide range of force levels generated by magnetic beads (sub-picowatts to several tens of picowatts), subnanometer precision in extension tracking, natural operation in constant-force mode, and simple instrumentation. Still, there are two major hurdles that have long hindered its application to short DNA molecules. First, standard magnetic beads used in MTs contain magnetic nanoparticles that are anisotropic and nonuniformly distributed. Under an external magnetic field, a magnetic bead favors
the alignment of its overall anisotropy axis with the applied field and thus experiences a rotational torque in addition to the pulling force to make an “off-axis” movement (17). This off-axis movement leads to an appreciable error in the measurement of molecular extensions, especially for short DNA tethers (18, 19). In addition, the estimation of applied magnetic force based on thermal fluctuations of a tethered bead becomes susceptible to many errors due to the short tether length and proximity of the bead to a surface (20, 21). Collectively, these inaccuracies affect the determinations of both force and extension in FECs, corrupting the intrinsic response of a short DNA tether. Furthermore, these two types of inaccuracies substantially vary across individual beads, rendering statistical analysis of pooled results problematic. Although there have been a few cases that interrogated short molecules with specialized schemes (10, 16, 20), a simple and generalizable method that allows nanomechanical measurements on short DNA with MTs is still lacking.

Here, we introduce a simple MT-based scheme that enables accurate profiling of FECs for short DNA, addressing two experimental challenges described above. First, we correct the force-dependent underestimation of extension resulting from the off-axis movements of magnetic beads (18, 19). The actual molecular extension is efficiently restored by considering the orientation and lateral displacement of a magnetic bead as functions of magnetic force. Next, a DNA hairpin is used as a force gauge to normalize the applied forces in multiple magnetic beads. We place the hairpin in a tether DNA and measure the force to unzip the identical hairpins in different bead-DNA constructs. This procedure yields force scaling factors for single magnetic beads. Rescaling the force axes of individual FECs with the scalers further homogenizes the FECs collected from multiple constructs, completing a two-step correction scheme.

Applying the developed method, we determined FECs of short dsDNA molecules down to 198 bp. In particular, application of the finite WLC (FWLC) model (9) to our FEC data yielded an essentially constant persistence length of ~50 nm in the entire contour length region from 10 kbp to 198 bp, confirming the invariability of the persistence length as an intrinsic material parameter of dsDNA. Our result also indicated extension of the experimental and theoretical limit in examining the dsDNA elasticity down to 198 bp. Last, we studied how the sequence contents of short DNA segments affected their elastic behavior. We observed a remarkable degree of sequence-dependent elasticity such that the persistence length of 500-bp dsDNA increased by 1.5-fold when the GC content doubled from 30 to 64%. In addition, this stiffening with increased GC base pairings completely disappeared upon CpG methylation. These results might imply the presence of “mechanical code” governing the nanomechanics of genomic DNA.

RESULTS

Distortion of short-DNA FECs in MTs by off-axis movements of magnetic beads

We first wanted to determine how correctly a standard FEC measurement in MTs probes the submicrometer elasticity of a short DNA tether. To this end, we synthesized a dsDNA construct that is roughly 1 kbp long with a contour length of ~350 nm (Fig. 1A, inset, and fig. S1A). This design contained a short hairpin structure in the middle used for the force calibration in each construct (see fig. S2 and note S1 for detailed effects of the hairpin on the mechanical parameters of dsDNA). We attached one end of the DNA to a glass surface and the other to a 1.4-μm-radius magnetic bead for the manipulation with MTs. In our MT setup (22), a pair of magnets above the sample generated upward magnetic forces in the range of 0 to 20 pN. To obtain FECs, we subjected bead-DNA constructs to a series of gentle force ramps (1 to 3 pN/s) while continuously tracking the bead positions at 100 Hz in all three dimensions.

To elucidate the force-extension behavior of the 1-kbp DNA construct, we collected FECs from multiple tethered beads. The presence of hairpin in our design yielded a unique ~30-nm transition signature around 13 pN (Fig. 1A), which we used to verify the sample identity. For simplicity, we aligned all the FECs so that their extensions at 10 pN coincided with one another. In this collection, we noticed a substantial heterogeneity in the resulting distribution of force-extension data throughout the force range we studied (Fig. 1A). For example, the mean extension values from two FECs often differed by more than 100 nm under 5 pN (Fig. 1A, for example, cyan versus blue). To quantitatively address this heterogeneity, we calculated the SDs of the mean extensions at discrete force levels (Fig. 1B, blue circles). The deviations were greater than 10 nm across the force regime that we explored and were more pronounced in the low-force regime, exceeding 30 nm at 1 pN. These deviation values underestimated the actual extent of dispersion because we artificially clamped the extensions at 10 pN for comparison. Overall, these results showed that the heterogeneities of FECs notably confounded the interpretation of genuine elastic responses of 1-kbp dsDNAs.

Because the high heterogeneity in FECs was unlikely due to the DNA mechanics itself, we hypothesized that this behavior would, at least in part, arise from the off-axis movements of magnetic beads relative to their tethers (Fig. 1C) (18, 19). Therefore, we sought to extract the true extension of a tether through a geometric consideration of the off-axis movement. During sample preparation for MTs, one end of a DNA molecule is attached to the surface of a magnetic bead at a random location, which we define as (x_{DNA}, y_{DNA}, z_{DNA}) (Fig. 1C, left). Independently of this attachment point, the magnetic bead bears an intrinsic anisotropy axis (referred to as a, brown dashed line in Fig. 1C) that is determined by the magnetic anisotropies of the embedded nanoparticles (17). When subjected to an external magnetic field B (along the x axis), the bead develops a magnetization, m, the direction of which energetically balances its alignment with both a and B (17, 18). Subsequently, the magnetic bead is rotated by an angle θ until m is fully aligned with B (Fig. 1C, from left to right). The z position of the bead (z_{bead}) then deviates from the end of DNA (z_{DNA}) by an offset z_{off}

\[ z_{off} = z_{DNA} - z_{bead} = R - R \cos \theta \]

where R is the bead radius. That is, this off-axis rotation leads to an underestimation of the DNA extension by z_{off}. a is generally slightly tilted from B, implying that the rotation angle θ and the resulting z_{off} are not constant but continuously change in response to varying force levels (18). In addition, z_{off} can show complicated behavior that varies across individual beads (19). Given the micrometer size of the beads, the offset z_{off} can be comparable to the length of short DNA tethers, suggesting that this underestimation must be corrected when determining the precise elastic properties of short DNAs. Because the measurements in MTs rely on tracking bead images, not the DNA itself, and the values for z_{off} and z_{DNA} are not readily available, a method to determine z_{DNA} and estimate z_{off} preferably in real time during bead tracking, is needed.
In situ correction of the off-axis movement for individual FECs

We noticed that \( x_{\text{off}} \), the lateral displacement of a magnetic bead \((x_{\text{bead}})\) from its tether attachment site \((x_{\text{DNA}})\), is geometrically related to the offset \( z_{\text{off}} \) via the following relation (note S2 and fig. S3, A and B):

\[
z_{\text{off}} = R \left( 1 - \sqrt{1 - \left( \frac{x_{\text{off}}}{R} \right)^2} \right)
\]

Because the position of the bead in the \( xy \) plane is precisely tracked in MT measurements, \( x_{\text{off}} \) provides a simple way for estimating \( z_{\text{off}} \). Such trigonometric relation has also been considered to model the bead offset in a double-tether MT experiment (23). Note that this way of measuring \( z_{\text{off}} \) is subject to two sources of errors: the measurement error in \( x_{\text{off}} \) and the dispersion in bead size, \( R \). We found, however, that both errors are relatively small compared to the magnitude of \( z_{\text{off}} \) (note S2 and fig. S3, C and D). Because \( x_{\text{off}} = x_{\text{bead}} - x_{\text{DNA}} \) by definition (Fig. 1C, bottom), we first examined the change in \( x_{\text{DNA}} \) (i.e., tether location) as a function of applied force. As previously reported (18, 19), \( x_{\text{DNA}} \) at a given force can be determined by slowly rotating the magnets (Fig. 1D). Because we worked with magnetic beads tethered by single DNA molecules, the beads were rotationally unconstrained and showed continuous circular motions instead of developing torsional strain in the tether. Consequently, the center of the circular trajectory in the \( xy \) plane was presumed to coincide with \((x_{\text{DNA}}, y_{\text{DNA}})\). We repeated this rotational measurement for a single magnetic bead as we increased the level of magnetic force. The bead traces tracked at different forces formed concentric circles, implying that the \( x \) and \( y \) positions of the tether DNA remained unchanged. The centers of the circles were kept nearly constant across the force regime we explored, changing less than 20 nm (Fig. 1D, inset).

We next examined the changes in \( x_{\text{bead}} \) as a function of applied tension. A representative trace of \( x_{\text{bead}} \) for a single bead is presented in Fig. 1E (left). Although individual beads showed disparate behaviors, we noticed a general trend: (i) \( x_{\text{bead}} \) was unstable under 1 pN likely because of bead-surface interaction; (ii) it reached an extreme value around 2 to 4 pN; and (iii) it changed its direction and asymptoted above 4 pN. This trend can be explained by the competition between two energy costs: the alignments of the magnetic moment \((m)\) with the external field \((B)\) and the anisotropy axis of the bead \((a)\) (17). Last, we noticed that Eq. 2 holds because the bead freely rotated about the \( x \) axis so that its \( y \) coordinate \((y_{\text{bead}})\) remained constant throughout the experiment (Fig. 1E, right).

By calculating \( x_{\text{off}} \) from the measured \( x_{\text{DNA}} \) and \( x_{\text{bead}} \) and plugging the \( x_{\text{off}} \) values into Eq. 2, we obtained the offset \( z_{\text{off}} \) as a function of applied tension (green curve in Fig. 1F). Before its application, \( z_{\text{off}}(F) \) was optionally filtered in force by averaging the data in 0.1-pN bins to reduce extra noise resulting from the correction procedure (Fig. 1F, black versus green curve on the left). Addition of \( z_{\text{off}}(F) \) to the bead coordinate \( z_{\text{bead}} \) restored the genuine force-extension relation of dsDNA described by the WLC elasticity (Fig. 1F, from blue to red), completing a simple algorithm for extension correction. The proposed correction scheme for the off-axis magnetic beads is summarized in fig. S3F.

We next combined the measurement of \( x_{\text{DNA}} \) with our routine FEC measurement (Fig. 1G). Here, we first rotated a target magnetic bead at a low magnetic force to obtain the tether location \( x_{\text{DNA}} \), which we showed to be kept largely constant at other force levels. Subsequent to the rotational measurement, we increased the magnetic force to record \( x_{\text{bead}} \) at each force level. Obtaining the difference between \( x_{\text{DNA}} \) and \( x_{\text{bead}} \) yielded estimates for \( x_{\text{off}} \) in real time as a function of force for individual FECs.

**Fig. 1.** Correction of extension for short DNA tethers in MTs. (A) FECs from 16 bead-tether constructs before extension correction. Inset: Cartoon of ~1-kbp construct. (B) Dispersion in FECs before (blue circles) and after (red squares) extension correction. SD values of mean extensions for the FECs in (A) and (H) are shown. Dotted line indicates 10 pN, where the extension was clamped. (C) Cartoon of a magnetic bead before (left) and after (right) reorientation. Dark brown: Embedded nanoparticles. a, anisotropy axis (dashed line); B, magnetic field (gray lines); m, magnetic moment (red); \( R \), bead radius; \( \theta \), angle of rotation. Bottom: Projection onto the \( xy \) plane. (D) Rotational measurements of \( x_{\text{DNA}} \) with fitting circles. Inset: Magnified view of the centers of circles. (E) Representative bead positions as functions of force. (F) Extension correction function (green) and its application (right). Black curve on top of green is the \( z_{\text{off}}(F) \) averaged in 0.1-pN bins. Dashed and dotted lines are WLC models with a closed and an open hairpin, respectively. (G) Representative bead trace during FEC measurements including extension correction. Blue ring: Rotational measurement (1 pN); vertical rainbow: FEC measurement (0.1 to 16 pN). (H) FECs after extension correction. The FECs in (A) were corrected and plotted in the same colors.

Shon et al., Sci. Adv. 2019;5:eaaav1697 12 June 2019 3 of 12
Last, we sought to see how much of the observed heterogeneity in FECs (Fig. 1, A and B) was resolved by reflecting the measured \( z_{\text{off}} \). When we corrected individual FECs with the corresponding \( z_{\text{off}} \) traces, the heterogeneity of FECs was dramatically reduced (Fig. 1H). The effect of the correction appeared in two force regimes. Under 5 pN, where the entropic stretching of DNA dominates the shape of FECs, the irregularities in extension due to the bead rotation, often leading to discontinuous changes or even a decrease in extension (cyan in Fig. 1A), were rectified. In the enthalpic stretching regime above 10 pN, the correction scheme adjusted the force-dependent increase in extension and brought the apparent slopes of multiple FECs in agreement with each other. This trend was confirmed by comparing the SDs in extension before and after the extension correction (Fig. 1B, from blue to red symbols). Overall, the correction procedure homogenized all FECs from different bead-tether constructs so that all results conform to a single WLC model (dashed and dotted lines in Fig. 1H).

**DNA hairpin–based force scaling factors for force normalization in MTs**

After improving the extension measurements on short DNA, we next reexamined our estimate of the magnetic force. As mentioned above, the 1-kbp DNA construct used in this work carried a short hairpin strand consisting of a 30-bp stem and a 4-nucleotide (nt) loop (Fig. S1A) (24, 25). The hairpins reproducibly displayed transition events above 10 pN, shuttling between the closed (base paired) and open (unzipped) states separated by ~30 nm (Fig. 2A).

We noted that the exact force at which the transition occurred apparently varied across individual bead-DNA constructs. For example, in the FECs for two bead-DNA constructs (Fig. 2A), one construct showed the unzipping around 14 pN (gray traces), while the same transition appeared around 12 pN for the other construct (black traces). The force values that we used here (and in Fig. 1) were based on our force calibration that assesses the magnetic force averaged over a sufficient number of beads as a function of bead-to-magnet distance \( d \) (Fig. S4; see note S3 for the detailed procedure). Thus, at a fixed bead-to-magnet distance, we assigned the same force value to all single magnetic beads disregarding potential differences in the generated magnetic force.

To systematically examine this force variation across multiple magnetic beads, we applied force ramps at a loading rate of 3 pN/s to measure the unzipping force \( \langle F_{\text{unzip}} \rangle \) (Fig. 2B). The \( F_{\text{unzip}} \) values collected from 28 different magnetic beads were broadly distributed in the range of 11 to 15 pN (Fig. 2C, top panel). In contrast, the \( F_{\text{unzip}} \) values from individual magnetic beads (Fig. 2C, individual rows at the bottom) were much narrowly distributed compared to the entire distribution. These results suggested that the main source of the broadening in \( F_{\text{unzip}} \) was the bead-to-bead variation in the generated magnetic force, rather than actual differences in \( F_{\text{unzip}} \) that would arise from stochastic crossings of an energy barrier for the unzipping of identical hairpins.

To quantitatively normalize the differences in force across beads, we used the DNA hairpin as an internal force gauge for single bead-DNA constructs. We defined a force scaling factor \( S(i) \) to describe the magnetic force exerted by the \( i \)th bead (19, 20, 26)

\[
F_{\text{unzip}}^{\text{scaled}} = \frac{F_{\text{unzip}}(i) \times S(i)}{S(i)}
\]

where \( F_{\text{unzip}}(i) \) is the unzipping force determined for the \( i \)th bead using the force calibration curve and \( F_{\text{unzip}}^{\text{scaled}} \) is the true unzipping force. A value of \( S(i) \) larger than 1 means that the \( i \)th bead generates a stronger force than average and vice versa. \( S(i) \) becomes independent of \( d \) when the magnetic moment \( m \) is saturated (|\( B \)| > 0.2 T) (20, 26) and is thus reduced to a simple proportionality constant in the pN regime we studied. We assumed that the unzipping force is governed only by the thermodynamic and kinetic nature of the hairpin strand, and therefore, \( F_{\text{unzip}}^{\text{scaled}} \) is constant for every bead-DNA construct. By determining \( F_{\text{unzip}}^{\text{scaled}} \) from the peak position (or the mean value) of the unzipping force distribution, we were able to estimate \( S(i) \) using the following equation

\[
S(i) = \frac{F_{\text{unzip}}^{\text{scaled}}}{\langle F_{\text{unzip}}^{\text{scaled}} \rangle} = \frac{\langle F_{\text{unzip}} \rangle}{F_{\text{unzip}}(i)}
\]

(4)

The force scaling factors obtained from Eq. 4 for 28 magnetic beads are shown in Fig. 2D. The 4% (SD) variation in the measured scalers agreed with other reports on bead-to-bead variability (20, 26). Last, applying the obtained scalers to the individual unzipping forces substantially narrowed their distribution (Fig. 2E versus Fig. 2C), validating the suggested scheme for correcting force variation across individual magnetic beads. The remaining variation in \( F_{\text{unzip}}^{\text{scaled}} \) mostly resulted from cycle-to-cycle variance in unzipping events (compare red distribution in Fig. 2E with in-bead distributions at the bottom).

**Consistency between force scalers from dynamic and equilibrium DNA hairpin measurements**

While the unzipping forces measured in our force-ramp experiments allowed efficient estimation of the force scaling factors, these results from dynamic measurements depended on the loading rate of force, therefore not reflecting equilibrium properties of the system. Thus, we wanted to confirm the validity of the force scalers in an independent measurement carried out under near-equilibrium conditions.

We performed force clamp experiments on single bead-DNA constructs by scanning the force range in which the repetitive unzipping and re-zipping transitions were observed (Fig. 2F) (24, 25). Before the analysis of transitions, we applied the off-axis correction that we developed in Fig. 1. As we show in the transition density plots examining the extension distributions before and after hairpin transition events \( (n = 9002) \), the unzipping and refolding transitions become substantially narrower upon the extension correction (fig. S5, A and B).

From the time-resolved traces of extension acquired at varying forces, the populations of the closed and open states of the DNA hairpin were measured. We observed a force-dependent shift in the two-state equilibrium (Fig. 2G) and determined the coexistence force \( F_{1/2} \) at which the closed and open states are equally populated (27). We reasoned that \( F_{1/2} \) is a good thermodynamic proxy that can gauge the force applied to an individual magnetic bead because it is governed solely by the hairpin sequence (24, 25). Thus, we collected \( F_{1/2} \) values from the 28 beads that were used in our dynamic \( F_{\text{unzip}}^{\text{scaled}} \) measurement and determined the individual scalers from \( S_{1/2}(i) \approx \langle F_{1/2} \rangle / F_{1/2}(i) \), analogously to Eq. 4. We checked the reliability of our thermodynamic results by cross-checking the hairpin opening distances obtained through four independent methods (details are given in note S4 and fig. S5), which all returned 27 to 28 nm (fig. S5, C to F). This interlocking consistency verifies the accuracy of our distance and force measurements that are thermodynamically conjugated to each other.

When we compared the two sets of scalers, \( S_{\text{unzip}}(i) \) and \( S_{1/2}(i) \), we found an almost perfect correlation between them (Fig. 2H), confirming the validity of the scaling factors we determined from dynamic force...
measurements. Because reliable determination of $F_{1/2}$ took longer than 10 min for each bead, we mainly used the dynamic measurement of $F_{\text{unzip}}$ (<1 min per bead) and resultant $S_{\text{unzip}}(i)$ unless otherwise noted.

We finally asked how the force scalers acquired from our method compare with those obtained from direct force measurements. To this end, we attempted to measure forces in the bead-tether constructs directly through the analysis of the power spectral density (PSD) (fig. S6), despite the difficulties with short tethers in force estimation (see note S3 for the detailed methods and discussion). A representative PSD for the Brownian motion of a bead perpendicular to the magnetic field (long-pendulum geometry or $y$ axis) is shown in fig. S6A with its fit to a coupled-fluctuation model (21). In the fitting procedure, relevant corrections for near-surface drag (fig. S7) and image blurring and aliasing were applied (28, 29), and the force ($F_{\text{PSD}}$) was treated as the only adjustable parameter (see eqs. S8 to S14 in note S3) (21). The force scaler $S_{\text{PSD}}(i)$ was then obtained by comparing $F$ at $d$ with the measured force $F_{\text{PSD}}$

$$S_{\text{PSD}}(i) = \frac{F_{\text{PSD}}(d; i)}{F(d; i)}$$  \hspace{1cm} (5)

in an analogous manner to Eq. 4. A scatter plot for the two series of scalers [$S_{1/2}(i)$ and $S_{\text{PSD}}(i)$] shows a decent positive correlation (fig. S6B), reassuring the validity of our hairpin-based force scaling. We suspect that the small discrepancy between the two estimates mostly resulted from the inaccuracy in $F_{\text{PSD}}$ due to the extensive corrections.

Two-step correction homogenizes short-DNA FECs

Equipped with the two-step correction schemes for our extension and force measurements developed above, we attempted to analyze FECs for 1-kbp dsDNA. We found that the hairpin positioned in the middle of 1-kbp dsDNA led to FECs with slightly shorter extension values than anticipated (fig. S2), which can be explained by the hairpin effectively dividing a DNA tether into two smaller halves (note S1 and fig. S2). Such a location-dependent effect of a defect in DNA has been described before (30). We thus moved the hairpin to the fixed boundary [i.e., the polyethylene glycol (PEG)–coated surface] to minimize this randomizing effect (Fig. 3A), which gave FECs with larger extension values for the 1-kbp dsDNA (fig. S2).

We collected FECs with this new pulling construct. With the scheme described in Fig. 1, we then corrected the underestimated extension of DNA due to the off-axis movement. This off-axis correction markedly
homogenized all the FECs collected from the 24 constructs (Fig. 3, B and C). Next, we applied the force scaling factor to normalize the force variation across individual magnetic beads. As described in Fig. 2, we rescaled the force level \( F \) by the obtained scalers \( S_{\text{unzip}} \) for each magnetic bead. This adjustment pinched the force window in which the hairpin transitions occurred, thereby further homogenizing the FECs (Fig. 3, C and D).

We finally applied the standard WLC model to the corrected FECs to obtain the effective persistence length \( L_{\text{WLC}}^p \) for the 1-kbp construct (see note S5 for the procedure). We first fitted the FECs from each construct individually to respective inextensible WLC models \((3, 31, 32)\), using the data under 8 pN. The distribution of the resulting \( L_{\text{WLC}}^p \) values became considerably narrower after the correction (Fig. 3E, green versus blue), centered around ~40 nm. Then, we attempted to fit all the FECs collected from multiple constructs to a single WLC model, and all data were successfully described by a global fit sharing a common \( L_{\text{WLC}}^p \) (Fig. 3F). The fit was improved throughout the entire force range. For example, the root mean square error (RMSE) at 1 pN in Fig. 3G decreased from 39 to 16 nm upon correction (fig. S8).

The \( L_{\text{WLC}}^p \) after full correction was 38.6 ± 1.4 nm [with 135 mM NaCl in 5 mM phosphate buffer, pH 7.4; error is 95% confidence interval (CI) of the fit], close to consensus values that range from 44 to 55 nm accepted for the persistence length value of dsDNA \((2, 3)\). Furthermore, we obtained a stretch modulus of 500 ± 100 pN (error, 95% CI) by including the data up to 16 pN and fitting them to an extensible WLC equation (Fig. 3F, inset on the right).

**Constant persistence length of dsDNA measured down to 198 bp**

After validating the developed scheme for FEC correction, we next applied this method to the investigation of the submicrometer elasticity of dsDNA. To this end, we first prepared dsDNA fragments of varying lengths (198 bp to 10 kbp) via polymerase chain reaction (PCR) using \( \lambda \) DNA as a template. To minimize sequence dependence, we chose target sequences such that their GC contents were close to 50% with no known intrinsically curved motifs present (table S1). As described above, the hairpin strand for force correction was placed at the bottom of the pulling construct. This design also simplified the synthesis of hairpin-containing tether because the construct could be prepared in a single PCR step using a primer with a preformed hairpin motif, instead of ligating multiple pieces of DNA. For all constructs with varying contour lengths, the hairpin structures were identical (fig. S1B).

We measured FECs for the prepared constructs in force-ramp experiments (Fig. 4A). We applied the two-step corrections to individual FECs. With short DNA tethers, it is typically difficult to distinguish proper bead-tether constructs from spurious ones or nonspecifically bound beads. The hairpin in the DNA construct left a clear fingerprint in FECs, which allowed us to distinguish genuine DNA-tethered beads, even for the dsDNA as short as 198 bp.

To analyze the length-dependent elasticity, we first normalized all FECs to the respective contour lengths of the tethers (Fig. 4B). In the normalized FECs, we found that the molecules longer than 1 kbp were nearly identical to each other in their elastic behavior (green, blue, purple, and black curves in Fig. 4B). However, the shortest 198-bp chain

---

**Fig. 3. Application of force-extension correction method on 1-kbp construct.** (A) Schematic of a 1-kbp construct with a force-calibrating hairpin at the end of the tether. (B to D) FECs from multiple constructs before correction (B), after extension correction (C), and after full correction including force scaling (D). Insets: Close-up views of the same curves. FECs from the same constructs are shown in matching colors across (B) to (D). (E) Box plots for individual WLC persistence lengths obtained before (B) and after (D) two-step correction (box, interquartile range; central mark, median; whiskers, the most extreme values are not considered outliers). (F) Heat map representations of the FECs in (B) to (D). Magenta dashed and dotted lines are inextensible WLC models with a closed and an open hairpin, respectively. Inset: Close-up view of the data after scaling (white box in the right panel). Black lines in the inset: Extensible WLC models. All results in this figure were obtained from 24 bead-tether constructs with 1-kbp tethers.
exhibited a marked deviation from the longer DNA tethers, where its extension below 10 pN was appreciably shifted to the reduced values (red curve in Fig. 4B). For a closer inspection, the distributions of extensions at 1 pN for the prepared constructs are shown in Fig. 4C. The centers of the distributions gradually shifted to lower extension values as the tether length decreased, with the intermediate 510-bp tether (orange) forming a distribution between the two limiting cases (red versus green/blue/purple). Note that the different sizes of hairpin transition in the normalized FECs (black arrow in Fig. 4B) resulted from the same unzipping distances of hairpin (~25 nm) normalized to the different tether lengths, so they were not associated with the tether elasticity. Likewise, the apparently larger noise with the 198-bp construct (red curve in Fig. 4B) also resulted from the division of similar magnitude of fluctuation (fig. S9A) by the short tether length.

Next, we fitted standard WLC models to the force-extension data, as shown for the representative 1-kbp construct in Fig. 4D (red curve; see fig. S10 for all results). We found that the persistence length obtained with the standard WLC model, \( \lambda_p^{\text{WLC}} \), decreased substantially as the contour length \( L_c \) shortened, reaching 13.9 ± 1.1 nm (error, 95% CI) with the 198-bp construct despite the fair quality in all fittings (blue circles in Fig. 4E and table S1). Thus, our precise measurement of FECs of short DNAs reproduced the previous observations that the standard WLC model estimates increasingly shorter persistence lengths as the contour length of the dsDNA becomes smaller than 1 kbp (9).

We next wondered whether it is valid to apply the standard WLC model to short DNA constructs examined here (e.g., 67 and 173 nm), which are comparable to or only three times longer than the persistence length assumed for infinitely long dsDNA (i.e., 40 to 50 nm). We thus used the FWLC model that considers (i) a finite length of the chain, (ii) boundary conditions at the ends of the finite chain, and (iii) rotational thermal fluctuations of the tethered bead (fig. S11A) (9). With the FWLC model used, the goodness of fit improved only slightly compared to the standard WLC (compare RMSE values in Fig. 4E). However, we found that the parameters estimated by FWLC were markedly different from those obtained from the standard WLC model. For example, with the 1-kbp construct, both the WLC and FWLC models fitted the data well (Fig. 4E, red and green curves), but the persistence length values estimated by FWLC (\( \lambda_p^{\text{FWLC}} \)) were significantly larger than those estimated by standard WLC (48.5 ± 1.1 nm versus 37 ± 2 nm).

We then applied the FWLC model to different tether lengths. We found that \( \lambda_p^{\text{FWLC}} \) essentially remains at a constant value of 46 ± 6 nm, which is also the limiting persistence length (\( \lambda_p^{\infty} \)) that both standard WLC and FWLC models estimate for an infinitely long chain (Fig. 4E). Thus, our results indicated a constant \( L_c \) across a wide range of contour length \( L_c \), confirming the notion that as an intrinsic material parameter of dsDNA, the persistence length is not expected to depend on \( L_c \). At the same time, the results push the limit of the chain length in which the FWLC framework is valid, from 1870 bp (9) to 198 bp, representing an extension by one order of magnitude.

In a detailed examination of the FWLC model, we found that the consideration of rotational fluctuations in a tethered bead was crucial in the estimation of the constant persistence length (fig. S11B). Thus, the gradual overshoot of \( \lambda_p^{\text{FWLC}} \) toward \( L_c = 198 \) bp presumably represents a need for a modification to the bead fluctuation calculations for their application to MTs, in particular, in the short \( L_c \) regime below 500 bp (Fig. 4E).

![Fig. 4. Measurements of short-DNA elasticity and modeling by WLC and FWLC models.](image)

(A) FECs for dsDNA with varying contour length. FECs were collected from multiple bead-tether constructs in each design (n = 14, 18, 13, 16, 18, and 6 for 198 bp, 510 bp, 1 kbp, 2 kbp, 4 kbp, and 10 kbp). Black dashed and dotted lines are WLC models for each tether length with a closed and open hairpin, respectively. (B) Normalized FECs for the graphs in (A). Black dotted line indicates 1 pN for the distributions in (C). (C) Distributions of extension data in (B) at 1 pN. Solid lines are fits to normal distributions. (D) Force-extension data (yellow) fitted with standard WLC (red) and FWLC (green) models. (E) Persistence length of dsDNA as a function of contour length obtained from standard WLC (blue circle) and FWLC (red triangle) models. Blue dashed line is a fit of blue circles to an empirical formula \( \lambda_p^{\text{WLC}} = \lambda_p^{\infty}(1 + aL_c / \lambda_p^{\infty}) \), with \( \lambda_p^{\infty} = 46 \) nm and \( a = 2.75 \) (9).
**High GC contents stiffen short dsDNA**

Regulatory elements of genes are often characterized by AT- or GC-rich sequences such as TATA boxes or CpG islands, respectively. When we examined the GC content in the λ-phage genome calculated with a 500-bp window, we found that the GC content level widely varies, ranging from 30 to 60%, even for these short segments of 500-bp dsDNA (Fig. 5A). Such different sequence compositions have been suspected to modulate the bending of DNA segments or their accessibility to DNA binding proteins (33, 34), but a paucity of proper methods makes the corresponding nanomechanical changes difficult to probe experimentally.

Thus, we asked whether GC-poor and GC-rich DNA segments show different behaviors in our precision force-extension measurements. We prepared 510-bp dsDNA constructs with the same design as above but with differing GC contents (30, 47, and 64%), all sampled from λ DNA (circles in Fig. 5A). The prepared constructs were examined with the same pulling method, and the resulting FECs were compared to each other after applying the same correction schemes as above.

The normalized FECs for the 510-bp dsDNA constructs with varying GC contents were largely similar to one another (Fig. 5B). However, the force-extension data formed distinct distributions when overlaid on each other, especially in the low-force region below 5 pN (Fig. 5C). For a clear comparison, we examined the distributions of extension at a representative force level of 1 pN (Fig. 5D). Notably, the GC-rich DNA (cyan) showed a noticeably increased extension compared to the other constructs.

We fitted the resulting FECs collected from multiple constructs (Fig. 5C) and obtained the values for $L_p^{\text{FWLC}}$: 49 ± 7, 52 ± 5, and 77 ± 14 nm (errors, 95% CI) with GC contents of 30, 47, and 64% (Fig. 5E). Therefore, it was clear from our results that a higher GC content resulted in a greater $L_p$ for short DNA fragments, a 1.6-fold increase in the range we probed.

To compare our results on short DNA with those for longer chains, we adopted a statistical model proposed to explain the experimental dependence of $L_p$ on GC content observed with genomic DNA preparations (35). We first evaluated the bending energies for dinucleotide pairs based on our 30% GC data and then used these values in evaluating the predicted $L_p$ at other GC content levels (35). This prediction (Fig. 5E, blue dashed line) is in quantitative agreement with our observations, validating the use of the above model for shorter duplex DNA.

**CpG methylation softens GC-rich dsDNA**

Methylation of the CpG islands is a prominent example of epigenetic regulation of these GC-rich regions. We finally questioned how CpG methylation would alter the elastic properties of short DNA segments. In particular, given the large increase in $L_p^{\text{FWLC}}$ we observed with the GC-rich dsDNA, we wondered whether our precision force-extension experiments would provide enough sensitivity to detect potential changes upon methylation. The 64% GC sequence we used met the usual criteria for the CpG islands (length > 200 bp, GC > 50%, and observed-to-expected CpG ratio > 60%), so its methylation would likely mimic the potential consequences of biological hypermethylation observed in cellular milieu.

To this end, we carried out enzymatic CpG methylation on the above 500-bp constructs with varying GC contents, which were originally prepared by PCR devoid of 5-methylcytosine (5-mC). We measured the efficiency of methylation on selected CpG sites using a methylation-sensitive restriction enzyme, which was found to be close to 100% (fig. S1C). Upon complete methylation, the expected numbers of 5-mCs for the three constructs are 7, 31, and 39, respectively (Fig. 5A, bottom). Then, the average spacing between 5-mCs in

---

**Fig. 5. Sequence dependence of short-DNA elasticity.** (A) GC content in λ-phage genomic DNA. Locations of 500-bp sequences with varying GC content are indicated with circles. Locations of CpG sites in each 500-bp construct are shown at the bottom. (B) Heat map representations of force-extension data measured for 510-bp dsDNA with 30% (magenta), 47% (orange), and 64% (cyan) GC content (n = 17, 18, and 22). (C) Color merge of the images in (B). White dotted line indicates 1 pN for the distributions in (D). (D) Distributions of extension data in (C) at 1 pN. Solid lines are fits to normal distributions, with their means indicated by the dashed lines. (E) FWLC persistence length of dsDNA as a function of GC content. Dashed line indicates a model dependence (33) adjusted for the current data on short DNA. (F) Heat map representations of force-extension data measured for CpG-methylated 510-bp dsDNA with 30% (magenta), 47% (orange), and 64% (cyan) GC content (n = 8, 9, and 10). White dotted line indicates 1 pN for the distributions in (G). (G) Distributions of extension data in (F) at 1 pN. Solid lines are fits to normal distributions, with their means indicated by the dashed lines. (H) Comparison of FWLC persistence length for the varying GC content before and after CpG methylation.
64% GC DNA is ~13 bp, and about 12-5 mCs are expected to be present within one unit of $L_p$ (~150 bp), likely sufficient to induce changes in the intrinsic mechanics.

Strikingly, the CpG methylation had a marked effect on the elasticity of GC-rich DNA. When the FECs were compared before and after the CpG methylation (Fig. 5F versus Fig. 5C), it was clear that the increase in extension observed for 64% GC DNA largely disappeared after the methylation. All FECs essentially collapsed onto one another, exhibiting no appreciable differences in the elasticity after CpG methylation. This overlapping of FECs was also prominent in the low-force regime around 1 pN (Fig. 5G versus Fig. 5D), suggesting that this phenomenon likely arises from the modulation of entropic stretchability. Consistent with this observation, the $L_p^{WLC}$ values obtained after the CpG methylation are essentially indistinguishable, converging to the global persistence length of dsDNA, namely, ~50 nm (Fig. 5H). The $L_p^{WLC}$ for the 30% GC did not change upon methylation as expected from the scanty distribution of CpG sites.

**DISCUSSION**

By examining the distortions of FECs occurring in conventional MT measurements, we found that most of the heterogeneity in FECs resulted from the off-axis displacements and the differential force scales exhibited by individual magnetic beads. We thus developed a two-step correction scheme, which restored the intrinsic force-extension relation of the tether dsDNAs, even for a 198-bp tether. Our correction scheme can be directly integrated into conventional FEC measurements with two simple requirements: the rotational measurement at a low-force level to determine the location of tether DNA and the inclusion of a short DNA hairpin in the tether. This method will be generalizable to wide ranges of tether length and bead size, and it will be most useful for short tethers and large beads. In particular, integration of the method into recently developed multiplexed SMFS setups (36–39) will streamline a high-throughput acquisition and analysis of single-molecule data by carefully accounting for small movements or variations for individual bead-tether constructs.

A successful incorporation of short tethers in SMFS systems promises an improvement of the measurement resolution (16, 20, 21). In SMFS, the spatial resolution is limited by thermal motions of a bead that depend on its tether length and stiffness (14). Consequently, a bead tethered by a shorter DNA is expected to reflect molecular events that occur within the bead–tether system in a more accurate manner. We verified this trend by measuring the noise levels in extension with varying tether lengths (fig. S9). Novel DNA-based tethers that are more rigid than bare duplex DNA have also been devised, potentially useful for this purpose (40–43). Obviously, an accurate characterization of the nanoscale mechanics of these short DNA structures will be a prerequisite for their wide application.

The force scaling method using hairpins will hopefully facilitate the use of short tethers in SMFS setups. While the magnetic force in an MT apparatus can be estimated directly from the thermal fluctuations of magnetic beads, the force needs to be determined at all the relevant positions of magnets. Thus, determining actual forces in this way not only is slow and laborious but also faces a multitude of challenges when using a short tether (note S3) (20, 21). Instead, the use of a hairpin in our method as a force standard quickly estimates the force scaling factor, allowing for a simple, first-order correction of the calibrated forces.

Note that the force-sensing hairpin, when placed in the middle of a DNA tether, was found to slightly affect the intrinsic mechanics of DNA (fig. S2). We theoretically ascribed this artifact to the changes in boundary conditions and experimentally circumvented it by placing the hairpin at the end of a tether. In the case of boundary-hairpin design, its FEC was not noticeably different from a bare DNA without a hairpin or single-stranded region. Consistently with this result, the intrinsic length-independent persistence length we obtained (46 nm) in the presence of a boundary hairpin implies that the potential artifact from this accessory region is largely negligible down to 198 bp. For more accurate mechanical measurements on short tethers, however, it would be advisable to verify the effect of hairpin and to minimize the length of single-stranded linkers. A comparative analysis of mechanical properties (as shown here for the sequence-dependent elasticity) would still yield meaningful results despite a small inaccuracy.

We expect that the hairpin-based force scaling scheme can be used in other applications. At the simplest level, the unzipping/rezipping force of the hairpin may be fine-tuned by varying the length and composition of the hairpin strand so that the transition occurs near the force regime of interest (24). In this vein, inclusion of G-quadruplex structures that unfold at higher forces than hairpins (>20 pN) (44) or utilization of overstretching transition of dsDNA (65 pN) (45) will be useful to span a wider force range. Furthermore, hairpin strands may be embedded in more complex systems like DNA origami (40, 42, 43) or protein-DNA hybrid (22). It will be particularly useful for novel designs of tethers because one cannot estimate force from bead fluctuations if the mechanical properties of these systems are unknown. Last, the method might also aid the use of homemade magnetic particles in MTs that are typically more polydispers in size (and thus in generated force) than those commercially available.

Although we focused on the utility of DNA hairpin as a force standard, unzipping distance of hairpins may serve additionally as length calibration standards in SMFS experiments. We implicitly exploited this aspect when comparing the unzipping distances extracted from several aspects of the transition (note S4). In this regard, a series of hairpins that differ in the stem length by only a few base pairs will provide a means for the precise calibration of nanopositioning devices (e.g., a piezo stage) used in SMFS methods.

Equipped with the two-step correction scheme for precise FEC measurements, we systematically studied the submicrometer elasticity of short dsDNA molecules as a function of contour length and GC composition. In particular, with the standard WLC equations used, we observed the decrease in the persistence length in the short dsDNA regime below 5 kbp. Notably, the observed relationship between the contour length and the WLC persistence length ($L_p^{WLC}$) followed an empirical formula previously derived from simulations and experiments on intermediate-length dsDNAs (9, 10, 15). This indicates that our observation is in line with previous observations using different experimental schemes and that the apparent decrease in $L_p^{WLC}$ in our measurements stems from the finite nature of short DNAs for the most part.

Because the short dsDNAs we interrogated were suspended between a magnetic bead and a glass surface, we needed to consider these immobile and impenetrable boundary conditions for correct modeling. With the FWLC model that considers the resulting confinement and altered fluctuations from the boundaries, we observed an essentially constant persistence length ($L_p^{FWLC}$) across the entire contour length region (0.2 to 100 kbp). Arguably, such molecules with constraints might resemble chromosomal DNA segments anchored by nucleosomes, adopting a “bead-on-a-string” conformation. In this regard, it will be interesting to extend this study to short stretches of...
DNA including nucleosome positioning sequence (46). Our force-extension methods will be well suited to this aim because such motifs are only ~150 bp in length (e.g., 147 bp for the Widom 601 sequence).

At the same time, we also observed a remarkable influence of the GC content on the elasticity of short dsDNAs. An increase in the GC content of 510-bp dsDNA from 30 to 64% was accompanied by a 1.6-fold increase in the effective persistence length. Recently, a high-throughput analysis of nanochannel-confined genomic sequences suggests that the persistence length of DNA increases as a function of GC content (35). In addition, a single-molecule interaction assay on short DNA duplexes indicated a stronger association between AT-rich sequences than GC-rich ones (47, 48). We suppose that the increased intramolecular contacts that occur transiently between the domains of AT-rich DNAs would effectively decrease the end-to-end extension, particularly in the low-force regime. This tendency will, in turn, lead to an increase in persistence length for GC-rich sequences where such AT-based interactions become less prominent, consistent with our results. However, a simpler explanation is also plausible in which the overall energetic stability of the GC-rich DNA confers the additional rigidity that manifests as an increase in persistence length. Another possibility is the difference in the intrinsic curvature of DNA that is known to be large for particular motifs (49), although discerning such static bends from dynamic flexibility in experiments would be challenging. Further tests on an expanded set of short DNA constructs will provide a more comprehensive view of the sequence-dependent elasticity of dsDNA.

It is remarkable that the steep increase of \( L_p \) with the GC content is largely abrogated by CpG methylation, pointing to a greater flexibility of GC-rich DNA upon methylation. This result is consistent with a previous measurement by optical tweezers on longer methylated DNA (50). Such an increase in flexibility may be again a manifestation of enhanced intramolecular associations, as suggested by single-molecule interaction assays and simulations (47, 48). Although such intersegmental contacts would be largely disrupted at high force levels applied in our MT experiments, such dynamics might still influence the force-extension profile that we probed around 1 pN. Because the FWLC-based persistence length estimations for short chains are highly sensitive to the force-extension data at low force levels, the stark change in \( L_p^\text{FWLC} \) upon methylation largely resulted from differential behaviors in this force regime around 1 pN.

How might these results relate to the DNA segments in cell nuclei with differing sequence composition? AT-rich regulatory regions are typically found devoid of nucleosomes, showing increased accessibility to DNA binding proteins (51). The intrinsic flexibility in these sequences may aid its binding to transcription factors, which often accompanies bending of target DNA (52). Note that the AT-rich sequence we excerpted from the \( \lambda \)-phage genome for our experiments included the upstream region of an open reading frame.

Conversely, mammalian promoters are frequently enriched in GC base pairs, a famous example of which is the CpG islands. It is tempting to postulate that the stiffness of GC-rich regions that can be additionally modulated by methylation would be a good candidate for dynamic regulation of DNA-protein binding. While hypermethylation of CpG islands is typically associated with repression of gene expression (53), the correlation between nucleosome occupancy, GC content, and its methylation level has not been fully understood yet (52, 54, 55). The complexity might be due to an intricate sequence-dependent interplay among CpG sites, poly(dA:dT) tracts, and nucleosome positioning sequences. All these players may conspire to orchestrate the binding of proteins such as histones and methyl-CpG-binding proteins but could also be influenced by their binding in turn. The \( L_p^\text{FWLC} \) value we measured shifted from 77 to 55 nm upon methylation, which corresponds to 230 and 160 bp, respectively. We note that this length regime of one to two persistence lengths corresponds to the length of DNA segments that are known to wrap around the histone octamers (146 bp). Thus, the change upon CpG methylation might be directly relevant for the facile regulation of DNA flexibility for the formation and remodeling of nucleosomes.

**MATERIALS AND METHODS**

**Synthesis of hairpin-containing dsDNA tethers**

For validation of the correction method (Figs. 1 and 2), we designed a ~1-kbp construct in which two 522-bp dsDNA tethers were bridged by a hairpin structure (fig. S1A). The tethers were prepared by PCR using biotin- and digoxigenin-modified primers for surface and bead attachment, respectively. The hairpin consisting of a 30-bp stem and a 4-nt loop (24) was separately synthesized by annealing and ligation of oligos. The hairpin strand and tethers were assembled by enzymatic digestion and ligation through their cohesive ends and verified in agarose gels. For the constructs used in the measurements of persistence length (Fig. 4), dsDNA fragments of varying length (198 bp to 4 kbp) and GC content (30 to 64%) were prepared by PCR and ligated with oligos that collectively formed a hairpin structure at one end of the dsDNA backbone (fig. S1B). Further details are in Supplementary Materials and Methods. For CpG methylation, 510-bp constructs with a hairpin at the biotin side were methylated at their CpG sites via CpG methyltransferase M.SssI (New England BioLabs). For each construct, ~800 ng of DNA was mixed with 240 \( \mu \)M S-adenosyl methionine and 8 U of M.SssI in the standard reaction buffer (NEBuffer 2) and incubated for 10 hours at 37°C. For the verification of methylation, the products were digested with methylation-sensitive restriction enzyme Bst UI (New England BioLabs), and the cleaved segments, if any, were visualized on a 2% agarose gel. The degree of protection by methylation was measured to be >90% in all cases.

**Sample chamber for MTs**

A flow cell was assembled from two glass coverslips passivated with PEG (Laysan Bio, M-SVA-5K) and spaced by a double-sided tape. A fraction (1 to 5%) of the PEG molecules were modified with biotin (Laysan Bio, BIO-SVA-5K) for the surface attachment of DNA molecules. Sequential injection of NeutrAvidin (Thermo Fisher Scientific, 31000), DNA constructs, and magnetic beads (Thermo Fisher Scientific, Dynabeads M-270) coated with anti-digoxigenin (Sigma-Aldrich, 1133089001) into the flow cell yielded bead-tether constructs that were subjected to the measurements by MTs. Further details are in Supplementary Materials and Methods.

**MT instrument**

The MT apparatus in this study was built on an inverted microscope (Olympus, IX73) similar to previously reported high-resolution MT setups (56–58). A pair of magnets (vertically aligned in opposite directions with a 1-mm gap) was placed above the stage holding a flow cell, and its vertical position and rotation were controlled by a translation stage (Physik Instrumente, M126) and a stepper motor (Autonics, A3K-S545W), respectively. The magnet axis was confirmed to be aligned to the imaging axis (within 1°) by following the motion of a free magnetic bead. Beads in a flow cell were illuminated by a red superluminescent
diode (QPPhotonics, QSDM-680-2) and imaged by a 100× oil-immersion objective (Olympus, UPlanSapo NA 1.40) and a high-speed complementary metal-oxide semiconductor (CMOS) camera (Mikrotron, EoSens MC-3082) grabbing 512 × 512 images at 4 kHz. The objective position was controlled by a piezo-controlled nanopositioner (Mad City Labs, Nano-F100S) to calibrate distances and to correct for drift. The images were recorded by a custom software written in LabVIEW (National Instruments), and the coordinates of beads were tracked in real time at up to 1.2 kHz. Unless necessary, measurements were performed at 100 Hz to reduce file size.

**Force measurements and calibration**

Magnetic forces were measured and calibrated as described in the literature (20, 28, 29). Forces were estimated from the PSDs for the Brownian motion in y (perpendicular to the magnetic field), applying corrections for near-surface viscosity (fig. S7) and blurring and aliasing in acquisition (note S3) (21). For calibration, we used 2.8-μm-diameter beads (Thermo Fisher Scientific, Dynabeads M-270) tethered by a 5.4-kbp dsDNA. The force versus magnet position data were fitted with a double-exponential function (fig. S4 and note S4) (20).

**FEC measurements**

Typical FEC measurements were performed by applying a forward (increasing) and a reverse (decreasing) force ramp in the range of 0.1 to 20 pN at a loading rate of 3 pN/s. The cycle was repeated for three times for each bead-tether construct. While the ramps were applied in both directions for completeness, only the data from reverse ramps were used when fitting FECs to a model. This choice ensures fast equilibrium of the bead position (59) and avoids secondary structures or nonspecific interactions at low forces.

**Correction of tether extension**

The off-centered attachment at a reference force (1 pN) was measured by rotating the magnet slowly (60°/s) (18, 19). The obtained x coordinates of bead (x_{\text{head}}) and DNA (x_{\text{DNA}}) were used to calculate σ_{\text{eff}} and generate a correction function, σ_{\text{eff}}(F), as described in the main text through Eqs. 1 and 2. The resulting function was then added to the raw z coordinates of the bead (x_{\text{head}}).

**Correction for variability in force**

Force-clamp measurements were performed by varying the force by 0.1 pN in the force regime where DNA hairpin transition occurred (24), each measurement lasting 10 s. Three such measurements for each bead were averaged to determine the coexistence force (F_{1/2}) and the corresponding force scalers as described in the main text. For determination of scalers from direct force analysis, PSDs were recorded at ~12 pN to avoid hairpins corrupting the intrinsic power spectra of the fluctuation.

**Data analysis**

Data from MT experiments were analyzed with custom software written in MATLAB (MathWorks). Methods for the fitting and the analyses of hairpin transitions are described in Supplementary Materials and Methods.

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at [http://advances.sciencemag.org/cgi/content/full/5/6/eaav1697/DC1](http://advances.sciencemag.org/cgi/content/full/5/6/eaav1697/DC1) Supplementary Materials and Methods

---

**REFERENCES AND NOTES**

1. J. P. Peters, L. J. Maher III, DNA curvature and flexibility in vitro and in vivo. Q. Rev. Biophys. 43, 23–63 (2010).
2. P. J. Hagerman, Flexibility of DNA. Annu. Rev. Biophys. Biophys. Chem. 17, 265–286 (1988).
3. M. D. Wang, H. Yin, R. Landick, J. Gelies, S. M. Block, Stretching DNA with optical tweezers. Biophys. J. 72, 1335–1346 (1997).
4. A. P. Fields, E. A. Meyer, A. E. Cohen, Euler buckling and nonlinear kinking of double-stranded DNA. Nucleic Acids Res. 41, 9881–9890 (2013).
5. C. Kim, O.-C. Lee, J.-Y. Kim, W. Sung, N. K. Lee, Dynamic release of bending stress in short dsDNA by formation of a kink and forks. Angew. Chem. Int. Ed. Engl. 54, 8943–8947 (2015).
6. M. J. Shon, A. E. Cohen, Nano-mechanical measurements of protein-DNA interactions with a silicon nitride pulley. Nucleic Acids Res. 44, e7 (2016).
7. T. E. Cloutier, J. Widom, Spontaneous sharp bending of double-stranded DNA. Mol. Cell 14, 355–362 (2004).
8. P. A. Wiggins, T. van der Heijden, F. Moreno-Herrero, A. Spakowitz, R. Phillips, J. Widom, C. Dekker, P. C. Nelson, High flexibility of DNA on short length scales probed by atomic force microscopy. Nat. Nanotechnol. 1, 137–141 (2006).
9. Y. Seol, J. Li, P. C. Nelson, T. T. Perkins, M. D. Betterton, Elasticity of short DNA molecules: Theory and experiment for contour lengths of 0.6-7 microm. Biophys. J. 93, 4360–4373 (2007).
10. Y.-F. Chen, D. P. Wilson, K. Raghunathan, J.-C. Meiners, Entropic boundary effects on the elasticity of short DNA molecules. Phys. Rev. E Stat. Nonlin. Soft Matter Phys. 80, 020903 (2009).
11. R. Vafabakhsh, T. Ha, Extreme bendability of DNA less than 100 base pairs long revealed by single-molecule cyclization. Science 337, 1097–1101 (2012).
12. T. T. Le, H. D. Kim, Probing the elastic limit of DNA binding. Nucleic Acids Res. 42, 10786–10794 (2014).
13. S. B. Smith, L. Finzi, C. Bustamante, Direct mechanical measurements of the elasticity of single DNA molecules by using magnetic beads. Science 259, 1122–1126 (1992).
14. K. C. Neuman, A. Nagy, Single-molecule force spectroscopy: Optical tweezers, magnetic tweezers and atomic force microscopy. Nat. Methods 5, 491–505 (2008).
15. M. Ribezić-Crivellari, F. Ritort, Force spectroscopy with dual-trap optical tweezers: Molecular stiffness measurements and coupled fluctuations analysis. Biophys. J. 103, 1919–1928 (2012).
16. N. Forns, S. de Lorenzo, M. Manosas, K. Hayashi, J. M. Huguet, F. Ritort, Improving signal/noise resolution in single-molecule experiments using molecular constructs with short handles. Biophys. J. 100, 1765–1774 (2011).
17. M. M. van Oene, L. E. Dickinson, F. Pedaci, M. Köber, D. Dulin, J. Lippert, N. H. Dekker, Biological magnetometry: Torque on superparamagnetic beads in magnetic fields. Phys. Rev. Lett. 114, 218301 (2015).
18. D. Klaus, R. Seidel, Torsional stiffness of single superparamagnetic microspheres in an external magnetic field. Phys. Rev. Lett. 102, 028302 (2009).
19. I. D. Vlamink, T. Henighan, M. T. J. van Loenhout, D. R. Burnham, C. Dekker, Magnetic forces and DNA mechanics in multiplexed magnetic tweezers. PLoS ONE 7, e41432 (2012).
20. H. Chen, H. Fu, X. Zhu, P. Cong, F. Nakamura, J. Yan, Improved high-force magnetic tweezers for stretching and refolding of proteins and short DNA. Biophys. J. 100, 517–523 (2011).
21. P. Daldrop, H. Brutzer, A. Hühle, D. J. Kauert, R. Seidel, Extending the range for force calibration in magnetic tweezers. Biophys. J. 108, 2550–2561 (2015).
SCIENCE ADVANCES | RESEARCH ARTICLE

22. M. J. Shon, H. Kim, T.-Y. Yoon, Focused clamping of a single neuronal SNARE complex by a complex under high mechanical tension. Nat. Commun. 9, 3639 (2018).
23. K. C. Neuman, G. Charvin, D. Bensimon, V. Croquette, Mechanisms of chiral discrimination by topoisomerase I. Nat. Protoc. 106, 6986–6991 (2016).
24. M. T. Woodside, W. M. Behnke-Parks, K. Larizadeh, K. Travers, D. Herschlag, S. M. Block, Nanomechanical measurements of the sequence-dependent folding landscapes of single nucleic acid hairpins. Proc. Natl. Acad. Sci. U.S.A. 103, 6190–6195 (2006).
25. M. T. Woodside, P. C. Anthony, W. M. Behnke-Parks, K. Larizadeh, D. Herschlag, S. M. Block, Direct measurement of the full, sequence-dependent folding landscape of a nucleic acid. Science 314, 1001–1004 (2006).
26. J. Lipfert, X. Hao, N. H. Dekker, Quantitative modeling and optimization of magnetic tweezers. Biophys. J. 96, 5040–5049 (2009).
27. J. Lipfert, B. Ono, S. B. Smith, I. Tinoco Jr., C. Bustamante, Reversible unfolding of single RNA molecules by mechanical force. Science 292, 733–737 (2001).
28. A. J. W. te Velthuis, J. W. J. Kerssemakers, J. Lipfert, N. H. Dekker, Quantitative guidelines for force calibration through spectral analysis of magnetic tweezers data. Biophys. J. 99, 1292–1302 (2010).
29. B. M. Lansdorp, O. A. Saleh, Power spectrum and Allan variance methods for calibrating single-molecule video-tracking instruments. Rev. Sci. Instrum. 83, 025115 (2012).
30. H. Chen, J. Yan, Effects of kink and flexible hinge defects on mechanical responses of short double-stranded DNA molecules. Phys. Rev. E 77, 041907 (2008).
31. C. Bustamante, J. F. Marko, E. D. Siggia, S. Smith, Entropic elasticity of lambda-phage DNA. Science 265, 1599–1600 (1994).
32. C. Bouchiat, M. D. Wang, J. F. Allemand, T. Strick, S. M. Block, V. Croquette, Estimating the persistence length of a worm-like chain molecule from force-extension measurements. Biophys. J. 76, 409–413 (1999).
33. M. E. Hogan, R. H. Austin, Importance of DNA stiffness in protein-DNA binding specificity. Nature 329, 263–266 (1987).
34. A. Pérez, C. L. Castellazzi, F. Battistini, K. Collinet, O. Flores, O. Deniz, M. L. Ruiz, D. Torrents, R. Eritja, M. Soler-López, M. Orozco, Impact of methylation on the physical properties of DNA. Biophys. J. 102, 2140–2148 (2012).
35. H.-M. Chuang, J. G. Reifenhberger, H. Cao, K. D. Dorfman, Sequence-dependent persistence length of long DNA. Phys. Rev. Lett. 119, 227802 (2017).
36. N. Ribbeck, O. A. Saleh, Multiplexed single-molecule measurements with magnetic tweezers. Rev. Sci. Instrum. 79, 094301 (2008).
37. I. De Vlaminck, T. Henighan, M. T. van Loenhout, I. Pfeiffer, J. Huijts, J. W. Kerssemakers, A. J. Katan, A. van Langen-Suurling, E. van der Drift, C. Wyman, C. Dekker, Highly parallel measurement of the full, sequence-dependent folding landscape of a nucleic acid. Proc. Natl. Acad. Sci. U.S.A. 106, 2629–2634 (2009).
38. P. A. Jones, S. B. Baylin, The fundamental role of epigenetic events in cancer. Nat. Rev. Genet. 3, 415–428 (2002).
39. R. Fenouil, P. Cauchy, F. Koch, N. Descostes, J. Z. Cabeza, C. Innocenti, P. Ferrier, S. Spicuagila, M. Gut, I. Gut, J. C. Andrau, CpG islands and GC content dictate nucleosome depletion in a transcription-independent manner at mammalian promoters. Genome Res. 22, 2399–2408 (2012).
40. C. K. Collings, P. J. Waddell, J. N. Anderson, Effects of DNA methylation on nucleosome stability. Nucleic Acids Res. 41, 2918–2931 (2013).
41. K. Kim, O. A. Saleh, A high-resolution magnetic tweezers for single-molecule measurements. Nucleic Acids Res. 37, e136 (2009).
42. B. M. Lansdorp, S. J. Tabrizi, A. Dittmore, O. A. Saleh, A high-speed magnetic tweezers beyond 10,000 frames per second. Rev. Sci. Instrum. 84, 044301 (2013).
43. D. Dulin, T. J. Cui, J. Crossen, M. W. Docter, J. Lipfert, N. H. Dekker, High spatiotemporal-resolution magnetic tweezers: Calibration and applications for DNA dynamics. Biophys. J. 109, 2113–2125 (2015).
44. M. Kruijoro, F. Chien, M. de Jager, J. van Noort, Subpiconeutron force spectroscopy using magnetic tweezers. Biophys. J. 94, 2343–2348 (2008).
45. I. Tinoco Jr., C. Bustamante, The effect of force on thermodynamics and kinetics of single molecule reactions. Biophys. Chem. 101–102, 513–533 (2002).
46. S. B. Smith, Y. Cui, C. Bustamante, Overstretching B-DNA: The elastic response of individual double-stranded and single-stranded DNA molecules. Science 271, 795–799 (1996).
47. A. Dittmore, D. B. McIntosh, S. Halliday, O. A. Saleh, Single-molecule elasticity measurements of the onset of excluded volume in poly(ethylene glycol). Phys. Rev. Lett. 107, 148301 (2011).

Acknowledgments: We thank D. Min for the technical assistance with MT instruments. We thank M. W. Kim and S. H. Kim for helpful discussions. Funding: This work was supported by the National Creative Research Initiative Program (Center for Single-Molecule Systems Biology to T.-Y.Y.; grant number: NRF-2011-0018352) funded by the National Research Foundation of Korea. M.J.S. was supported by the BK21 Plus Program from the Korean Ministry of Education. Author contributions: M.J.S. and T.-Y.Y. wrote the manuscript.

Additional data related to this paper are available upon a reasonable request.

Submitted 22 August 2018
Accepted 3 May 2019
Published 12 June 2019
10.1126/sciadv.aav1697

Citation: M. J. Shon, S.-H. Rah, T.-Y. Yoon, Subpiconeutron elasticity of double-stranded DNA revealed by precision force-extension measurements with magnetic tweezers. Sci. Adv. 5, eaav1697 (2019).