

Optical Trapping Nanometry of Hypermethylated CpG-Island DNA

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ABSTRACT Cytosine methylation is a key mechanism of epigenetic regulation. CpG-dense loci, called “CpG islands”, play a particularly important role in modulating gene expression. Methylation has long been suspected to alter the physical properties of DNA, but the full spectrum of the evoked changes is unknown. Here we measured the methylation-induced nanomechanical changes in a DNA molecule with the sequence of a CpG island. For the molecule under tension, contour length, bending rigidity and intrinsic stiffness decreased in hypermethylated dsDNA, pointing at structural compaction which may facilitate DNA packaging in vivo. Intriguingly, increased forces were required to convert hypermethylated dsDNA into an extended S-form configuration. The reduction of force hysteresis during mechanical relaxation indicated that methylation generates a barrier against strand unpeeling and melting-bubble formation. The high structural stability is likely to have significant consequences on the recognition, replication, transcription, and reparation of hypermethylated genetic regions.

INTRODUCTION

The chemical structure of DNA defines its nanomechanical properties, thereby strongly influencing the efficiency of DNA packaging and the activity of DNA-based mechanoenzymes. Methylation of cytosines in CpG dinucleotides of the genomic DNA is one of the most important epigenetic modifications in higher organisms (1–3). CpG-rich regions called “CpG islands” precede >70% of genes in human cells, which points at crucial, yet not fully understood regulatory functions (4). CpG islands in active promoter regions are mostly unmethylated, while in most of the loci with smaller CpG density these dinucleotides are methylated. Hypermethylation in gene promoter CpG islands is usually associated with suppressed expression of the associated gene (5). Recently it has been shown that in addition to CpG sites other, non-CpG cytosines may also be methylated and that they may also play a regulatory role in transcriptional silencing and differentiation of mammalian cells (6–10). Alterations in DNA methylation state by either hyper- (5,11) or hypomethylation (12,13) have been correlated with neoplastic transformation and aberrant embryonic

development. Cytosine methylation has long been suspected to modulate DNA nanomechanical properties, thereby influencing the binding of associated proteins and nucleosomes (14–19). However, often contradictory findings were observed about the methylation-induced nanomechanical changes in DNA (18,20–24). Initial cyclization kinetics experiments have not detected changes in the flexibility of exhaustively methylated DNA sequences (20). By contrast, a reduced local flexibility was measured in DNA oligonucleotides containing selectively methylated CpG sites (21). Different magnitudes of contour-length reduction and stiffening of methylated DNA were found by measuring the equilibrium shape of surface-adsorbed molecules with atomic force microscopy (AFM) (22,23,25). By using molecular force assay and AFM-based pulling experiments, it has been shown that methylation significantly affects strand separation forces and thus DNA mechanical stability (24). Furthermore, DNA flexibility in chromatin may either increase or decrease depending on the nucleosomal positioning relative to the CpG dinucleotides (16,26,27). Altogether, prior findings indicate that methylation alters the mechanical properties of DNA. However, the overall spectrum of structural and dynamic alterations and the precise mechanisms of methylation-induced nanomechanical changes in DNA are still unclear.

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Optical trapping nanometry (optical tweezers) is a sensitive method for characterizing the mechanical properties and force-driven transitions of individual DNA molecules (28–30). The ends of a DNA molecule can be captured with various available chemical techniques, and the molecule's structure is not constrained by interactions with surfaces. Upon stretching a double-stranded (ds) DNA molecule with optical tweezers, in the force range of 0–10 pN the molecule first extends at the expense of reducing its configurational entropy while the end-to-end distance asymptotically approaches the contour length. The measure of the molecule's bending rigidity is the persistence length, which may be obtained by fitting the experimental data with the wormlike chain (WLC) model of entropic elasticity (31). In the force range of 10–60 pN, in the so-called enthalpic regime, dsDNA extends due to the distortion of the bonds holding its structure together. The measure of axial elasticity in this regime is the stretch modulus (or intrinsic stiffness), which can be obtained by fitting the experimental data with a model of extensible wormlike chain (32,33). At ~65 pN dsDNA, cooperatively extends within a narrow force range to ~1.7 times its length (30). Three processes appear to proceed simultaneously within a torsionally unconstrained dsDNA molecule during this overstretch transition (34,35): strand unpeeling, melting bubble formation, and transition into a so-called S-form configuration. Although the exact structure of S-form DNA is not known, it is hypothesized to be an unwound helix, much like a ladder, with basepairing intact. At forces >65 pN the molecule displays the mechanical properties of either the S-form DNA, or, if strand separation has occurred, single-stranded (ss) DNA. Upon relaxing from the overstretched state, DNA recovers its structure very rapidly. Increasing levels of strand unpeeling and bubble formation, however, can impede the structural recovery, which is manifested in the appearance of force hysteresis (30,34). By cyclically stretching and relaxing a dsDNA molecule with optical tweezers, the complex spectrum of its nanomechanical behavior may thus be characterized.

In this work we explored the effect of methylation on the nanomechanical properties of a 3312-bp-long piece of DNA that met the criteria of a CpG island, by using optical trapping nanometry in the force range of 0–100 pN with high spatial (~1 nm) and force resolution (~0.2 pN). The use of both enzymatic and chemical methylation allowed us to assess the contribution of both CpG and the additional non-CpG cytosines to DNA nanomechanics. Hypermethylation led to the structural compaction, increased bending and axial compliance and the stabilization of the dsDNA structure under tension. Furthermore, we find that in the overstretched state methylated DNA is longer than the non-methylated, suggesting that it attains, to our knowledge, a novel extended S-form. Hypermethylation-induced changes are thus likely to influence DNA packaging and the rates of DNA-based mechanoenzymatic processes.

MATERIALS AND METHODS

Sample preparation

A 3312-bp-long sequence was selected from λ -phage DNA by using a CpG island search algorithm (36,37). Three different samples were prepared: a nonmethylated sequence for use as control (DNA_{nm}), an enzymatically methylated sample (DNA_{em}), and a chemically methylated one (DNA_{cm}). DNA_{nm} was prepared with standard PCR employing DreamTaq polymerase (Fermentas, Vilnius, Lithuania). Primers (5'-biotin-CGTGCCGTGTGCAG and 5'-NH₂-CACCGCTGGCGTTCA) were designed with the Primer3 tool (38) using the entire λ -phage genome sequence available in the GeneBank (39) database (for further detail, see the [Supporting Material](#)). The resulting reaction mixtures were purified from agarose gel (1% agarose, Tris-acetate-EDTA buffer, 10 V/cm). The final product was isolated with a QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) and eluted with ultrapure water. DNA concentration was determined by measuring absorbance at 260 nm (NanoDrop 1000; Thermo Fisher Scientific, Waltham, MA). Concentration was typically ~30 ng/ μ L. Purity, according to the 260:280 nm absorbance ratio, was ~1.8 in all cases. DNA_{em} was prepared by methylating DNA_{nm} with M.SssI CpG methyltransferase (Thermo Fisher Scientific) by mixing 33 μ L of 40 ng/ μ L DNA solution, 4 μ L of 10 \times M.SssI Buffer, 2 μ L of enzyme solution, and 1 μ L of 5 mM S-adenosyl methionine. Reaction mixtures were incubated at 37°C for 30 min, and the reaction was stopped by raising the temperature to 65°C. DNA_{em} was purified and quantified similarly to DNA_{nm}. Methylation efficiency was assessed by digestion with the methylation-sensitive *HpaII* restriction enzyme (Fermentas) and by pyrosequencing. The tests indicated that nearly all (~90%) cytosines in CpG sites became methylated, which corresponds to 15% of all cytosines in the sequence (see the [Supporting Material](#)). In DNA_{cm} all cytosines were methylated (except for 10 cytosines in the primers) regardless of whether C was in a CpG site or not. DNA_{cm} was prepared by adding m⁵CTP, instead of CTP, to the standard PCR mixture. The reaction was optimized by adding 6–8% DMSO to the reaction mixture (see the [Supporting Material](#)). Because the level of methylation (i.e., hypo- or hypermethylation) is expressed as a relative measure (i.e., in comparison to the usual methylation level of the given genetic region), and because 90% of the CpG cytosines were methylated in DNA_{em} and all of the cytosines were methylated in DNA_{cm}, we consider both of them hypermethylated.

Optical trapping nanometry

Nanomechanical manipulation was carried out with a custom-built, dual-beam counterpropagating optical tweezers apparatus (40–43) in constant velocity mode (in some experiments force was kept constant; see the [Supporting Material](#)). The NH₂ end of the DNA molecule was covalently linked to a 2.5 μ m carboxylated latex bead (Kisker Biotech, Steinfurt, Germany). The other end of the molecule was attached to a 3.0 μ m carboxylated latex bead coated with streptavidin, which captured the biotinyl group. One of the beads was held in the optical trap while the other one with a micropipette embedded in a custom-built flow chamber mounted on a close-loop piezoelectric stage (Nano-PDQ375; Mad City Labs, Madison, WI). DNA molecules were stretched by moving the micropipette away from the trap with a constant rate (typically 500 nm/s) with a step resolution of ~1 nm. Trap stiffness was ~0.2 pN/nm. Instrument control and data acquisition were managed by using custom-written LabView routines using (LabView v.7.1; National Instruments, Austin, TX). Force was measured by calculating the change in photonic momentum (44), with a resolution of ~0.2 pN and acquisition rate of 1–5 kHz. Buffer condition was 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA. To identify the zero-extension position in the displacement data, the micropipette-bead was pressed gently against the trapped bead either before or after the experiment. Molecular extension (z) was calculated by correcting displacement (s) according to force (F) and trap stiffness (κ) as

$$z = s - \frac{F}{\kappa}. \quad (1)$$

Data were processed with the softwares LabView and IgorPro (v.6.2.2.2; WaveMetrics, Lake Oswego, OR) and were smoothed to 100 Hz with a median filter.

AFM

The topographical structure of surface-adsorbed DNA molecules was assessed with noncontact-mode AFM (Cypher; Asylum Research, Santa Barbara, CA) using silicon cantilevers (AC160TS; Olympus, Tokyo, Japan; nominal tip radius 7 nm, resonance frequency ~300 kHz). Samples were deposited on freshly cleaved mica in a buffer containing 10 mM NaCl, 4 mM HEPES (pH 8), and 2 mM MgCl₂. After 10 min of incubation, the mica surface was washed gently with a stream of ultrapure water and dried with a flow of high purity N₂. Data acquisition and analysis were carried out with the IgorPro 6 software suite (WaveMetrics) and custom modules from Asylum Research (see also Fig. S5).

Force-field molecular dynamics simulation

The modeled DNA molecule was a 30-bp section taken from the experimentally studied sequence (see the Supporting Material) and contained a total 23 cytosines (8 in CpG and 15 non-CpG cytosines). In the hypermethylated model molecule (mDNA_{cm}) all of the cytosines were methylated on ⁵C, whereas in the control (mDNA_{nm}) none of them were. The structures were built with Maestro (45) in ds B-DNA conformation. TIP3 water boxes with 65 × 65 × 220 Å size were added (46). Simulations were carried out with the CHARMM36 force field (47) using the NAMD 2.10 program (48). After equilibration, steered molecular dynamics simulations were performed with 150 pN constant force applied for 15 ns under NVT conditions. Ten sets of force-time data were averaged for both mDNA_{nm} and mDNA_{cm}.

Data analysis

Each force (F) versus extension (x) curve obtained in the optical trapping nanometry experiments was fitted, by using the Marquardt-Levenberg nonlinear least squares method, with the extensible wormlike-chain (eWLC) equation (32,33)

$$x = L_0 \left[1 - \frac{1}{2} \left(\frac{k_B T}{F L_p} \right)^{1/2} + \frac{F}{K} \right] \quad (2)$$

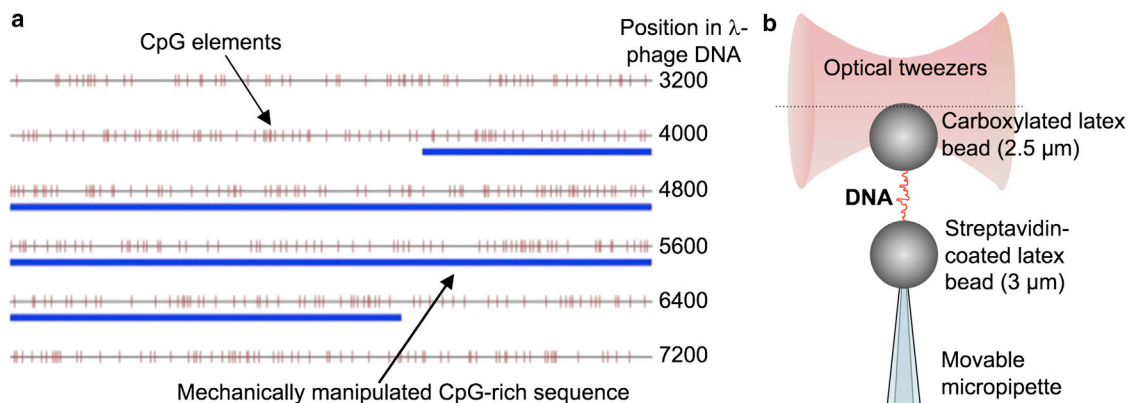


FIGURE 1 Nanomechanical manipulation of a CpG island. (a) Layout of λ -phage DNA from position 2400 to 7200 illustrating the distribution of CpG elements (vertical bars). The CpG island chosen for our experiments is underlined in blue. (b) Schematics of the optical trapping nanometry experiment. To see this figure in color, go online.

to obtain the contour length (L_0), persistence length (L_p), and intrinsic stiffness (stretch modulus, K) of the DNA molecules. In this equation, k_B is Boltzmann's constant, and T is absolute temperature. The force-versus-extension curves for ssDNA and S-form DNA were simulated according to established procedures (35) adapted for the contour length of our constructs and for the ionic conditions or our experiments. The analysis of AFM data (for contour and persistence lengths) is detailed in the Supporting Material.

RESULTS

Hypermethylated DNA constructs

The DNA sequence chosen for nanomechanical measurements was cloned from λ -phage DNA (Fig. 1 a). Sequence selection was based on the criteria of the CpG island: GC content > 50%, total length > 200 bp, and observed-to-expected CpG dinucleotide ratio > 0.6 (37). The chosen 3312-bp-long sequence contained CpG elements with an average frequency of 1/9 basepairs (49–51) (Figs. 1 a and S1). While the control, nonmethylated sequence (DNA_{nm}) lacked methylated cytosines altogether, the enzymatically methylated sequence (DNA_{em}) contained 300 methylated CpG sites (~90% of CpG cytosines, ~15% of all cytosines; see the Supporting Material). All except 10 (in the two primers) of the 1942 cytosines were methylated in the chemically methylated sequence (DNA_{cm}), prepared with PCR containing m⁵C in the reaction mix (Figs. S2–S4). The structural features of the different DNA constructs were analyzed by using AFM (Figs. S5–S7). The contour length of the methylated forms was significantly smaller than that of the nonmethylated (Fig. S6, d and e; Table 1). The persistence length of the methylated forms, measured with two different types of shape analysis (Fig. S7), was significantly greater than that of the nonmethylated (Table 1).

Nanomechanics of differentially methylated dsDNA

We mechanically manipulated single molecules of dsDNA captured in a torsionally open geometry to maximize the

TABLE 1 Structural and Mechanical Parameters of Nonmethylated, Enzymatically, and Chemically Hypermethylated DNA Obtained in AFM and Optical Tweezers Measurements

	Nonmethylated DNA	Enzymatically Methylated DNA	Chemically Hypermethylated DNA
dsDNA contour length (theoretical, 3.4 Å/bp)	1126.1	—	—
dsDNA contour length (nm) (AFM)	1089.4 ± 2.8 <i>n</i> = 32	1056.8 ± 2.9 <i>n</i> = 38	1059.3 ± 2.0 <i>n</i> = 45
dsDNA persistence length (nm) (AFM, mean-square-separation)	66.9 ± 0.3 <i>n</i> = 32	78.8 ± 0.7 <i>n</i> = 46	79.4 ± 0.4 <i>n</i> = 58
dsDNA persistence length (nm) (AFM, orientation correlation)	60.7 ± 0.2 <i>n</i> = 34	71.2 ± 0.3 <i>n</i> = 46	71.8 ± 0.3 <i>n</i> = 58
dsDNA contour length (nm) (optical tweezers, eWLC fit)	1142.5 ± 1.6 <i>n</i> = 34	1143.3 ± 1.6 <i>n</i> = 36	1132.2 ± 2.7 <i>n</i> = 23
dsDNA persistence length (nm) (optical tweezers, eWLC fit)	44.6 ± 1.5 <i>n</i> = 34	41.2 ± 1.5 <i>n</i> = 35	39.1 ± 1.8 <i>n</i> = 23
dsDNA stretch modulus (pN) (optical tweezers, eWLC fit)	1828.5 ± 52.5 <i>n</i> = 34	1602.9 ± 53.2 <i>n</i> = 36	1514.5 ± 66.3 <i>n</i> = 23
Overstretch force (pN) (at 1.5 μm extension)	64.1 ± 0.2 <i>n</i> = 33	63.9 ± 0.2 <i>n</i> = 17	66.2 ± 0.3 <i>n</i> = 22
Overstretch transition slope (pN/μm) (linear fit)	3.2 ± 0.1 <i>n</i> = 30	3.6 ± 0.2 <i>n</i> = 14	5.0 ± 0.5 <i>n</i> = 15
S-DNA contour length (nm) (eWLC fit)	1978.7 ± 8.7 <i>n</i> = 33	2010.3 ± 5.4 <i>n</i> = 35	2024.8 ± 8.9 <i>n</i> = 16
Hysteresis length (nm) (along overstretch transition)	160.4 ± 11.3 <i>n</i> = 60	96.1 ± 8.7 <i>n</i> = 105	11.4 ± 2.3 <i>n</i> = 138
Hysteresis area (pN/nm)	1898.3 ± 448.28 <i>n</i> = 17	688.57 ± 259.93 <i>n</i> = 18	320.76 ± 30.824 <i>n</i> = 14

Errors refer to standard error of the mean except for the AFM-based persistence length data (standard deviation of the fit; see the [Supporting Material](#)). The value *n* refers to the number of DNA molecules.

conformational degrees of freedom. One of the 5' ends contained an NH₂ group, by which DNA was covalently attached to a carboxylated latex bead. The opposite 5' end contained biotin, by which this end was attached to a streptavidin-coated latex bead. Individual DNA molecules were manipulated with force-measuring optical tweezers (40–43) by stretching and relaxing them with a constant velocity of 500 nm/s (Fig. 1 *b*). Nanomechanical data were collected in the force range of 0–100 pN. Fig. 2 shows raw and superimposed smoothed stretch force-versus-extension data for DNA_{nm} (Fig. 2 *a*), DNA_{em} (Fig. 2 *b*), and DNA_{cm} (Fig. 2 *c*).

The global appearance of the force-curves was similar for each DNA form. At low forces, a nonlinear force response was observed, and at 65 pN a force plateau appeared that corresponds to the cooperative transition, which converts dsDNA into an overstretched state (30). Further stretch resulted in a sharp increase of force. To reveal the fine detail of differences in the nanomechanical behavior of the different DNA forms, we overlaid their consensus force-versus-extension curves (Fig. 3) and calculated the relevant polymer-chain parameters by fitting raw data with the extensible wormlike-chain (eWLC) model (Eq. 2) (Table 1). In the entropic regime (0–10 pN), subtle differences were observed between the nonmethylated and methylated forms of dsDNA (Fig. 3 *b*). The contour and persistence lengths of dsDNA_{em} were not significantly different from those of dsDNA_{nm}. In contrast, both the contour length and the persistence length were significantly reduced in dsDNA_{cm}

(Table 1). Significant changes were detected in the enthalpic regime of elasticity (10–60 pN), which describes extensibility beyond the contour length of the random, entropic polymer coil (Fig. 3 *c*). The stretch modulus of both dsDNA_{em} and dsDNA_{cm} was significantly smaller than that of dsDNA_{nm} (Table 1).

Force-induced overstretch transition

In the overstretch region (Fig. 3 *d*) of the force-versus-extension curves intriguing differences were observed between the nonmethylated and methylated DNA forms. Differences were observed in three parameters: overstretch force, cooperativity and the length of the overstretch transition. While the overstretch force (measured at 1.5 μm extension that corresponds approximately to the midpoint of the transition) was not significantly different between DNA_{nm} and DNA_{em}, significantly greater forces were required to progress through this transition in the case of DNA_{cm} (Figs. 3 *d* and 4 *a*; Table 1). To estimate the cooperativity of the process, we measured the slope of the line fitted to the overstretch transition. The slope was significantly greater in both DNA_{em} and DNA_{cm} than in DNA_{nm} (Fig. 4 *b*; Table 1).

Finally, and most strikingly, the length of the transition was greater than that of DNA_{nm} in both methylated DNA forms, which resulted in an overstretched form of DNA with an extended contour length (Figs. 3 *e* and 4 *c*; Table 1). To explore the nature of the overstretched state, we

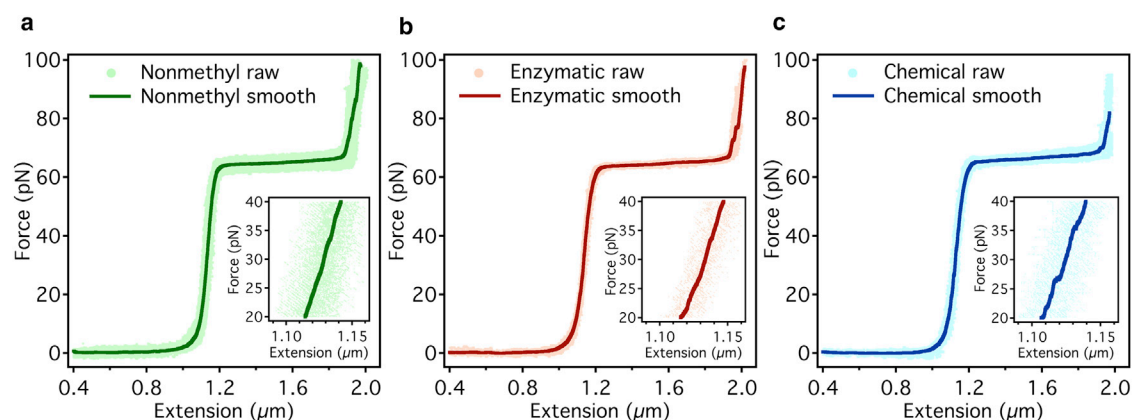


FIGURE 2 Force-versus-extension curves for the three different DNA forms. Raw data with superimposed smoothed data are shown. Smoothing was carried out by local averaging within a window the size of which was $\sim 1\%$ of the total dataset. (a) Nonmethylated DNA (DNA_{nm}). Raw data for 30 force-versus-extension curves containing a total number of 247,000 datapoints are shown (light points). Smoothing window was 2000 points wide. (b) Enzymatically methylated DNA (DNA_{em}). Raw data for 17 force-versus-extension curves containing a total number of 65,000 datapoints are shown (light points). Smoothing window was 500 points wide. (c) Chemically hypermethylated DNA (DNA_{cm}). Raw data for 15 force-versus-extension curves containing a total number of 101,000 datapoints are shown (light points). Smoothing window was 1000 points wide. (Insets) Enlarged regions of the plots merely to indicate the dispersion of the data. In generating the above plots, only stretch data were used. To see this figure in color, go online.

compared representative force-versus-extension curves with theoretical data for S-form DNA, ssDNA and 2ssDNA (two ssDNA molecules held in parallel) (Fig. 5, *d* and *e*). According to the fit, overstretched DNA_{nm} resembled S-DNA, whereas overstretched methylated DNA forms were similar, apparently, to a ssDNA (Fig. 5, *d* and *e*). The 2ssDNA model showed a behavior entirely different from each of the different DNA constructs. Importantly, a truly single-stranded state is not possible under our experimental conditions, because DNA was captured at its opposite 5' ends in a torsionally open geometry. To investigate whether the overstretched state of methylated DNA corresponds to an alternative structure, we carried out repetitive stretch-relaxation cycles interrupted with pauses at high, clamped forces (Figs. 5 and S8–S11). During stretch, only the cooperative overstretch transition was observed, and additional distinct transitions were never detected. During relaxation, different mechanical behavior was observed depending on the methylation state. Whereas in DNA_{nm} a large hysteresis was typically observed (Fig. 5 *a*), force hysteresis was reduced in DNA_{em} (Fig. 5 *b*) and it was essentially absent in DNA_{cm} (Fig. 5 *c*). When held at a high constant force (>70 pN, 5 s), the extension of the DNA_{nm} and DNA_{em} slowly increased as a function of time, whereas that of the chemically hypermethylated form (DNA_{cm}) remained essentially constant (Fig. S11).

Molecular dynamics simulation

To assess the structural basis of the nanomechanical differences between nonmethylated and hypermethylated dsDNA, steered molecular dynamics simulations were carried out (Fig. 6, Movie S1). Constant-force (extension versus time) rather than constant-velocity (force versus

extension) simulations were performed for three reasons. First, because of its smaller computational demand, statistically meaningful number of simulation runs could be obtained that allowed averaging the data that contained large fluctuations. Second, the problems associated with the vast differences between loading rates in simulated versus experimental loading rates in constant-velocity manipulations could be minimized. Third, because of the small size (30 bp) of the model DNA molecules (mDNA), detectable length differences were anticipated only between the overstretched transitions of the nonmethylated (mDNA_{nm}) and chemically hypermethylated (mDNA_{cm}) forms, where a large length change occurs across a narrow force range (see Fig. 3 *d*). Molecular structure snapshots of the simulation (Fig. 6 *a*) indicate that mDNA_{cm} retains a greater helicity than mDNA_{nm} .

The averaged extension-versus-time curves (Figs. 6 *b* and S9) indicate that the largest difference between the simulated nanomechanical behavior of mDNA_{nm} and mDNA_{cm} occurs in the 2–4 ns interval, which corresponds to 50–60% molecular extension. In this regime, the extension of mDNA_{cm} under the same force is smaller than that of mDNA_{nm} , indicating that greater force is required to stretch hypermethylated DNA than the nonmethylated form. Considering that the 2–4 ns region corresponds to the overstretch transition (compare to Fig. 3, *a* and *d*), the simulation supports the experimental observations and provides a glimpse at the differences between the structures of the DNA forms.

DISCUSSION

We have investigated the effect of methylation on DNA nanomechanics by using a 3312-bp model dsDNA that

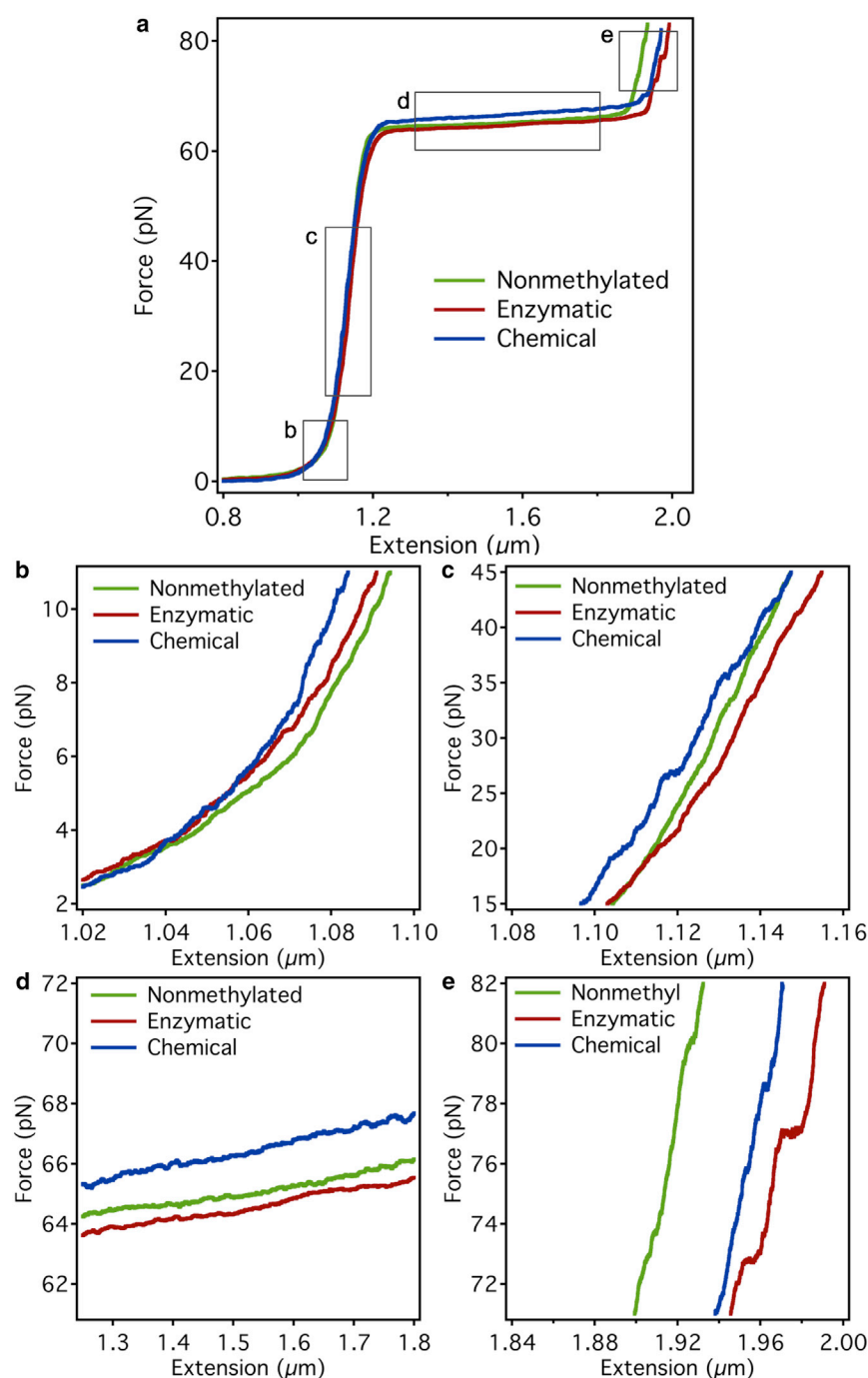


FIGURE 3 Comparison of nanomechanical data in different force regimes. (a) Overview of the consensus force-versus-extension curves of the different DNA constructs. (Boxes) Regions magnified in (b)–(e). (b) Region of dsDNA entropic elasticity. (c) Region of dsDNA enthalpic elasticity. (d) Overstretch transition. (e) Region of the elastic behavior of overstretched DNA.

fulfilled the criteria of a CpG island, as opposed to the short arbitrary sequences in prior studies (18,21,24,52). To maximize the effect of methylation, a PCR-based chemical protocol was used in addition to enzymatic treatment. The degree of cytosine modification reached hypermethylation levels in both DNA_{em} and DNA_{cm}. A further advantage of DNA_{cm} lies in the fact that it enables the assessment of non-CpG cytosine methylation as well. The structural features of the DNA constructs were characterized with AFM. The contour length of all three constructs was shorter

than theoretically predicted (assuming 3.4 Å/bp for B-DNA) in accordance with previous findings (53). In addition, according to our analysis, the contour length of the methylated DNA constructs was 3% shorter than that of the nonmethylated form (Fig. S6; Table 1). Contour-length reduction in surface-adsorbed molecules of methylated DNA has been observed before (22). The persistence length of the surface-adsorbed methylated forms increased significantly (Fig. S7; Table 1) as reported in Kaur et al. (22) and Cassina et al. (25). The observed stiffening is thought to be due to the

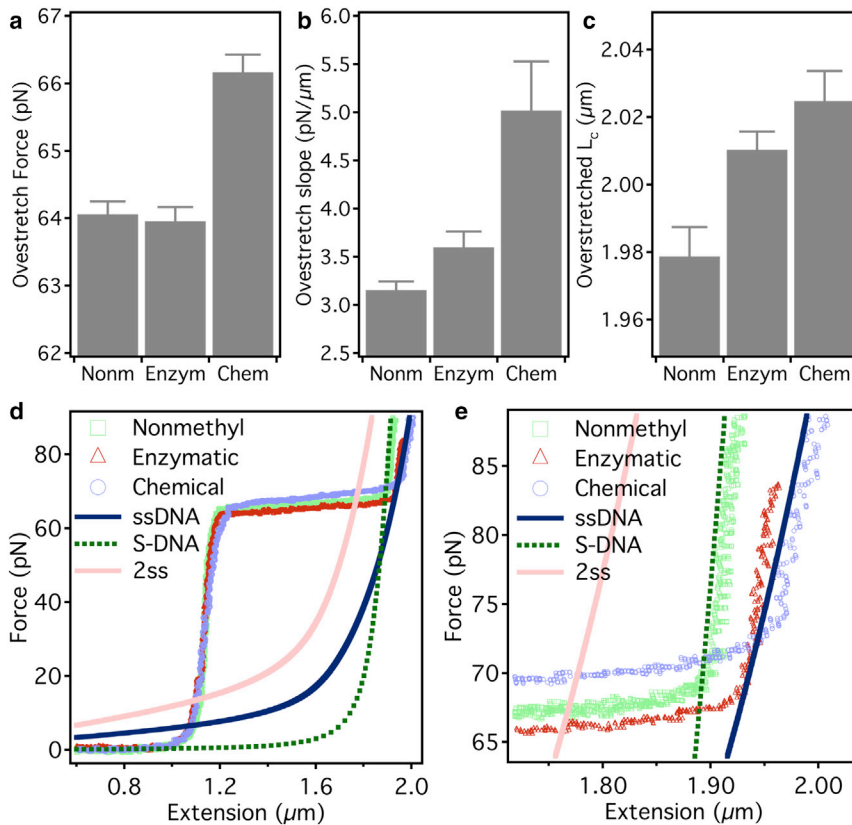


FIGURE 4 Overstretch behavior of dsDNA. (a) Overstretch force, measured at 1.5 μm extension. (b) Overstretch slope measured by fitting a line on the entire overstretch transition. (c) Contour length of the overstretched DNA measured by fitting the eWLC equation on the force data. Stretch modulus of 2700 pN was systematically used in these fits. Detailed statistics are listed in Table 1. (d) Comparison of experimental data with theoretical models of ssDNA, 2ssDNA, and S-DNA. (e) Enlarged view of (d) to highlight differences. To see this figure in color, go online.

dehydration of the hydrophobic hypermethylated DNA on the substrate interface (22). Under tension, however, the DNA molecule may display nanomechanical parameters different from those extracted by using shape analysis (54). Therefore, we exposed the DNA constructs to a wide range of forces that covered both the entropic and enthalpic elasticity regimes and the overstretch transition. Previous studies reported on the effect of methylation either only on its entropic elasticity or strand separation forces (22–24). Here, we were able to sensitively investigate the full spectrum of differences between the nanomechanics of non-methylated and methylated DNA.

In the force regime of entropic elasticity (0–10 pN), hypermethylation-evoked nanomechanical changes were subtle, but significant. The contour length of dsDNA_{cm}, as obtained from the optical tweezers data, was reduced by 1% (Table 1), indicating that hypermethylation indeed leads to an axial compaction of the DNA structure. In contrast to the AFM data, however, the persistence length of dsDNA_{cm} became reduced, indicating that, apparently, methylation leads to an increase in the flexibility of the DNA chain under tension. Thus, smaller energy is required to bend dsDNA, which is likely to influence chain packaging by nucleosomes. We note that the persistence length of the nonmethylated DNA construct was much smaller when measured with optical tweezers than with AFM. Such a difference has been noted before and has been attributed to a high (>50%) GC

content (54). The 59% GC content of the DNA construct used in our experiments thus explains the persistence-length differences in DNA_{nm} observed with the different methods. The increased flexibility of methylated DNA under tension is in contrast with some cyclization kinetics (18,21) and AFM (22,25) experiments which reported the stiffening of methylated DNA. We speculate that in cyclization kinetics a strong sequence dependence relative to the small size of the molecules (55), and in AFM experiments surface constraints and electrostatic effects (56) contribute to the observed differences. Furthermore, the differences may be reconciled by the increased enthalpic compliance observed here for the methylated DNA constructs (see Fig. 3 c and text below), which results in enhanced extensibility in the enthalpic regime of forces.

In the enthalpic regime of elasticity (10–60 pN) significantly smaller stretch moduli were determined for the methylated forms of DNA than for dsDNA_{nm}. Enthalpic or intrinsic elasticity is commonly attributed to the elastic distortion of bonds along DNA (28). It has been suggested that methylation generates angular distortions in the DNA backbone (20), which are then straightened during stretch. The smaller stretch moduli observed for the methylated DNA forms indicate that smaller forces are required for extending DNA in the 10–60 pN regime. Presumably, the greater axial compliance of methylated DNA further aids its packaging by nucleosomes.

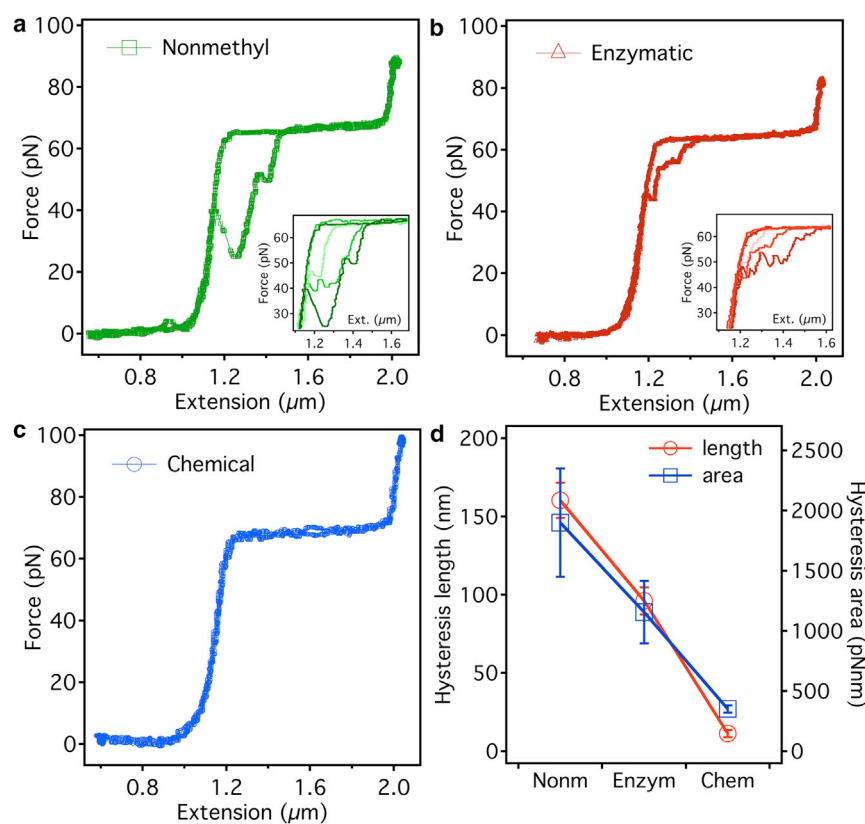


FIGURE 5 Analysis of force hysteresis. (a)–(c) Force-versus-extension curves obtained in stretch and relaxation cycles for a DNA_{nm}, DNA_{em} and DNA_{cm} molecule. Single nanomechanical cycles are shown for each construct. (Insets in a and b) Magnified views of several force-extension cycles to indicate the hysteresis shape. (d) Magnitude of hysteresis, measured as the length of the hysteretic region along the extension axis and as hysteresis area, as a function of methylation status. Detailed statistics are listed in Table 1. To see this figure in color, go online.

The largest effect of hypermethylation was observed in the overstretch transition of the CpG island. Recently it has been shown that three processes may simultaneously occur during this process: strand unpeeling (denaturation) resulting in an apparent ssDNA behavior, melting-bubble formation that corresponds to local regions of partial inside-strand separation, and transition from the B-form into an S-form DNA (34,35,57). While the exact structure of S-DNA is still unknown, it is most commonly thought to resemble an unwound helix with a straightened configuration in which the coupling between the complementary strands is maintained (35). Because of the torsionally open geometry of our single-molecule manipulation experiment, all of the transitions were allowed to take place during stretch. However, we have not observed the distinct, stepwise, sawtoothlike transitions characteristic of strand unpeeling in any of the DNA constructs (34,58). The absence of strand unpeeling is most likely caused by the overwhelming GC content of the constructs (59%), which tends to stabilize the double-stranded configuration (58,59). Comparison of the experimental data with theoretical models (Fig. 4, d and e) suggested that the overstretched state of DNA_{nm} is S-form DNA. The systematic presence of force hysteresis in DNA_{nm} indicates, furthermore, that melting-bubble formation was also present. Thus, the configuration of overstretched DNA_{nm} is a combination of S-DNA and melting bubbles. The gradual disappearance of force hysteresis

caused by methylation (Fig. 5) suggests that the number of melting bubbles became progressively reduced, and the overstretched state of DNA_{em} and DNA_{cm} is dominated by S-form DNA. The lack of progressive extension of DNA_{cm} in high-force-clamp (>70 pN) experiments (Figs. S11–S14) indicates that melting-bubble formation is strongly inhibited by methylation. In hypermethylated DNA, two significant differences were systematically observed in comparison with DNA_{nm}: the slope of the transition was increased, and the overstretched state was longer. Furthermore, in DNA_{cm}, hence due to non-CpG cytosine methylation, greater forces were required to evoke the overstretch transition. The elevated transition force indicates that a larger overall energy barrier needs to be surpassed during overstretch. Force-field molecular dynamics simulations on a short segment of the experimentally manipulated DNA (Fig. 6) supported the findings. Because the model DNA is ~ 1000 -fold shorter than the one experimentally manipulated, the ~ 5 Å greater extension of mDNA_{cm} than that of mDNA_{nm} during the overstretch transition (Fig. 6 b, in the 2–4 ns time interval) corresponds remarkably well to the ~ 0.5 μm greater extension of DNA_{nm} than that of DNA_{cm} in the optical tweezers experiment (Fig. 3 d, at ~ 65 pN). The increased overall energy barrier is likely due to stronger intrachain interactions caused by the increased hydrophobicity of the methylcytosines. The increased strand separation forces measured, by using molecular force assay, for

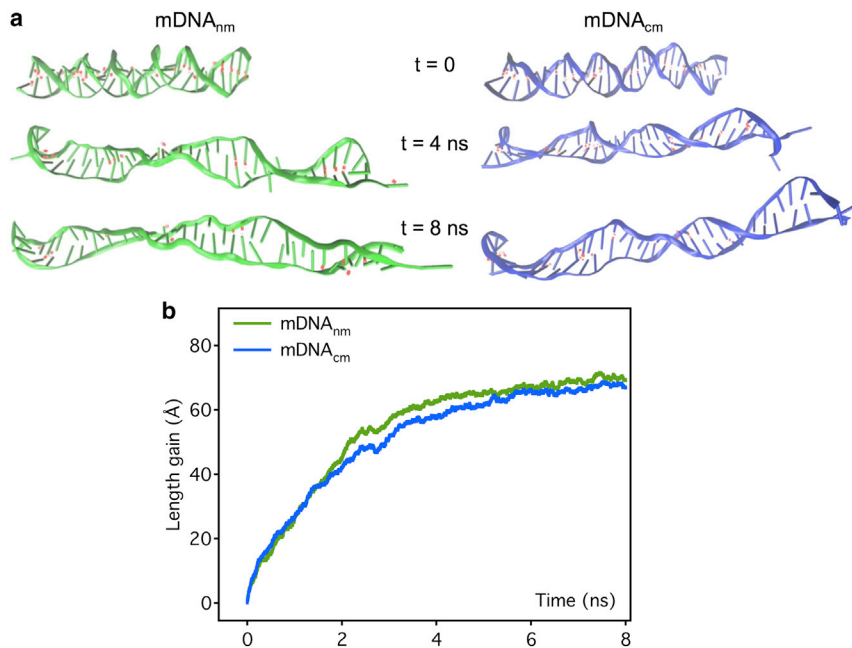


FIGURE 6 Force-field molecular dynamics simulation of nonmethylated and hypermethylated model DNA (mDNA) constructs. (a) Structures of the nonmethylated (mDNA_{nm}) and hypermethylated (mDNA_{cm}) model DNA molecules at the highlighted time points during constant-force (150 pN) simulation. (b) Length-versus-time functions for mDNA_{nm} and mDNA_{cm}. The curves were obtained by averaging 10 independent simulation runs. For further statistics, see the [Supporting Material](#). To see this figure in color, go online.

more extensively methylated dsDNA (24) and the increased thermal stability of non-CpG-methylated dsDNA support this idea (60). The increase in the slope of the overstretch transition indicates that cooperativity became reduced. Thus, the number of sites along the DNA strand at which B-S transition coincidentally occurs becomes reduced upon methylation. Comparison of the experimental data with theoretical models (Fig. 4, *d* and *e*) suggested that the overstretched state of both DNA_{em} and DNA_{cm} resembles ssDNA. However, because of the lack of unpeeling transitions, the minimal or totally absent force hysteresis and the torsionally open geometry of the manipulated DNA, we exclude the possibility that the overstretched state of methylated DNA is true ssDNA. Rather, an extended S-form DNA is present. Assuming that the transition is homogenous along the DNA molecule, the extension involves a 0.14 Å/bp lengthening relative to S-form DNA. The structure of this extended S-DNA is not known. We speculate that a higher hydrogen bond distortion and a decreased helicity of the S-DNA form may underlie the extension transition. The significant reduction of hysteresis (Fig. 5) indicates that interstrand bonds are strengthened significantly. Possibly, the electron-donor properties of the methyl group enhance base-stacking interactions that stabilize strand pairing in the stretched state. The longer-lived base pairing in the simulated mDNA_{cm} molecule (Fig. 6; [Movie S1](#)) supports this possibility. Furthermore, a basepaired but overstretched DNA conformation has been demonstrated in a GC-rich construct (61).

In conclusion, a putative novel form of extended S-DNA has been detected in the hypermethylated CpG island studied here. While the hypermethylated dsCpG island may be

packaged into a more compact configuration, it appears to be more difficult to separate its strands. Methylation of non-CpG cytosines apparently provide an additional stabilizing mechanism. Because strand separation is important in DNA replication, transcription, and repair, hypermethylated CpG islands may present a thermodynamic and kinetic barrier for mechanoenzyme action. Slowing or halting mechanoenzymatic processes at hypermethylated CpG islands may thus contribute to a finely tuned spatial pattern of gene expression.

SUPPORTING MATERIAL

Supporting Materials and Methods, Supporting Results, fourteen figures, and one movie are available at [http://www.biophysj.org/biophysj/supplemental/S0006-3495\(16\)34334-X](http://www.biophysj.org/biophysj/supplemental/S0006-3495(16)34334-X).

AUTHOR CONTRIBUTIONS

C.I.P. performed research, analyzed data, and wrote the article; P.B. performed research; G.F. performed research; R.K. designed research and wrote the article; and M.K. designed research, analyzed data, and wrote the article.

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