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Most Methylation-Susceptible DNA Sequences in Human Embryonic Stem Cells Undergo a Change in Conformation or Flexibility upon Methylation

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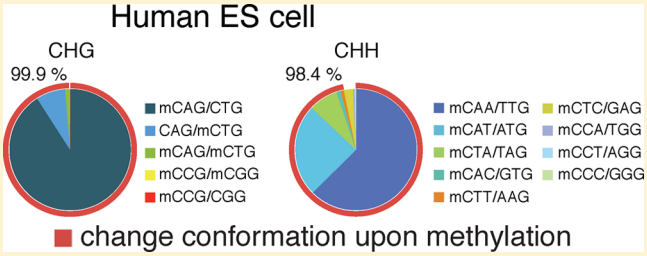
Supporting Information

ABSTRACT: DNA methylation in eukaryotes occurs on the cytosine bases in CG, CHG, and CHH (where H indicates non-G nucleotides) contexts and provides an important epigenetic mark in various biological processes. However, the structural and physical properties of methylated DNA are poorly understood. Using nondenaturing polyacrylamide gel electrophoresis, we performed a systematic study of the influence of DNA methylation on the conformation and physical properties of DNA for all CG, CHG, and CHH contexts. In the CG context, methylated multimers of the CG/CG-containing unit fragment migrated in gels slightly faster than their unmethylated counterparts. In the CHG context, both homo- and hemimethylation caused retarded migration of multimers of the CAG/CTG-containing fragment. In the CHH context, methylation caused or enhanced retarded migration of the multimers of CAA/TTG-, CAT/ATG-, CAC/GTG-, CTA/TAG-, or CTT/AAG-containing fragments. These results suggest that methylation increases DNA rigidity in the CG context and introduces distortions into several CHG and CHH sequences. More interestingly, we found that nearly all of the methylation repertoires in the CHG context and 98% of those in the CHH context in human embryonic stem cells were species that undergo conformational changes upon methylation. Similarly, most of the methylation repertoires in the *Arabidopsis* CHG and CHH contexts were sequences with methylation-induced distortion. We hypothesize that the methylation-induced properties or conformational changes in DNA may facilitate nucleosome formation, which provides the essential mechanism for alterations of chromatin density.



DNA methylation is an important epigenetic mark in various biological processes, including gene expression, DNA−protein interactions, transposable element suppression, cell proliferation, embryogenesis, parental imprinting, X chromosome inactivation, differentiation, and oncogene repression.[1−8] This mechanism is utilized by major eukaryotic groups such as animals, plants, and many fungi. In mammals, DNA methylation occurs almost exclusively on the cytosine bases in the symmetric CG context. This methylation is estimated to occur in ∼70−80% of CG dinucleotides throughout the genome.[9] However, non-CG methylation also occurs in embryonic stem (ES) cells, with a recent study showing that nearly one-quarter of all methylations identified in human ES cells was in a non-CG context (CHG and CHH).[10] In plants, DNA methylation commonly occurs in the symmetric CG, symmetric CHG, and asymmetric CHH contexts.[11] In *Arabidopsis thaliana*, the genomewide methylation levels are approximately 22−24, 7−8, and 2% in the CG, CHG, and CHH contexts, respectively.[12,13] DNA methylation in plants also predominantly occurs on transposons and other repetitive DNA elements, in contrast to that in mammals.[14]

Regardless of the biological significance of DNA methylation, knowledge of the structural and physical properties of methylated DNA is limited.[15] Using an *Eco*RI site-containing fragment, $(CGCGAATTCG)_3$, cyclization kinetics experiments and Monte Carlo simulations have shown that cytosine methylation in the symmetric CG context does not affect the bend magnitude or direction but lowers the bending flexibility by ∼30% and causes underwinding in the methylated regions from 10.5 to 11.0 bp per turn.[16] Similarly, a recent study using a molecular force assay and single-molecule force spectroscopy showed a strong methylation dependence of strand separation.[17] An electrophoretic analysis using the ligation products of the 10 bp *Bam*HI oligomer CGGGATCCCG suggested that the methylation of the most central Cs induces a small curvature of the helix axis.[18] Furthermore, another group focused on the effect of cytosine methylation on the

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Table 1. Unit DNA Fragments Used in This Study and Previous Studies[a]

Context	Name	Sequence	
		Current study	Previous studies
	Standard	CAGACTCAGTCAGTAGTCAGT TGAGTCAGTCATCAGTCAGTC	
CG	CG/CG	CATGCGTCAGTCAGCGTCAGT CGCAGTCAGTCGCAGTCAGTA	
	mCG/mCG	CATG^{me}C GTCAGTCAG^{me}C GTCAGT C G^{me}CAGTCAGTC G^{me}CAGTCAGTA	(C) CGCGAATT^{me}C G^{me}C G^{me}C GAATT^{me}C G^{me}C G^{me}C GAATTCG GCGCTTAA G^{me}C G^{me}C G^{me}CTTAA G^{me}C G^{me}C G^{me}CTTAAGC
	mCG/CG	CATG^{me}CGTCAGTCAG^{me}CGTCAGT C GCAGTCAGTC GCAGTCAGTA	(H) CAAAAA^{me}CGCG TTTT GCGCGT
			(H) CAAAAACG^{me}CG TTTTGC GCGT
	CG/mCG	CATGC GTCAGTCAGC GTCAGT CG^{me}CAGTCAGTCG^{me}CAGTCAGTA	(H) CAAAAAGC^{me}CG TTTTCG GCGT
			(H) GGCCA^{me}CGTGACCTGA^{me}CGTAC GGT GCACTGGACT GCATGCC
CHG	CAG/CTG	CCTACAGCCAGCTACAGCCAG TGTCGGTCGATGTCGGTCGGA	
	mCAG/mCTG	CCTA^{me}CA GCCAGCTA^{me}CA GCCAG T GT^{me}CGGTCGAT GT^{me}CGGTCGGA	
	mCAG/CTG	CCTA^{me}CAGCCAGCTA^{me}CAGCCAG T GTCGGTCGAT GTCGGTCGGA	
	CAG/mCTG	CCTACA GCCAGCTACA GCCAG TGT^{me}CGGTCGATGT^{me}CGGTCGG	
	mCAG/mCTG$_{14\text{-}7}$	CCTA^{me}CA GCCAGCTACAGC^{me}CA G T GT^{me}CGGTCGATGTCG GT^{me}CGGA	
	mCAG/mCTG$_{4\text{-}6\text{-}4\text{-}7}$	CCTA^{me}CA GC^{me}CA GCTA^{me}CA GC^{me}CA G T GT^{me}CG GT^{me}CGAT GT^{me}CG GT^{me}CGGA	
	CCG/CGG	GACGTGACCGTGACTGACCGT CACTGGCACTGACTGGCACTG	
	mCCG/mCGG	GACGTGA^{me}CC GTGACTGA^{me}CC GT CACT GG^{me}CACTGACT GG^{me}CACTG	
	mCCG/CGG	GACGTGA^{me}CCGTGACTGA^{me}CCGT CACT GGCACTGACT GGCACTG	(H) CAAAAAG^{me}CCG TTTTC GGCGT
	CCG/mCGG	GACGTGACC GTGACTGACC GT CACTGG^{me}CACTGACTGG^{me}CACTG	
CHH	CAA/TTG	ACTGCAACTGACTGCAACTGC CGTTGACTGACGTTGACGTGA	
	mCAA/TTG	ACTG^{me}CAACTGACTG^{me}CAACTGC C GTTGACTGAC GTTGACGTGA	(H) ^{me}CAAAAACACA TTTTGTGTGT
			(H) ^{me}CAAAAACGCG TTTTGCGCGT
	CAT/ATG	TGCACATGCATGCACATGCAC TGTACGTACGTGTACGTGACG	
	mCAT/ATG	TGCA^{me}CATGCATGCA^{me}CATGCAC T GTACGTACGT GTACGTGACG	(H) GGCCA^{me}CATGACCTGA^{me}CATAC GGT GTACTGGACT GTATGCC
			(H) GGCCT^{me}CATGACCTGT^{me}CATAC GGA GTACTGGACA GTGTGCC
	CAC/GTG	ACTGCACATGCATGCACATGC CGTGTACGTACGTGTACGTGA	
	mCAC/GTG	ACTG^{me}CACATGCATG^{me}CACATGC C GTGTACGTAC GTGTACGTGA	(H) CAAAAA^{me}CACA TTTT GTGTGT
			(H) CAAAAACA^{me}CA TTTTGT GTGT

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Table 1. continued

Context	Name	Sequence	
		Current study	Previous studies
	CTA/TAG	CGTGCTACTGACTGCTACTGA CGATGACTGACGATGACTGCA	
	mCTA/TAG	CGTG^{me}CTACTGACTG^{me}CTACTGA C GATGACTGAC GATGACTGCA	
	CTT/AAG	GCAGCTTGCATGCACTTGCAT CGAACGTACGTGAACGTACGT	
	mCTT/AAG	GCAG^{me}CTTGCATGCA^{me}CTTGCAT C GAACGTACGT GAACGTACGT	(H) GGCCT^{me}CTTGACCTGT^{me}CTTAC GGA GAACTGGACA GAATGCC
	CTC/GAG	AGTGCTCATGCATGCTCATGC CGAGTACGTACGAGTACGTCA	
	mCTC/GAG	AGTG^{me}CTCATGCATG^{me}CTCATGC C GAGTACGTAC GAGTACGTCA	
	CCA/TGG	CTGACCACTGACTGCCACTGA TGGTGACTGACGGTGACTGAC	
	mCCA/TGG	CTGA^{me}CCACTGACTG^{me}CCACTGA T GGTGACTGAC GGTGACTGAC	
	CCT/AGG	GACACCTGCATGCACCTGCAT TGGACGTACGTGGACGTACTG	
	mCCT/AGG	GACA^{me}CCTGCATGCA^{me}CCTGCAT T GGACGTACGT GGACGTACTG	
	CCC/GGG	CATGCCCATGCATGCCCATGA CGGGTACGTACGGGTACTGTA	
	mCCC/GGG	CATG^{me}CCCATGCATG^{me}CCCATGA C GGGTACGTAC GGGTACTGTA	(D) CGG GAT^{me}CCCG GCC^{me}CTA GGGC

[a] ^{me}C indicates 5-methylcytosine. C, Nathan and Crothers;[16] H, Hodges-Garcia and Hagerman;[19] D, Diekmann.[18] We include the data for CCG/mCGG in the CHG category for the sake of convenience.

conformation of curved DNA structures formed by an $A_5 \cdot T_5$ tract (A-tract),[19] and their electrophoretic analysis suggested that methylation up to 3 bp from an A-tract can alter the conformation of the net curved structure.

DNA bends have various biological functions,[20,21] and the flexibility profile of DNA can serve as a signal in gene expression.[22,23] Therefore, scrutinizing the effects of DNA methylation would help to clarify the mechanism underlying the biological effects of DNA methylation. We performed a systematic study of the influence of DNA methylation on the conformation and physical properties of DNA. Using nondenaturing polyacrylamide gels, the electrophoretic behavior of ligation products of DNA fragments containing hemimethylated or homomethylated CG, CHG, or CHH sequences was analyzed. In some cases, methylation induced formation of DNA curvature, which was generally clearer in longer fragments. In other cases, methylation generated additive effects on curved conformations. This study also suggested that DNA methylation confers rigidity to certain DNA sequences.

EXPERIMENTAL PROCEDURES

Oligonucleotides. The oligonucleotides listed in Table 1 were purchased from Operon or Takara as 5′-phosphorylated forms. Annealing was performed in 50 mM Tris-HCl and 10 mM $MgCl_2$. Generally, double-stranded (ds) DNA molecules were purified by electrophoresis on 20% nondenaturing polyacrylamide gels.

DNA Ligation. The annealed duplexes were ligated using T4 DNA ligase (NEB Japan) in the buffer provided. The 20 μL ligation reaction mixtures typically contained 2.5 μM DNA with 50 units of DNA ligase and were incubated at 16 °C for 60 min. The ligation products were treated with bacterial alkaline phosphatase (Takara) and 5′-end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Toyobo). A 20 bp DNA marker ladder (Takara) was also dephosphorylated and 5′-end-labeled, as described above. The labeled DNA was purified using a MicroSpin G-25 column (GE Healthcare).

Gel Electrophoresis. The ligation ladder products were analyzed on 9 or 12.5% nondenaturing polyacrylamide gels (29:1 acrylamide:bisacrylamide ratio) in 0.5× TBE buffer [45 mM Tris-borate and 1.0 mM EDTA (pH 8.3)]. Gels were conditioned with a prerun at 100 V (5 V/cm) at 4 or 50 °C for 60 min, prior to sample loading. Electrophoresis was performed in 0.5× TBE buffer at 100 V (5 V/cm), at 4 or 50 °C, using a circulating temperature control system. After electrophoresis, the gels were dried and exposed to an imaging plate, which was then scanned using an FLA-7000 fluorescent image analyzer (GE Healthcare).

Determination of Rs. The mobilities of the ligation ladder products were analyzed using Multi-Gauge (Fujifilm), and the relative sizes were calculated. The relative size, Rs, is the ratio of the apparent size (in base pairs) as determined from the gel mobility to the actual size (in base pairs). The apparent sizes of the ligation ladder products were determined on the basis of the mobilities of the standard (Table 1) ligation ladder products.

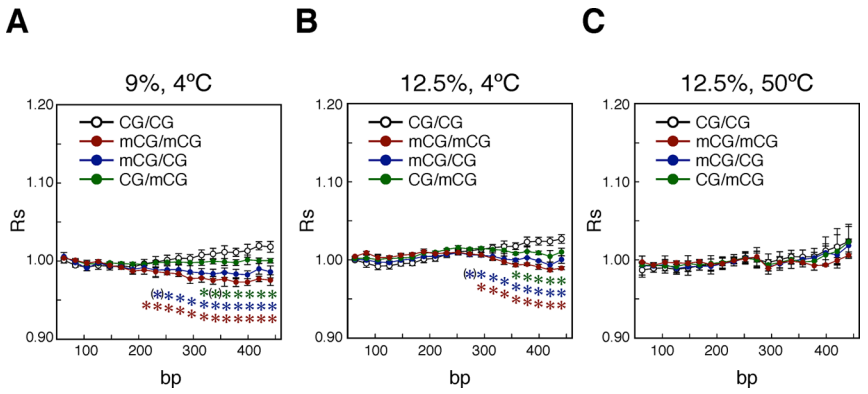

Figure 1. Electrophoretic behavior of methylated and unmethylated CG multimers. The values represent the means ± standard deviation (SD) of triplicate determinations. Using 9% (A) or 12.5% (B and C) nondenaturing polyacrylamide gels, electrophoreses were performed at 4 °C (A and B) and 50 °C (C). The Rs value is the ratio of the apparent size of a fragment to its actual size.[28] Data points are indicated using the following colors: white for CG/CG, red for mCG/mCG, blue for mCG/CG, and green for CG/mCG. *P* values were calculated between the methylated and corresponding unmethylated multimers and are shown using the same colors as those indicating fragment species. $^{*}P < 0.05$; $^{(*)}P \geq 0.05$ to <0.1.

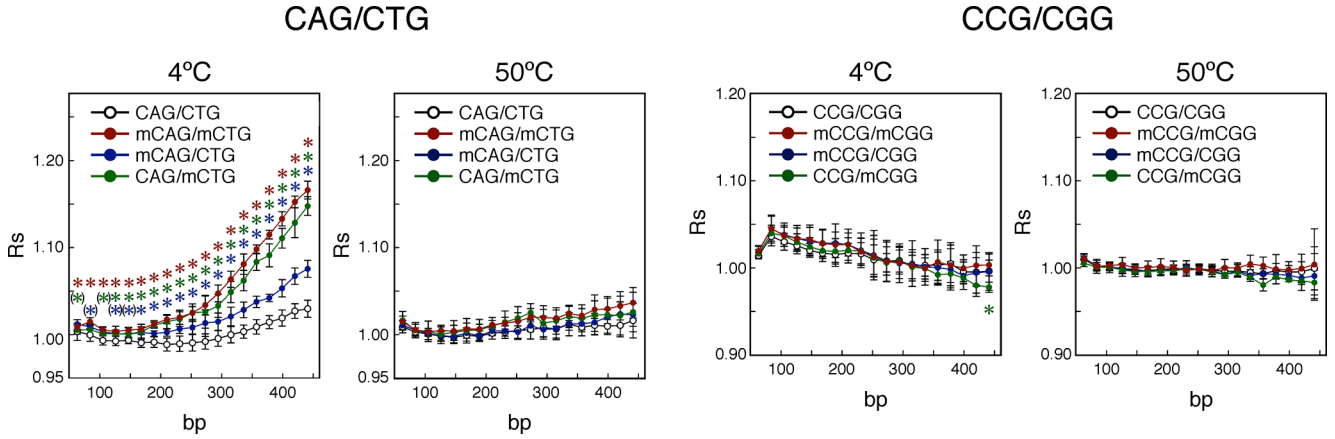

Figure 2. Electrophoretic behavior of methylated and unmethylated CHG multimers. The values represent the means ± SD of triplicate determinations. The electrophoretic conditions were the same as those for panels B and C of Figure 1. Data points are indicated by the following colors: white for CAG/CTG and CCG/CGG, red for mCAG/mCTG and mCCG/mCGG, blue for mCAG/CTG and mCCG/CGG, and green for CAG/mCTG and CCG/mCGG. *P* values were calculated between the methylated and corresponding unmethylated multimers and are shown using the same colors as those indicating fragment species. $^{*}P < 0.05$; $^{(*)}P \geq 0.05$ to <0.1.

Analysis of Occupancy Ratios. The numbers of in vivo occurrences of CHG and CHH sequences and the methylated forms were calculated using the NCBI Build 36/HG18 (for human) and TAIR 7 (*Arabidopsis*) databases, data for human DNA methylomes, and epigenome maps of *Arabidopsis*. For the human CHG and CHH sequences, the files mc_h1_r1.tar.gz and h1_c_basecalls.tar.gz, which contain data for the positions and frequencies of methylcytosine in human ES cells, were downloaded from http://neomorph.salk.edu/human_methylome/data.html. We screened for the positions of one or more occurrences of methylcytosine and counted the numbers of methylated CHG and CHH sequences. For the *Arabidopsis* CHG and CHH sequences, the number of occurrences of methylated sequences was obtained from Supplementary Table S5 of Lister et al.,[13] in which the number of occurrences of methylated CHG sequences is shown as the number of occurrences of the following sets: mCAG/CTG and mCAG/mCTG, CAG/mCTG and mCAG/mCTG, and mCCG/CGG and mCCG/mCGG. On the basis of these numbers, the occupancy ratios (%) were calculated.

RESULTS

We constructed a series of DNA fragments with phased 5-methylcytosines (^{me}Cs) (Table 1). Generally, the positions of the ^{me}Cs alternated at 10 and 11 bp intervals to make the ^{me}Cs occur with a periodicity of 10.5 bp on average, which is the helical repeat length of DNA. This construction allowed sensitive electrophoretic detection of conformational changes in DNA induced or enhanced by cytosine methylation.[24,25] At first, using nondenaturing polyacrylamide gel electrophoresis, we compared the electrophoretic behavior of methylated DNA fragments with that of their unmethylated counterparts for all biologically possible methylation contexts.

CpG Methylation Enhances Migration Speed during Electrophoresis. The electrophoreses were first performed at 4 °C using 9% polyacrylamide gels (Figure 1A and Figure S1 of the Supporting Information). The Rs value (apparent size/actual size, with size in base pairs) indicates the extent of the electrophoretic anomaly of a given fragment. An Rs value of >1.0 corresponds to retarded migration of the fragment, and a value of <1.0 corresponds to fast migration, compared with the expected migration for the fragment size. The retarded migration is a hallmark of curved (bent) DNA structures, and

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it is enhanced by low temperatures and normalized by high temperatures.[25−31] Rigid DNA migrates faster than normal DNA.[30,32]

In the CG context, the Rs values for CG/CG multimers gradually increased with fragment size, and long multimers showed Rs values of >1.0 (for unit fragment structures, see Table 1). Thus, these results indicated that the parental CG/CG long multimers have slightly curved conformations. mCG/mCG and mCG/CG multimers longer than ∼200 bp migrated faster than their unmethylated counterparts. CG/mCG multimers longer than ∼250 bp behaved similarly. However, the Rs values for CG/mCG multimers were ∼1.0 irrespective of their size, indicating that retarded migration caused by curved conformations was well balanced by the faster migration caused by the increased rigidity of DNA upon methylation. The difference in the electrophoretic behavior between the unmethylated and methylated fragments became smaller when 12.5% polyacrylamide gels were used (Figure 1B). Interestingly, in 12.5% gels, the retarded migration (CG/CG multimers) was slightly enhanced and the faster migration (mCG/mCG and mCG/CG multimers) was slightly suppressed, as compared with those phenomena in 9% gels. The former result agrees well with the reports that retarded migration caused by curved DNA structures is enhanced with higher polyacrylamide concentrations in gels.[30,31,33−35] To the best of our knowledge, the latter result is the first finding. The anomalies observed at 4 °C were largely normalized in electrophoresis at 50 °C (Figure 1C).

CAG/CTG Methylation Causes Conformational Changes in DNA. The CHG context comprises CAG, CTG, and CCG, and the first two of these sequences form dsDNA. Both homo- and hemimethylation of CAG/CTG caused markedly retarded migration of the resulting fragments (Figure 2 and Figure S2 of the Supporting Information). The extent of the electrophoretic anomaly increased with fragment size. mCAG/mCTG multimers showed the most retarded migration, and CAG/mCTG multimers also showed a similar migration. However, the extent of retarded migration of these methylated fragments dramatically decreased at 50 °C, strongly indicating that methylation generated DNA curvatures for CAG/CTG-containing fragments. To confirm this hypothesis, we examined the electrophoretic behavior of multimers of mCAG/mCTG$_{14-7}$ and mCAG/mCTG$_{4-6-4-7}$. The unit sequences of these molecules have a nucleotide sequence identical to the mCAG/mCTG sequence, but the ^{me}Cs in mCAG/mCTG$_{14-7}$ are out of phase with the DNA helical repeat length of 10.5 bp; mCAG/mCTG$_{4-6-4-7}$ contains twice as many ^{me}Cs as mCAG/mCTG and mCAG/mCTG$_{14-7}$, and ^{me}Cs occur with a periodicity of <10.5 bp (Table 1). As shown in Figure 3, although the mCAG/mCTG$_{4-6-4-7}$ multimers still exhibited slight anomalies, the extent of the electrophoretic anomaly considerably decreased, even at 4 °C. Thus, it seems safe to conclude that methylation of CAG/CTG generated some conformational distortion at the methylation site and that the repeated distortion caused pronounced curvature of the DNA in mCAG/mCTG and CAG/mCTG (Figure 2). On the other hand, in another CHG context (we include data for CCG/mCGG in this category for convenience, although it is a CG methylation), the mCCG/mCGG and mCCG/CGG multimers showed almost the same behavior as the corresponding unmethylated CCG/CGG multimers (Figure 2). In the case of long CCG/mCGG multimers, however, slightly faster migration was detected (though only to an extremely small extent), which was consistent with the data shown in Figure 1.

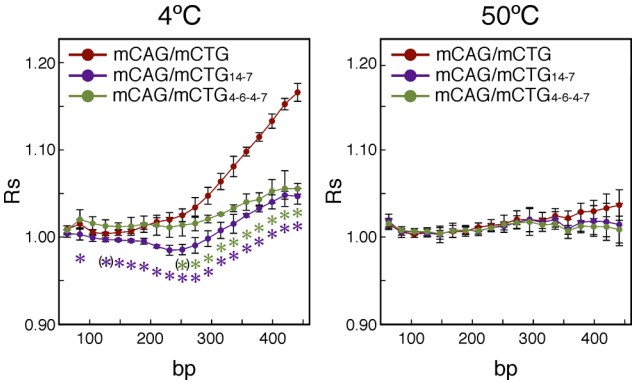

Figure 3. mCAG/mCTG$_{14-7}$ and mCAG/mCTG$_{4-6-4-7}$ multimers show reduced extents of electrophoretic anomaly compared with mCAG/mCTG multimers. The values represent the means ± SD of triplicate determinations. The mCAG/mCTG$_{14-7}$ and mCAG/mCTG$_{4-6-4-7}$ multimers have ^{me}Cs positioned differently from those in mCAG/mCTG multimers (Table 1). The electrophoretic conditions were the same as those for panels B and C of Figure 1. Data points are indicated using the following colors: red for mCAG/mCTG, purple for mCAG/mCTG$_{14-7}$, and light green for mCAG/mCTG$_{4-6-4-7}$. Data for the mCAG/mCTG multimers are from Figure 2. *P* values were calculated between mCAG/mCTG$_{14-7}$ and mCAG/mCTG multimers and between mCAG/mCTG$_{4-6-4-7}$ and mCAG/mCTG multimers and are shown using the same colors as those indicating fragment species. $^{*}P < 0.05$; $^{(*)}P \geq 0.05$ to <0.1.

Methylation Introduces Distortions into Some CHH Members. The CHH context comprises the following nine sequences: CAA, CAT, CAC, CTA, CTT, CTC, CCA, CCT, and CCC. These sequences in dsDNA generate only hemimethylated products upon methylation. As shown in Figure 4, electrophoretic analyses indicated that long multimers of CAA/TTG, CTA/TAG, CTT/AAG, CTC/GAG, and CCC/GGG have curved DNA conformations; i.e., they showed clear electrophoretic retardation at 4 °C but behaved almost normally at 50 °C (Figure 4). Among these constructs, CAA/TTG and CTT/AAG have periodic $A_2{\cdot}T_2$ or $T_2{\cdot}A_2$ tracts in phase with the DNA helical repeat length of 10.5 bp, which is a sufficient condition for constructing curved DNA structures.[25,33,36−39] Thus, they formed curved DNA structures and showed electrophoretic retardation. However, the reason for the electrophoretic retardation of long multimers of CTA/TAG, CTC/GAG, and CCC/GGG at a low temperature is difficult to understand. This issue will be discussed later.

Upon methylation, the CHH members exhibited two different "phenotypes". For the CAA/TTG, CAT/ATG, CAC/GTG, CTA/TAG, and CTT/AAG multimers, hemimethylation caused or enhanced the retarded migration of the fragments. However, when electrophoreses were performed at a higher temperature (Figure 4), the electrophoretic anomalies almost disappeared and no substantial differences were observed between the methylated fragments and their unmethylated counterparts. Thus, these results suggest that the retarded migration or enhanced retardation was caused by generation of curved conformations or enhancement of previously existing curved DNA conformations. In contrast, for the CTC/GAG, CCA/TGG, CCT/AGG, and CCC/GGG multimers, there was almost no change in electrophoretic behavior compared with that of the unmethylated counterparts,

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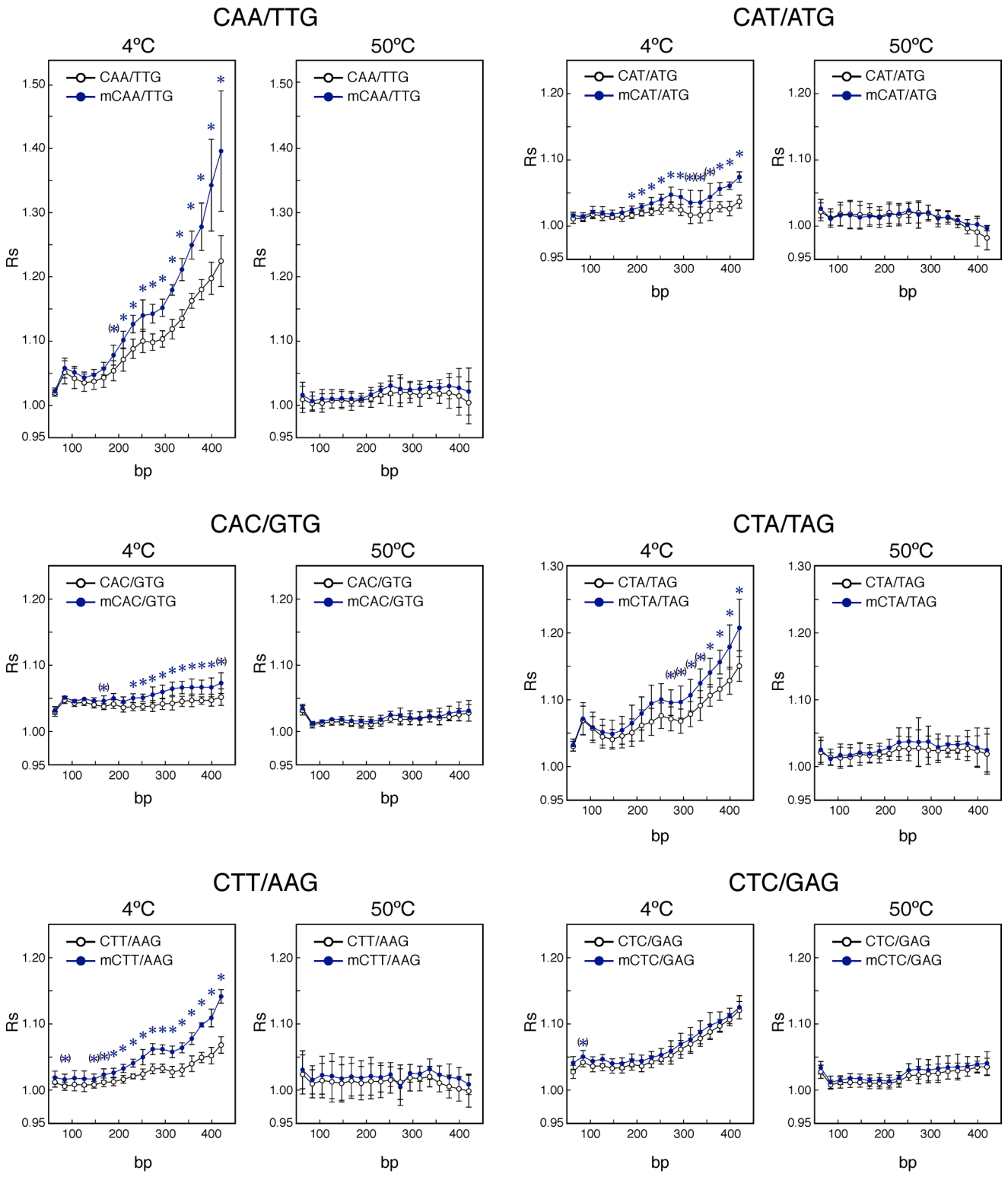

Figure 4. continued

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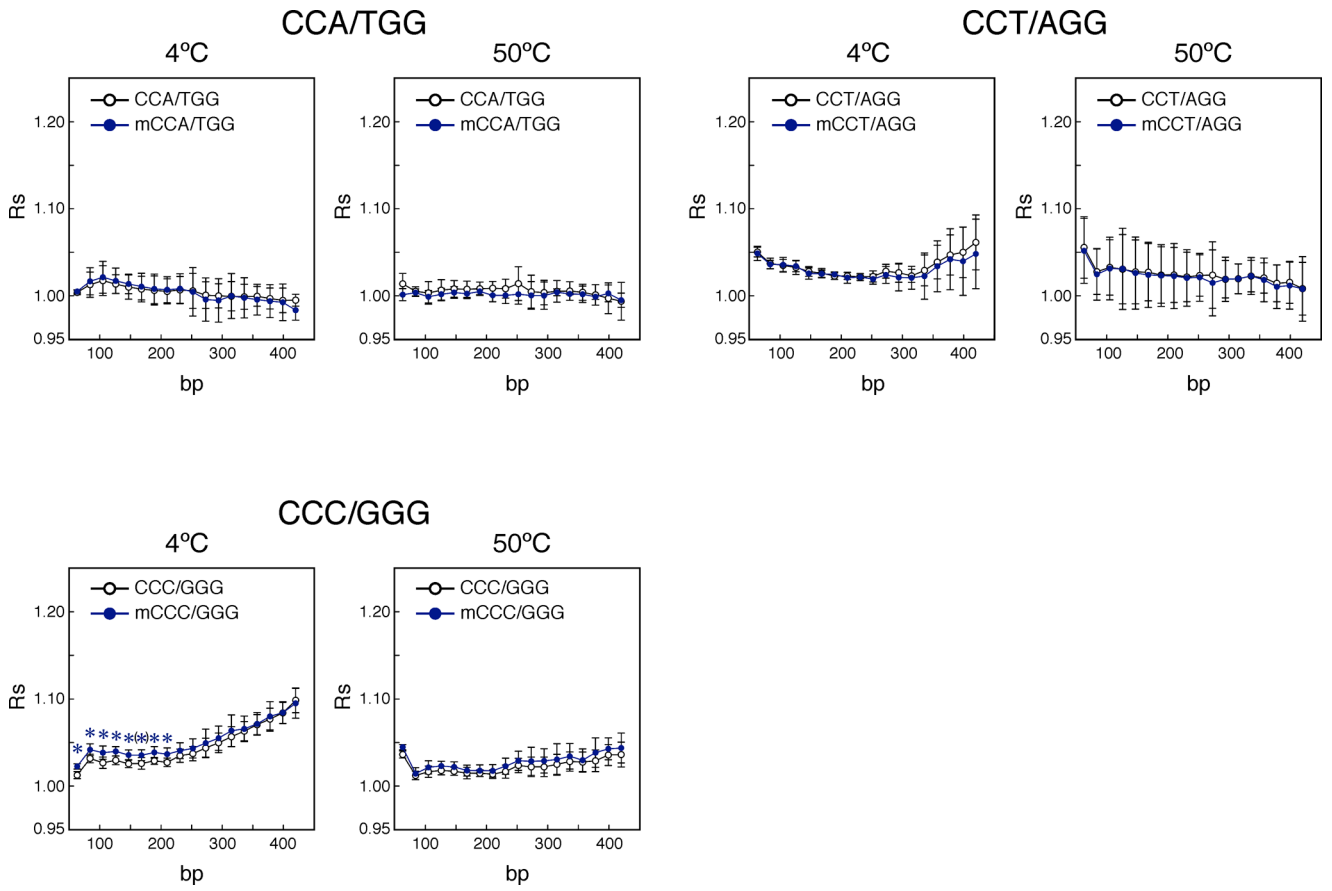

Figure 4. Electrophoretic behavior of methylated and unmethylated CHH multimers. The values represent the means ± SD of triplicate determinations. The electrophoretic conditions were the same as those for panels B and C of Figure 1. The CHH context generates only hemimethylated products. Data points are indicated using the following colors: white for unmethylated fragments and blue for methylated fragments. *P* values were calculated between the methylated and corresponding unmethylated multimers and are shown using the same colors as those indicating fragment species. $^{*}P < 0.05$; $^{(*)}P \geq 0.05$ to <0.1.

indicating that no substantial conformational change was introduced by methylation.

Frequency of in Vivo Methylation and Conformational Changes in DNA. The CHG and CHH contexts comprise 12 sequences in total, including two contained within CAG/CTG. Electrophoretic analyses suggested that the CAG/CTG, CAA/TTG, CAT/ATG, CAC/GTG, CTA/TAG, and CTT/AAG sequences changed their conformations upon methylation. Therefore, we analyzed the relationship between this phenomenon and the sequence preference for DNA methylation in vivo. The preferred sequences have been reported for humans and *Arabidopsis* by Lister et al.[10,13] At first, as the basis for this analysis, the frequencies of occurrence of the CHG and CHH members on the human and *Arabidopsis* genomes were examined, using the NCBI Build 36/HG18 (for humans) and TAIR 7 (for *Arabidopsis*) databases (Figure 5A). The results of Lister et al. are summarized in Figure 5B (modified presentation). Very interestingly, nearly all of the methylation repertoires in the CHG context and 98% of the repertoires in the CHH context found in human ES cells were those that showed conformational changes upon methylation (Figure 5B). Similarly, most of the methylation repertoires were distortion-induced sequences upon methylation in *Arabidopsis*. These findings suggest that methylation-induced conformational changes in DNA may have some biological function.

DISCUSSION

This study examined the effect of DNA methylation on the conformation and physical properties of DNA. CG/CG methylation resulted in the fast migration of multimers (Figure 1), whereas methylated CAG/CTG, CAA/TTG, CAT/ATG, CAC/GTG, CTA/TAG, and CTT/AAG sequences caused retarded migration of multimers (Figures 2 and 4). These results suggest a change in the physical properties of the DNA and a change in the DNA conformation, respectively. Interestingly, with regard to the CHG and CHH contexts, most of the sequences that are actually methylated in vivo underwent a change in conformation upon methylation (Figure 5).

Intrinsic Structures of the Unit Fragments. Before discussing the change in DNA conformation and the properties associated with methylation, we must discuss the conformation of the unmethylated fragments. Among the fragments generated in this study, the long multimers of unmethylated CAA/TTG, CTA/TAG, CTT/AAG, CTC/GAG, and CCC/GGG are thought to have curved DNA conformations (Figure 4). The CAA/TTG and CTT/AAG multimers have phased $A_2{\cdot}T_2$ and $T_2{\cdot}A_2$ tracts, respectively (Table 1), and each tract occurs repeatedly on the multimers with an average periodicity of 10.5 bp. Thus, these multimers should have curved conformations. However, the other three multimers lack an A_n (or T_n) tract sequence. The retarded migration of the long

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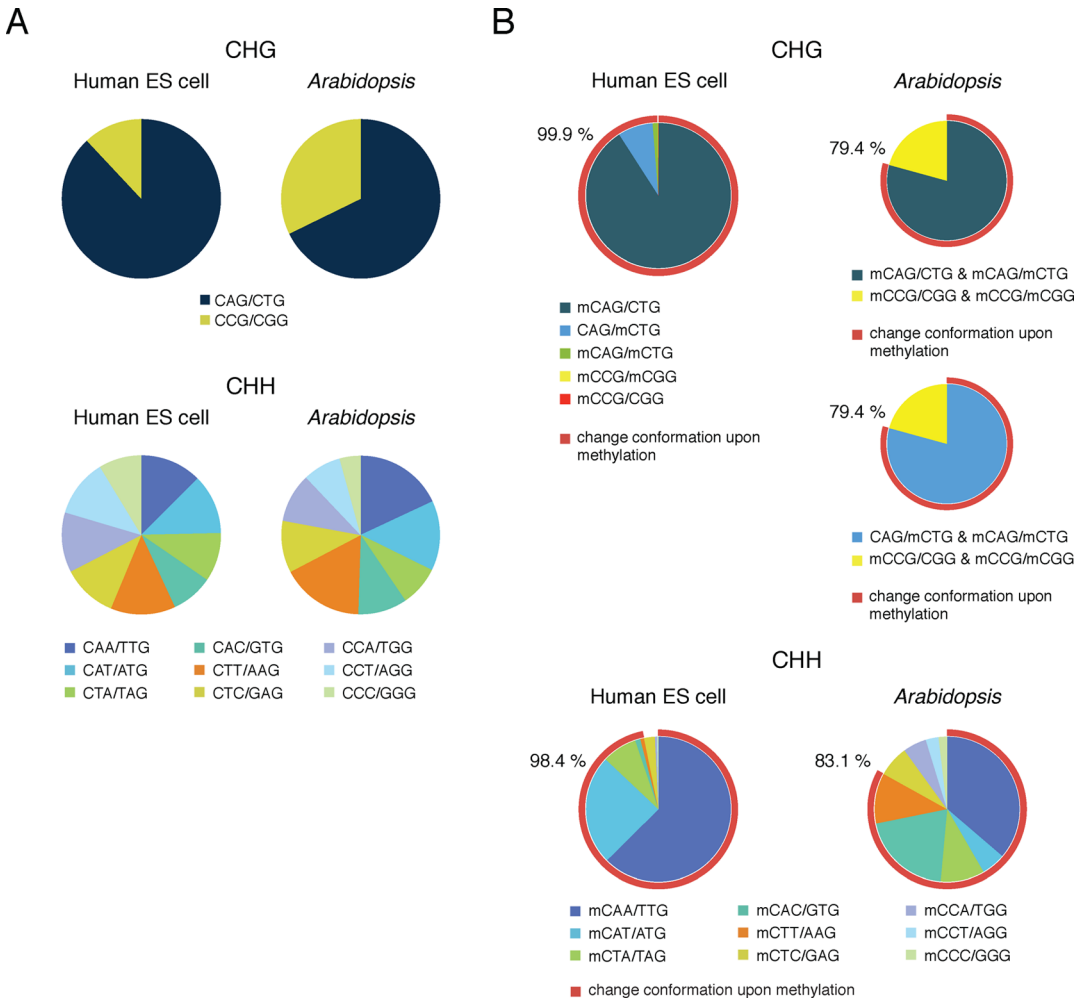

Figure 5. Most methylation target sequences in the CHG and CHH contexts of human ES cells and *Arabidopsis* are those that change their conformations upon methylation. (A) Occupancy ratio (%) of each sequence relative to the total sequences in the CHG or CHH context in the human and *Arabidopsis* genomes. (B) Occupancy ratios (percent) of methylated forms relative to total methylated sequences belonging to the CHG or CHH context. Except for the data for the CHG context of *Arabidopsis*, each percentage for the methylation repertoire was obtained from Lister et al.[10,13] For the *Arabidopsis* CHG context, only the percentages for the data sets could be obtained.[13] The sequences that undergo changes in their conformations upon methylation are shown using red arcs placed outside of the pie charts, along with their percentages.

multimers may be explained, as follows. The extent of retarded migration observed for curved DNAs usually depends on the polyacrylamide concentration in the gel and increases as the concentration is increased.[30,31,33−35] This study used 12.5% gels to enhance the sensitivity of the gel analyses. Thus, these experimental conditions and the very long repeats of the unit fragments may have allowed detection of minor conformational distortions "hidden" in the CTA/TAG, CTC/GAG, and CCC/GGG fragments. Furthermore, it seems that CG/CG (Figure 1), CCG/CGG (Figure 2), CAT/ATG, CAC/GTG, and CCT/AGG (Figure 4) also have such minor distortions within their unit fragments.

Methylation of CpG Increases DNA Rigidity, and That of CHG and CHH Induces DNA Bends in Several Cases. Using cyclization kinetics experiments and Monte Carlo simulations, Nathan and Crothers examined the difference between methylated and unmethylated DNAs with CG sequences (Table 1) and concluded that methylation reduces the bending flexibility and induces underwinding of the methylated nucleotides.[16] The first part of this conclusion agrees with the results obtained from analyses using solid-state nuclear magnetic resonance,[40] molecular dynamics calculations,[41] and Fourier transform infrared spectroscopy.[42] Furthermore, more recently, molecular dynamics simulations suggested that methylation makes CG steps stiffer, especially in terms of roll and tilt deformations.[43]

The mCG/mCG, mCG/CG, and CG/mCG multimers longer than ~200−250 bp showed faster migration than their unmethylated counterparts (Figure 1). Such a migration tendency was also observed for CCG/mCGG long multimers, although the anomaly was extremely small (Figure 2). At present, we cannot clearly understand why the effect of methylation on CCG/CGG is very subtle. One possible reason is a neighboring sequence effect. This putative effect is also seen in the different behavior between mCG/CG and CG/mCG multimers (Figure 1A). However, this issue needs to be investigated further. Fast migration in electrophoresis is reportedly caused by increased rigidity of the DNA.[30,32] Thus, the results indicate that these multimers are endowed with rigidity by methylation, agreeing well with the previous studies described above. Hodges-Garcia and Hagerman[19] found no substantial anomaly in the electrophoretic behavior of 5′-GGCCA^{me}CGTGACCTGA^{me}CGTAC-3′ (only the Watson strand is shown) (Table 1) multimers in 9% nondenaturing

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polyacrylamide gels at room temperature and speculated that the methylation effect was too subtle to be detected using a mobility shift assay. Considering that the major difference in the electrophoretic conditions between the current study [9% gels, 4 °C (Figure 1A)] and the study by Hodges-Garcia and Hagerman was the temperature, this parameter seems to be very important for the electrophoretic detection of DNA rigidity.

Methylation-induced or -enhanced retarded migration of DNA fragments was observed for CAG/CTG, CAA/TTG, CAT/ATG, CAC/GTG, CTA/TAG, and CTT/AAG (Figures 2 and 4). There has been no previous report of the effects of methylation on CAG/CTG and CTA/TAG sequences. However, for the other four sequences, a similar study was performed by Hodges-Garcia and Hagerman.[19] The unit DNA fragments are listed in Table 1. The CAT/ATG and CTT/AAG fragments in our study have sequences similar to the corresponding unit fragments in their study. For CAT/ATG, almost the same effect of methylation was found between the two studies, but for CTT/AAG, the effects of methylation were different: a clear effect was detected in our study (Figure 4), while almost no effect was detected in the earlier study, in which electrophoreses were performed at room temperature using 9% gels.[19] This difference seems to be due to the electrophoretic conditions, such as polyacrylamide gel concentrations and temperatures. To confirm this, we also performed electrophoretic analyses using 9% gels at 4 and 25 °C. In the 25 °C experiment, the difference in the electrophoretic behavior between unmethylated and methylated CTT/AAG fragments became very slight (but did not disappear) (Figure S3 of the Supporting Information). In conclusion, we think that our electrophoretic conditions could allow the detection of methylation-induced conformational changes occurring on CTT/AAG fragments.

The sequences of the CAA/TTG and CAC/GTG fragments in this study also differed from those of the corresponding fragments of 5′-^{me}CAAAAACACA-3′ (here, we refer to this as #1), 5′-^{me}CAAAAACGCG-3′ (#2), 5′-CAAAAA^{me}CACA-3′ (#3), and 5′-CAAAAACA^{me}CA-3′ (#4) used by Hagerman's group (Table 1). Multimers #2 and #3 and the corresponding multimers in our study showed the same behavior. However, multimers #1 and #4 behaved slightly differently from the corresponding multimers in our work, because these multimers migrated slightly faster than their parental unmethylated counterparts.[19] Hagerman's group used the ratio of the mobilities of the methylated species relative to their unmethylated counterparts to detect DNA conformational changes caused by methylation. Therefore, conformational changes induced by methylation would have enhanced or reduced the extent of retarded migration of the multimers. This is because the multimers of unmethylated fragments #1−#4 all formed curved conformations. It is known that when the planarity of a given curvature is enhanced, the extent of retarded migration is also enhanced, but when it is reduced, the extent of retarded migration is reduced.[44] Thus, the essence of the data from Hagerman's group is that methylation introduced local distortions into the DNA, which agrees well with our current observations. Finally, we note that the data profiles of the mCCC/GGG and corresponding CCC/GGG multimers in Figure 4 suggest that conformational changes occurred upon methylation, in agreement with Diekmann.[18] However, we did not include this sequence in the "conformation-changed" group, because the difference between the profiles was slight compared with those of other members of this group.

Biological Relevance of Methylation-Induced Changes in the Properties and Conformations of DNA. This study showed that methylation in the CG context increases DNA rigidity and suggests that methylation introduces subtle distortions into CAG/CTG, CAA/TTG, CAT/ATG, CAC/GTG, CTA/TAG, and CTT/AAG sequences. Interpretation of this result in terms of the context and in vivo frequency of methylation led to several interesting observations. In the CG context, methylation induces property changes; in the CHG context, nearly all (human ES cells) or ∼80% (*Arabidopsis*) of the in vivo methylated sequences are those that undergo a change in conformation upon methylation, and in the CHH context, 98% (human ES cells) or 83% (*Arabidopsis*) of in vivo methylated sequences are those that undergo a change in conformation upon methylation (Figures 1 and 5B). The conformational and mechanical properties of DNA influence nucleosome positioning and the manner in which DNA is organized in chromatin.[21] DNA methylation and histone modifications are considered to affect chromatin density.[10,45] In addition, a recent study suggested that CpG methylation may generate a more tightly wrapped nucleosome structure.[46] Chromatin density and nucleosome properties should influence the accessibility of relevant genomic regions and modulate genetic events.[45] Finally, we hypothesize that the methylation-induced properties or conformational changes in DNA may facilitate nucleosome formation, which provides the essential mechanism for alterations of chromatin density.

■ ASSOCIATED CONTENT

Supporting Information

Figures S1−S3. This material is available free of charge via the Internet at http://pubs.acs.org.

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