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Regulation of DNA Methylation Using Different Tensions of Double Strands Constructed in a Defined DNA Nanostructure

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Abstract: A novel strategy for regulation of an enzymatic DNA modification reaction has been developed by employing a designed nanoscale DNA scaffold. DNA modification using enzymes often requires bending of specific DNA strands to facilitate the reaction. The DNA methylation enzyme *Eco*RI methyltransferase (M.*Eco*RI) bends double helix DNA by 55°−59° during the reaction with flipping out of the second adenine in the GAATTC sequence as the methyl transfer reaction proceeds. In this study, two different double helical tensions, tense and relaxed states of double helices, were created to control the methyl transfer reaction of M.*Eco*RI and examine the structural effect on the methylation. We designed and prepared a two-dimensional (2D) DNA scaffold named the "DNA frame" using the DNA origami method that accommodates two different lengths of the double-strand DNA fragments, a tense 64mer double strand and a relaxed 74mer double strand. Fast-scanning atomic force microscope (AFM) imaging revealed the different dynamic movement of the double-strand DNAs and complexes of M.*Eco*RI with 64mer and 74mer double-strand DNAs. After treatment of the double strands in the DNA frame with M.*Eco*RI and the subsequent digestion with restriction enzyme *Eco*RI (R.*Eco*RI), AFM analysis revealed that the 74mer double-strand DNA was not effectively cleaved compared with the 64mer double-strand DNA, indicating that the methylation preferentially occurred in the relaxed 74mer double-strand DNA compared with that in the tense 64mer double strand. Biochemical analysis of the methylation and specific digestion using a real-time PCR also supported the above results. These results indicate the importance of the structural flexibility for bending of the duplex DNA during the methyl transfer reaction with M.*Eco*RI. Therefore, the DNA methylation can be regulated using the structurally controlled double-strand DNAs constructed in the DNA frame nanostructure.

Introduction

DNA-binding proteins including modifying enzymes first bind to nonspecific sites and diffuse along the DNA to search out specific sequences and then modify the corresponding sites.[1] DNA modification often needs structural changes at the target DNA strands, such as DNA bending, for the reaction to proceed. Among the DNA modifications, DNA methylation plays a central role in maintaining the biological system by protecting it from invasion by external genomes and epigenetic activation/deactivation of the transcription seen in the differentiation and reprogramming of the cell.[2] DNA methyltransferases introduce methyl groups to the C5 and N4 positions of cytosine and the N6 position of adenine in the presence of *S*-adenosyl-L-methionine (SAM).[2] *Eco*RI methyltransferase (M.*Eco*RI) bends double helix DNA by 55°−59° with flipping out of the second adenine in the GAATTC sequence as DNA methylation proceeds.[3,4] DNA bending with M.*Eco*RI was observed at the single-molecule level using DNA−gold nanoparticle conjugates containing a target sequence, which was monitored by measuring the distance between two nanoparticles.[5] From the structural point of view, if the DNA bending is artificially controlled, DNA methylation should be suppressed or promoted. For example, the artificial DNA bending by 30° using a tense alkyl linker connected by a disulfide linkage showed promotion of the binding of HMG-D protein to duplex DNA.[6] If the different tensions of the double helices, tense and relaxed double-strand DNAs, are prepared, the activation or deactivation of the methyl transfer reactions for specific DNA strands can be controlled and examined.

For the preparation of structurally controlled double-strand DNAs, versatile scaffolds have been designed and prepared by utilizing the methods developed in DNA nanotechnology.[7−12] The DNA origami method made possible the construction of

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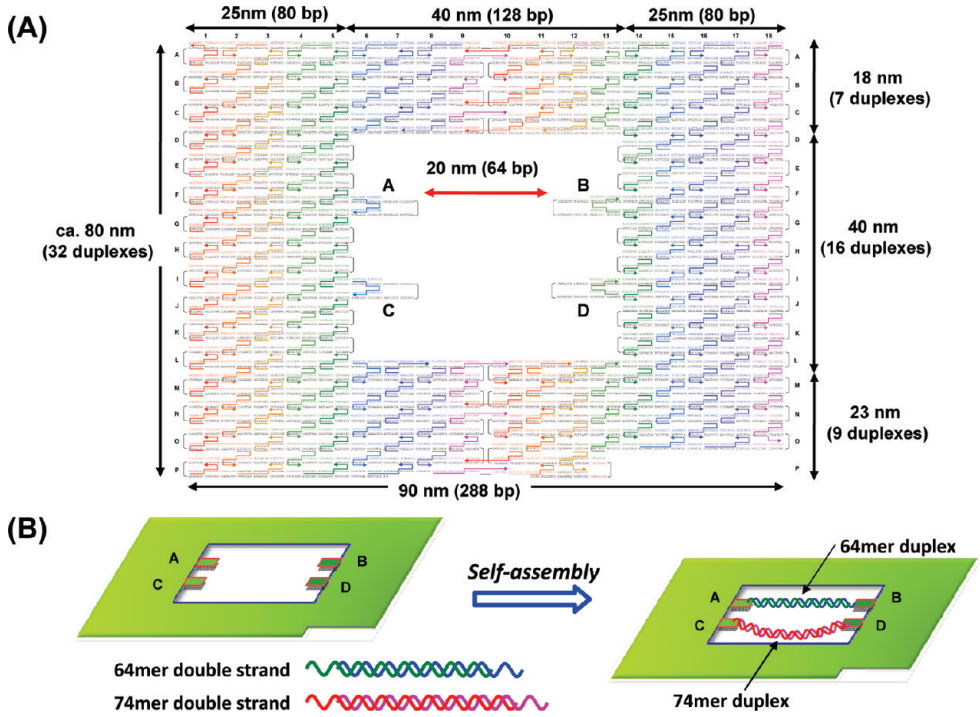

Figure 1. DNA frame structure and the incorporation of double-strand DNAs into the DNA frame. (A) The dimensions of the DNA frame structure with the vacant area inside the frame and the four connection sites A, B, C, and D. (B) Scheme for the incorporation of two different double-strand DNAs by self-assembly using the connection sites.

addressable two-dimensional (2D) and three-dimensional (3D) scaffolds for the production of complicated patterns and for placing and arranging functional molecules and nanomaterials.[13−16] Selective positioning of the functional molecules and nanoparticles has been achieved on the DNA origami scaffold system.[17] The method is valuable for the preparation of predesigned 2D DNA structures, which leads to the defined assembly of middle-sized nanostructures.

Here we report the creation of two different sets of double helical tensions, tense and relaxed states of double helices, to examine the effect on DNA methylation by M.*Eco*RI using these structurally controlled duplex DNAs. To prepare the two duplex DNA states, we designed and prepared the 2D DNA nanostructure, named the "DNA frame," using the DNA origami method. A DNA frame (80 nm × 90 nm) is a rectangle with a vacant rectangle area (40 nm × 40 nm) inside, in which four connection sites were introduced for hybridization of the two different double-strand DNAs (Figure 1A). The length of the double strands fitted in the DNA frame is 64mer double-strand DNA, which corresponds to the same length of the duplex parallel to the frame (Figure 1B). We also introduced a 74mer double-strand DNA, which can allow bending by 60° at the target sequence. We introduced 64mer and 74mer double strands into the frame, meaning that the 64mer double-strand DNA just fitted as a tense strand in the frame, while the 74mer double strand is a more relaxed duplex that can allow bending of the double-strand DNAs. Using these tension-controlled double strands in the DNA frame, we intended to control the DNA methylation and examine the effect of the DNA bending on the methylation reaction.

Results and Discussion

DNA Frame Formation and Incorporation of Double-Strand DNAs. DNA frame formation was carried out using M13mp18 single-stranded DNA and sequence-designed DNA strands (staple strands) in a buffer containing Tris buffer (pH 7.6), EDTA, and Mg^{2+}. The mixtures were annealed from 85 to 15 °C by decreasing the temperature at a rate of 1.0 °C/min. After annealing, the DNA structures were observed by a fast-scanning atomic force microscope (AFM) in the same buffer solution.

AFM images of DNA frame structures are shown in Figure 2. After self-assembly by annealing, the predesigned shape of DNA frame structure presented in Figure 1A was observed (Figure 2A, B), and all the imaged structures maintained the

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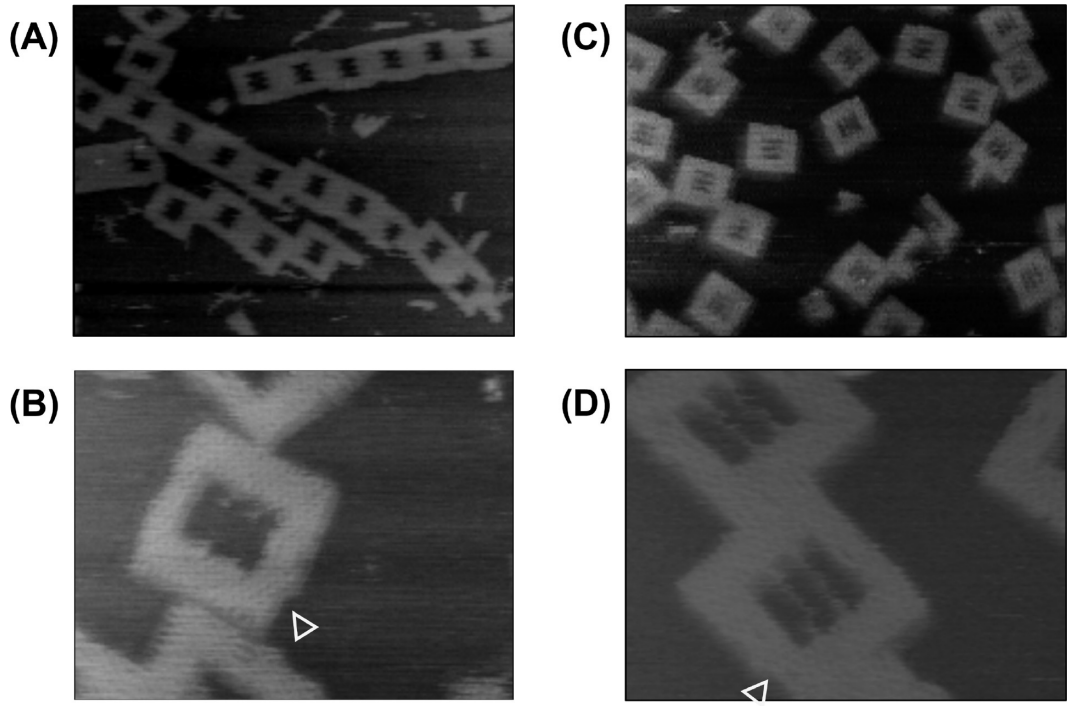

Figure 2. AFM images of the DNA frame and the DNA frame having two double-strand DNAs. (A, B) AFM image of self-assembled DNA frame and the expanded image. (C, D) AFM image of DNA frame carrying two different double-strand DNAs and the expanded image. A triangle in the figure shows a right-bottom lacking corner of the DNA frame (orientation marker). Image size: (A), (C) 1000 nm × 750 nm; (B), (D) 300 nm × 225 nm.

designed shapes. Most of the DNA frames further assembled as extended structures, in which multiple frames connected by π-stacking interactions. The size of the outside edges of the DNA frame structure were 107 ± 5 nm × 101 ± 4 nm, which are a little bit larger than the expected size (90 nm × 80 nm). Four connection sites were observed in the inside vacant area, and the dimensions were 45 ± 3 nm × 43 ± 3 nm, which are also slightly larger than the original design (40 nm × 40 nm). The positions of the four connection points were identified by the absence of the corner of the right bottom side of the DNA frame (orientation marker).

To introduce further double-strand DNAs into the DNA frame, we assembled the two different double-strands. A tense 64mer double-strand and a relaxed 74mer double-strand were introduced between the connection sites A−B and C−D, respectively. To selectively hybridize these strands into the connection points, we introduced corresponding 16mer single strands to these strands, which have an overhang at both 5′-sides (Table S1). Hybridization of the two duplexes was achieved in a solution containing the DNA frame and two duplexes (2 equiv) by heating at 40 °C and then cooling to 15 °C at a rate of 1.0 °C/min. After hybridization, AFM images were obtained in the same buffer solution. Using this hybridization condition, two double-strand DNAs were incorporated into the DNA frame, and 95% of the frames were observed as a two-duplex attached form and 5% as a one-duplex attached form (113 frames analyzed) (Figure 2C, D). These results indicate that the two duplexes are long enough to fit into these connection sites and are easily incorporated into the frame structure by sequence-selective hybridization.

Fast-Scanning AFM Imaging of Double-Strand DNAs and M.*Eco*RI Binding. Detailed analysis of the tense 64mer double-strand and relaxed 74mer double-strand DNAs were performed using fast-scanning AFM imaging, which can successively acquire one AFM image per second.[18] Dynamic movement of the duplexes was observed by successive scanning. Images of a typical strand movement in both 64mer and 74mer double-strand DNAs are shown in Figure 3A. Dynamic movement of the 74mer double strand was observed in the DNA frame, while the movement of the 64mer double strand was modest. This result indicates that the 74mer double strand had more freedom of movement compared with the 64mer double strand. From the trajectory of the 74mer double-strand DNA, the center of the duplex moved in a radius of <7 nm. The results also indicate that the 74mer double-strand can bend ∼60° in the DNA frame nanostructure.

The binding of M.*Eco*RI to the double-strand DNAs in the DNA frame was observed by AFM. The DNA frame was placed on the mica surface in the buffer, the mica plate was rinsed by the buffer to remove excess staples and specific double strands, and then M.*Eco*RI solution was applied onto the mica plate. The binding of M.*Eco*RI to the double strand in the DNA frame was observed, and the height of the M.*Eco*RI binding site was 3.6 nm, which was 2.5 nm higher than that of the double strands (1.1 nm) (Figures 3B, 3C, and S2). The binding of M.*Eco*RI to the duplexes was 26% of the DNA frame, which is roughly consistent with the reported K_d value (43 nM) of M.*Eco*RI at the concentration of 10 nM DNA frame (108 frames analyzed)

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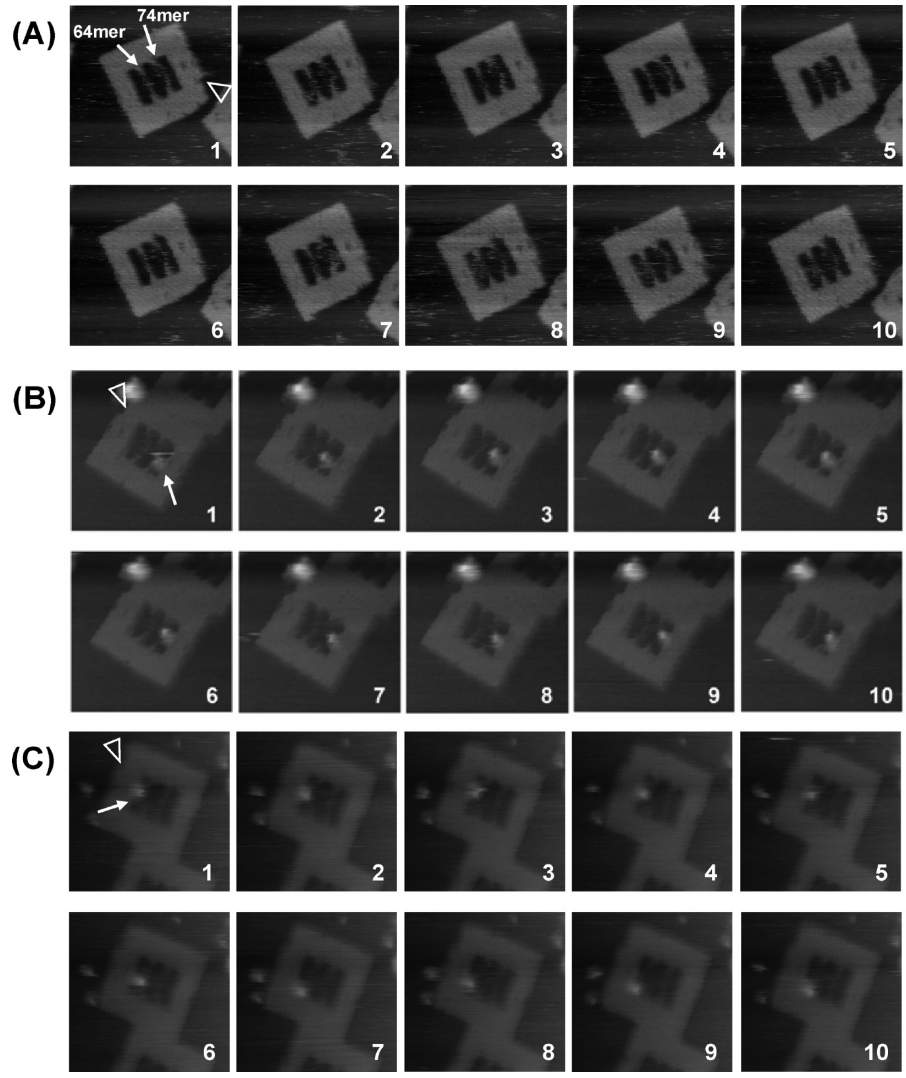

Figure 3. Successive fast-scanning AFM images of two double-strand DNAs in the DNA frame and M.*Eco*RI binding to the double strands in the DNA frame. One image was obtained every 1 s. (A) 64mer and 74mer double strands in the DNA frame. (B, C) Single M.*Eco*RI binding to the 64mer and 74mer double strands in the DNA frame, respectively. An arrow in the figure indicates M.*Eco*RI. A triangle in the figure shows a right-bottom lacking corner of the DNA frame (orientation marker), and 64mer and 74mer represent the 64mer and 74mer double-strand DNAs, respectively. The numbers in the figures are the lapsed times (s) of the successive images. Image size 225 nm × 225 nm.

(Figure S3).[19] The ratios of the M.*Eco*RI binding for the 64mer and 74mer double strands were 13% and 87%, respectively, indicating the preferential binding to the 74mer double strand (Figures 3B, 3C, and S3). The reason for these observations is that the 74mer double strand may more easily accommodate the structural change induced by the binding of M.*Eco*RI to the duplex.

M.*Eco*RI binding to the DNA strand was directly observed by fast-scanning AFM. Successive images were obtained as one image for 1 s. When M.*Eco*RI bound to the 74mer double strand, the movement of the single M.*Eco*RI was observed in the DNA frame (Figure 3C). The distance of the movement from the center of the connectors C and D in the frame was <26 nm, indicating that the 74mer double strand is flexible enough for M.*Eco*RI to smoothly move on the duplex DNA. On the other hand, when M.*Eco*RI bound to the 64mer double strand, the movement of the M.*Eco*RI was relatively small (Figure 3B). The distance of the movement of M.*Eco*RI on the 64mer double strand from the center of the connectors A and B in the frame was <6 nm. These results indicate that the 74mer double strand is flexible enough to change the duplex structure, unlike the 64mer double-strand, when M.*Eco*RI bound to the duplexes in the DNA frame.

Effect of Tensions of Double-Strand DNAs in the DNA Frame on DNA Methylation. We next examined the M.*Eco*RI methylation and R.*Eco*RI cleavage to identify DNA methylation in the DNA frame by AFM imaging. Methylation of the tense 64mer double-strand and relaxed 74mer double-strand DNAs in the DNA frame was performed by treatment with M.*Eco*RI in the presence of *S*-adenosyl-L-methionine (SAM). After successive treatment with R.*Eco*RI, the methylation was analyzed by observing the preserved double-strand DNAs by AFM. When the target sequence was methylated, the sequence is not cleaved by R.*Eco*RI, indicating that the DNA strand in the DNA frame is preserved. On the other hand, when the target sequence is not methylated, the double-strand DNA cleaved by R.*Eco*RI should have remained in the DNA frame. The DNA frames after treatment with enzymes were analyzed by AFM (Figure 4). First, R.*Eco*RI completely cleaved both DNA strands in the frame under our experimental conditions (Figure S4). Although

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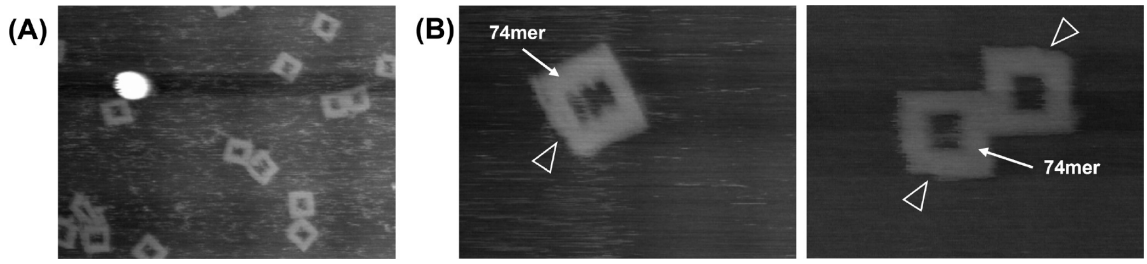

Figure 4. AFM images of DNA double-strand cleavage in the DNA frame after treatment with M.*Eco*RI followed by R.*Eco*RI. Wide image (A) and expanded images (B) of the reaction products. A triangle represents an orientation marker in the DNA frame, and 74mer represents the 74mer duplex DNA. Image sizes: (A) 1000 nm × 750 nm; (B) 500 nm × 375 nm.

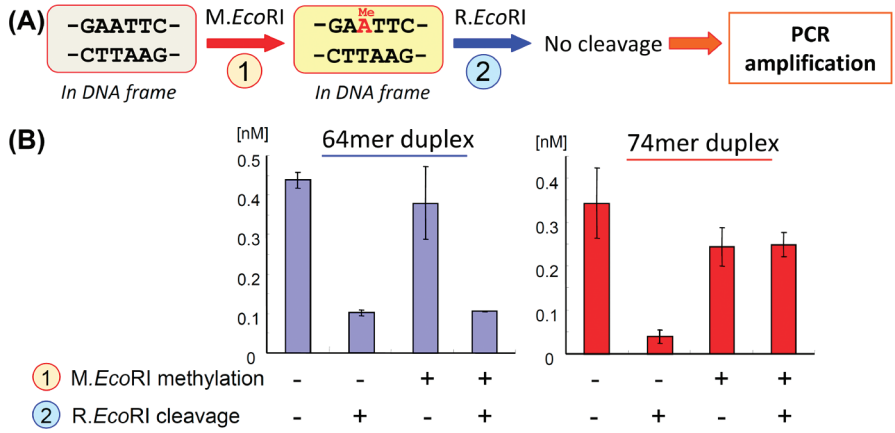

Figure 5. Quantification of the methylation reaction with M.*Eco*RI in the DNA frame. (A) Scheme for the method for the biochemical analysis of the methylation. Two DNA strands were treated with M.*Eco*RI in the first step and then treated with R.*Eco*RI in the second step. Finally, the specific sequence of the samples was quantified by real-time PCR. (B) The results of real-time PCR for the estimation of the initial concentration of the 64mer and 74mer double strands in the DNA frame after treatment with enzymes. Without M.*Eco*RI treatment, R.*Eco*RI cleavage gave a clear difference in the results (compare the two left bars). The data were collected from three independent experiments. Primers used for the real-time PCR are listed in Table S2.

R.*Eco*RI needs unwinding of the duplex by 25° for binding,[20] both strands may unwind like a usual duplex. Second, after treatment with M.*Eco*RI, no cleavage of the duplexes in the DNA frames was observed (Figure S5). Finally, after reaction with M.*Eco*RI and subsequent treatment with R.*Eco*RI, 43% of the 74mer double strands and 13% of the 64mer double strands were preserved (Figure 4), in which 118 frames were analyzed. The results show that the 74mer double strand was not as effectively cleaved as the 64mer double strand and the relaxed 74mer duplex is a better substrate for methylation of M.*Eco*RI than the tense 64mer duplex.

Biochemical Analysis of the Methylation in the DNA Frame by Real-Time PCR. To examine the effect of the tension of the double-strand DNAs on methylation, we performed the biochemical analysis of the methylation in the DNA frame after gel purification. The tense 64mer and relaxed 74mer double strands in the DNA frame were treated with M.*Eco*RI. Then methylation was analyzed by cleavage with R.*Eco*RI, and the initial concentration of the preserved duplex DNAs was estimated by real-time PCR (Figure 5). The scheme for the analysis of methylation is shown in Figure 5A. In our strategy, when the target sequence is methylated, the sequence is not cleaved by R.*Eco*RI (Figure S5), meaning that the full-sized incorporated duplex DNAs should be amplified by PCR using two specific primers. On the other hand, when the target sequence is not methylated, cleavage of the target duplex DNAs by R.*Eco*RI should result in no amplification of the target duplex DNA by PCR. For this experiment, we employed a gel-purified DNA frame carrying two duplexes to avoid background PCR amplification due to the contamination of the free target double strands (Figure S6). When the purified DNA frame carrying two duplexes were amplified by using the specific primers for 64mer and 74mer strands, target PCR products were obtained from both combinations of primers (Figure S7). When the duplex DNAs were treated with M.*Eco*RI in the absence of the DNA frame, target PCR products were obtained from methylated duplexes, indicating that methylation did not reduce the PCR amplification (Figure S8).

To estimate the initial concentration of the target DNA in the DNA frame by real-time PCR, we performed control experiments for comparison in the presence and absence of M.*Eco*RI and R.*Eco*RI. In the control reactions using 64mer and 74mer double strands in the DNA frame without M.*Eco*RI, both strands were amplified at initial concentrations of 0.34−0.44 nM. On the other hand, after treatment of these strands with R.*Eco*RI, the initial concentration dropped to 0.04−0.10 nM. These results indicate that the R.*Eco*RI cleavage worked to reduce the PCR amplification, which was comparable to the low initial concentration of target double strands. After treatment with M.*Eco*RI, if the target sequence is methylated, subsequent R.*Eco*RI cleavage should be prohibited, resulting in the preserved concentration of target double strands. In the case of the 64mer double strand treated with M.*Eco*RI, the initial concentration of the corresponding strand after R.*Eco*RI cleavage dropped from 0.38 to 0.10 nM. On the other hand, when the 74mer double strand was treated with M.*Eco*RI and further

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cleaved with R.*Eco*RI, the initial concentration remained unchanged at 0.25 nM with and without R.*Eco*RI cleavage. These results indicate that the 74mer double strand in the frame was strongly methylated under this reaction condition.

Bending of the duplex DNA for methyl transfer to the N6-position of the specific adenine is required for the reaction to proceed.[4,5] In our DNA frame system, tensed and relaxed double strands can be created by using the defined length in the nanostructural DNA scaffold. The relaxed 74mer double strand can accommodate M.*Eco*RI to bind and bend the target sequence. On the other hand, the tense 64mer double strand allows binding of the M.*Eco*RI, but this strand is a poor substrate for bending, resulting in poor methylation of the target sequence.

Conclusion

We have designed and prepared a DNA frame nanostructure using the DNA origami method and created two different double helical tensions in the frame structure to control and examine the effect of the tensions on DNA methylation of *Eco*RI methyltransferase. The methylation reaction can be controlled using two structurally fixed types of double-strand DNAs constructed in the DNA frame nanostructure. We have shown the importance of DNA strand relaxation in allowing double helix bending during the methylation reaction of M.*Eco*RI. To the best of our knowledge this is the first demonstration of the role of DNA flexibility on an enzymatic modification reaction. This method can be used for other DNA-modifying enzymes and DNA-repair enzymes that bend the double helix during the enzymatic reaction. In addition, the DNA frame structure could be a versatile scaffold to observe the single-molecule behavior of DNA-binding enzymes, including binding to DNA, searching the specific sequence, catalyzing the reaction substrate, and dissociation from the DNA. The method can be extended to the direct observation of various enzymatic phenomena in the designed nanoscale space.

Materials and Methods

Design of DNA Frame Structure. The DNA sequence design of the DNA frame structure followed the rules of the DNA origami method. The sequence of the M13mp18 single-stranded DNA was used, and the staple strands (most of them are 32mer) were assigned for the formation of the designed DNA frame. The DNA frame lacks the right bottom corner for identification of the orientation of the frame (orientation marker). The sequences and positions of the staple strands are listed in the Supporting Information.

DNA Frame Formation and Introduction of the Double-Strand DNAs. The DNA frame was assembled in a 20 μL solution containing M13mp18 single-stranded DNA (New England Biolabs) 10 nM, staple strands (226 strands) 50 nM (5 equiv), 20 mM Tris buffer (pH 7.6), 1 mM EDTA, and 10 mM $MgCl_2$. The mixture was annealed from 95 to 15 °C at a rate of −1.0 °C/min. Hybridization of the two duplexes was achieved in the solution containing a DNA frame and two duplexes (2 equiv) by heating at 50 °C and then cooling to 15 °C at a rate of 1.0 °C/min using a thermal cycler. The sequences of the staple strands are listed in the Supporting Information.

Fast-Scanning AFM Imaging of the DNA Frame. AFM images were obtained on a fast-scanning AFM system (Nano Live Vision, RIBM, Tsukuba, Japan) using a silicon nitride cantilever (Olympus BL-AC10EGS). The sample (2 μL) was adsorbed on a freshly cleaved mica plate for 5 min at room temperature and then washed three times with the same buffer solution. Scanning was performed in the same buffer solution.

Observation of Binding of the M.*Eco*RI to the Target Double-Strand DNAs in the DNA Frame. The binding of the M.*Eco*RI to the duplexes in the DNA frame was examined in a solution containing *Eco*RI methyltransferase (2 units, New England Biolabs), 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM $MgCl_2$, and 1 mM DTT. The solution mixtures (2 μL) were added onto the DNA frame with two double strands adsorbed on the mica plate, and the mica plate was washed three times using the same buffer to remove the excess M.*Eco*RI. Scanning was performed in the same buffer solution.

Methylation of the Target Double-Strand DNAs in the DNA Frame. A DNA frame having both 64mer and 74mer double strands was purified by 1.0% agarose gel electrophoresis, and the corresponding band was cut and recovered using a Freeze 'N Squeeze DNA gel extraction column (BioRad) by following the manufacture's protocol (Figure S5). The methylation reaction was performed in a 10 μL solution containing the gel-purified DNA frame with two double strands, M.*Eco*RI, 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 0.08 mM SAM at 37 °C for 1.5 h. Then R.*Eco*RI (2 units) was added to the reaction mixture, and the mixture was incubated at 37 °C for 1.5 h.

Quantification of Methylation in the DNA Frame by Real-Time PCR. Real-time PCR and subsequent calculations were performed with a 7300 Real-Time PCR System (Applied Biosystems), which detects the signal emitted from fluorogenic probes during PCR. Real-time PCR was performed with FastStart Universal SYBR Green Master (Roche). The PCR mixture contained 1× FastStart Universal SYBR Green Master, 300 nM forward and reverse primers, and the gel-purified DNA frame carrying two double strands in a total volume of 25 μL. Each 40 PCR cycle was programmed as a 15 s denaturation step at 95 °C and 1 min hybridization step, with probes and primers and target DNA strands at 58 °C. The standard curve was obtained using 500, 100, 20, 4, and 0.8 pM DNA templates. The incorporated double-strand DNAs and primer sequences are listed in Table S2.

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Supporting Information Available: Experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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