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Microarray-based method to evaluate the accuracy of restriction endonucleases *Hpa*II and *Msp*I

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Abstract

A double-strand DNA (ds DNA) microarray was fabricated to analyze the structural perturbations caused by methylation and the different base mismatches in the interaction of the restriction endonucleases *Hpa*II and *Msp*I with DNA. First, a series of synthesized oligonucleotides were arrayed on the aldehyde-coated glass slides. Second, these oligonucleotides were hybridized with target sequences to obtain ds DNA microarray, which includes several types of double strands with the fully methylated, semi-methylated, and unmethylated canonical recognition sequences, semi-methylated and unmethylated base mismatches within the recognition sequences. The cleavage experiments were carried out under normal buffer conditions. The results indicated that *Msp*I could partially cleave methylated and semi-methylated canonical recognition sequences. In contrast, *Hpa*II could not cleave methylated and semi-methylated canonical recognition sequences. *Hpa*II and *Msp*I could both cleave the unmethylated canonical recognition sequence. However, *Hpa*II could partially cleave the sequence containing one GG mismatch and not cleave other base mismatches in the corresponding recognition site. In contrast, *Msp*I could not recognize the base mismatches within the recognition sequence. A good reproducibility was observed in several parallel experiments. The experiment indicates that the microarray technology has great potentials in high-throughput identifying important interactions between protein and DNA.

Keywords: DNA microarray; Restriction endonucleases; Methylation; Single base mismatches

Protein–DNA interaction is an essential event in many biological processes, such as transcription, replication, restriction, and modification. The sequence selectivity of DNA binding proteins plays an important role in controlling these processes in the cell. Understanding how DNA binding proteins to select the correct DNA sequence from the nonspecific sequences has been attracting more and more interest in recent years. Most of the studies have concentrated on crystal structural analysis for proteins bound to their specific DNA sequences [1]. These have increased our understanding in working mechanisms of DNA binding protein on the molecular level, but it is still not sufficient in our knowledge of how specific and nonspecific sequences are distinguished in the cell.

Among DNA binding proteins, restriction endonucleases are perhaps the most extreme in their DNA selectivity [2]. Restriction endonucleases constitute a bacterial defense system against foreign DNA (e.g., bacteriophage DNA) by cleaving it within a defined recognition sequence [3,4]. Their corresponding modification enzymes protect the chromosomal DNA by methylation of the sequence. An essential requirement of restriction enzymes is their high accuracy: cleavage at sites protected by methylation or at sites which deviate from the canonical sequence would induce recombination and repair events which may lead to mutations. Some works have been made in understanding the molecular basis of the specificity of restriction enzymes, mainly due to chemical modification studies, in which the structural elements of the DNA substrates needed for efficient cleavage were identified [5]. These works have further increased our understanding to restriction enzymes. Research on the effects of sequence variations

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of the canonical recognition sites on the cleavage efficiency of restriction endonucleases is important for understanding its specificity. These sequence variations include both a single base pair change and mismatches within the recognition site. The former can result in over a million-fold decrease in activity [6,7], and the latter can impart a local destabilization on a double helix and affect the deformability and dynamic properties of the DNA at the mismatched site [8]. Thielking et al. [9] have shown that oligonucleotides with a mismatch in the *Eco*RI site are cleaved more slowly by *Eco*RI than cognate oligonucleotides (canonical *Eco*RI site) but faster than oligonucleotides with a star site (sequences deviating by one base pair from the canonical sequence). For *Eco*RV, the double-strand cleavage of star substrates is at least 5 orders of magnitude slower than cleavage of the canonical substrate. In contrast, most of the mismatch substrates are accepted more readily [10]. Recently, we evaluated the effect of CA mispairs on the accuracy of restriction endonuclease *Bst*UI [11]. The results showed that *Bst*UI could partially cleave the sequences containing one CA mispair in the corresponding recognition site. In contrast, almost no digestion was obtained for the sequence containing two CA mispairs in its recognition site. In addition, the position of CA mispair within the *Bst*UI recognition site had little effect on the cleavage efficiency of restriction enzymes. However, some of the restriction endonucleases have no ability to catalyze the cleavage of mismatch-containing recognition sites in DNA [12,13]. The above studies provided a new insight into the accuracy analysis of restriction endonucleases, even if there still remain many unclear and controversial results due to the limited experimental data. Therefore, it is very important to develop some useful methods to analyze the accuracy of restriction endonucleases or other DNA binding proteins.

Here, a double-strand DNA microarray method was developed to analyze the accuracy of restriction endonucleases *Hpa*II and *Msp*I. *Hpa*II is a pair of isoschizomers with *Msp*I and they have the same recognition site (CC*GG). However, *Hpa*II is sensitive to mammalian CpG methylation and *Msp*I cannot be blocked by CpG methylation. A series of synthesized oligonucleotides were immobilized on the aldehyde-coated glass slides to fabricate an array. These oligonucleotides can be combined with complementary methylated and unmethylated sequences to obtain a double-strand DNA microarray which includes the canonical methylated and unmethylated recognition sequences and difference base mismatches within the recognition sequences. Then cleavage experiments were carried out under normal buffer conditions on chip. It can be applied to analyze the accuracy and sensitivity of restriction endonucleases by comparing fluorescent intensity change before and after cleavage.

Materials and methods

Preparation of oligonucleotides. The methylated and unmethylated oligonucleotide probes and targets used in this study were synthesized and purified by Shengyou (Shanghai, China) and are summarized in Table 1. The oligonucleotide targets were labeled with Cy3 fluorescence dye (Table 1).

Preparation of aldehyde-coated glass slides. The glass slides were cleaned for 1 h in a solution consisting of 1/3 hydrogen peroxide (30%) and 2/3 sulfuric acid (18 M) [14], rinsed three times in deionized distilled water, left for 10 min in boiling deionized distiller water, and dried under an argon flow. The cleaned glass slides were silanized with 2% of 3-aminopropyltriethoxysilane (Sigma) dissolved in 95% acetone for 2 min, washed twice with acetone, and baked for 45 min at 75 °C. The silanized slides were activated with 5% glutaraldehyde in 0.11 M PBS (pH 7.0) for 2 h, washed thoroughly with distilled water, and dried. The aldehyde-coated slides were stored at 4 °C and used in 15 days.

Manufacture of the single-strand DNA microarray. The probes were suspended in sodium carbonate buffer (0.1 M, pH 9.0) to a final concentration of 80 μM. Approximately 0.05–0.1 pmol of each oligonucleotide was printed in quadruplicate as microdots (100 μm in diameter) on the aldehyde-coated glass slides using a PixSys5500 microarrayer (Cartesian Technology). The distance between two spots (center-to-center) was 300 μm. After spotting, the glass slides were incubated in a humid chamber at room temperature overnight and then at 37 °C for 2 h. The slides were washed thoroughly in 0.1% SDS. After further treatment with a $NaBH_4$ solution for 15 min, the slides were ready for hybridization.

Hybridization of oligonucleotide target to fabricate ds DNA microarray. The labeled targets T1 or T2 were mixed with an equal amount with T3 (control), respectively, and suspended in hybridization solution (containing 35% formamide, 0.5% SDS, 2.5× Denhardt's, and 4× SSPE). The microarray hybridization was conducted in a moist hybridization chamber under a coverslip at 42 °C for 2 h. After hybridization, the slide was rinsed and washed at room temperature with 2× SSC–0.1% SDS and 0.1× SSC–0.1% SDS for a total of 15 min, respectively, and then dried by centrifugation at 600 rpm for 5 min.

The ds DNA microarray has been fabricated after hybridization, which contains 10 types of double strands with the methylated, semi-methylated, and unmethylated canonical recognition sequences (CMG, CSG, and CG), semi-methylated, and unmethylated base mismatches within the recognition sequences (ASG, GSG, TSG, AG,

Table 1
Oligonucleotide probes and target sequences used in this study

Code	Sequences
CMG	5′-NH_2-$(T)_{10}$- TTGAGCAAC<u>CC^{M}GG</u>AGTCTGGA-3′
CG	5′-NH_2-$(T)_{10}$-TTGAGCAAC<u>CCGG</u>AGTCTGGA-3′
AG	5′-NH_2-$(T)_{10}$-TTGAGCAAC<u>C**A**GG</u>AGTCTGGA-3′
GG	5′-NH_2-$(T)_{10}$-TTGAGCAAC<u>C**G**GG</u>AGTCTGGA-3′
TG	5′-NH_2-$(T)_{10}$-TTGAGCAAC<u>C**T**GG</u>AGTCTGGA-3′
C	5′-NH_2-$(T)_{10}$-GTGAGGTCAGATGTGGATCGA-3′
T1	5′-Cy3-TCCAGACTCCGGGTTGCTCAA-3′
T2	5′-Cy3-TCCAGACTCC^{M}GGGTTGCTCAA-3′
T3	5′-Cy3-TCGATCCACATCTGACCTCAC-3′

Underlining indicates the canonical *Hpa*II or *Msp*I recognition sequence. Bold indicates the bases that are different from the canonical recognition sequence. C indicates the negative control, which does not contain *Hpa*II or *Msp*I recognition sequence. T1 or T2 is perfectly matched with CMG or CG. T3 is perfectly matched with C. T1, T2, and T3 are all labeled with Cy3 fluorescence dye. C^M indicates the methylated cytosine. T1 or T2 was mixed with an equal amount with T3 to cohybridize with DNA microarray.

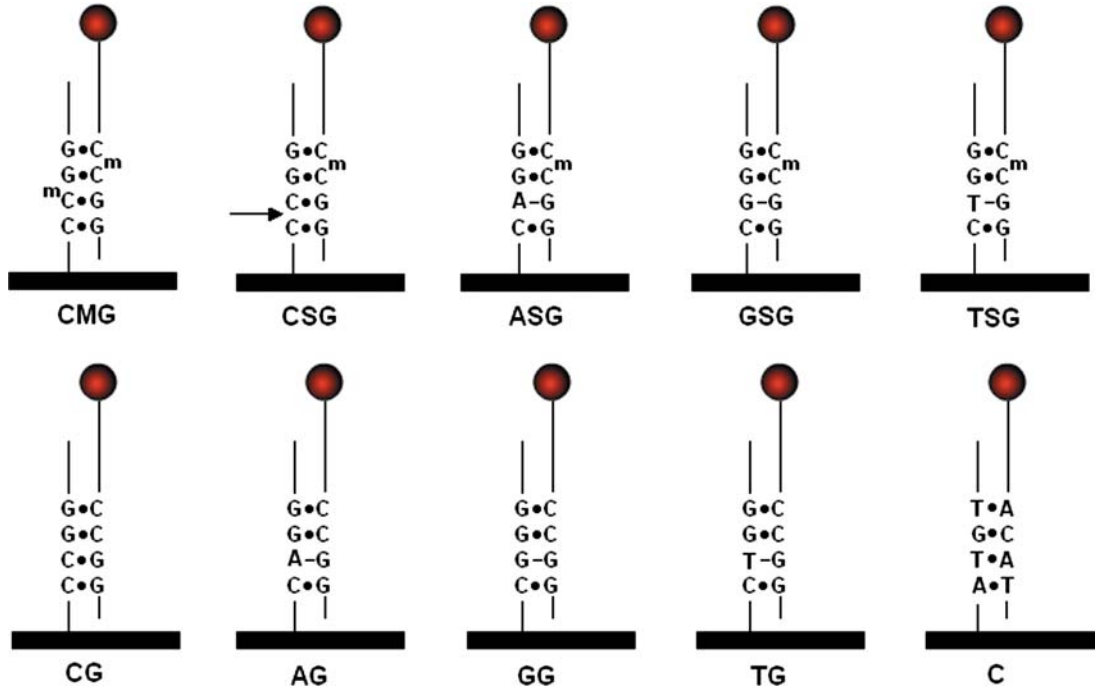

Fig. 1. Ten types of duplexes in DNA microarrays. CMG, CSG, and CG indicate the full methylated, semi-methylated, and unmethylated canonical *Hpa*II or *Msp*I recognition sequences, respectively. ASG, GSG, and TSG indicate the semi-methylated sequences containing the different base mismatches within the recognition site, respectively. AG, GG, and TG indicate the unmethylated sequences containing the different base mismatches within the recognition site, respectively. C indicates negative control not containing the *Hpa*II or *Msp*I recognition sequence. Arrow shows the cleavage position of *Hpa*II or *Msp*I.

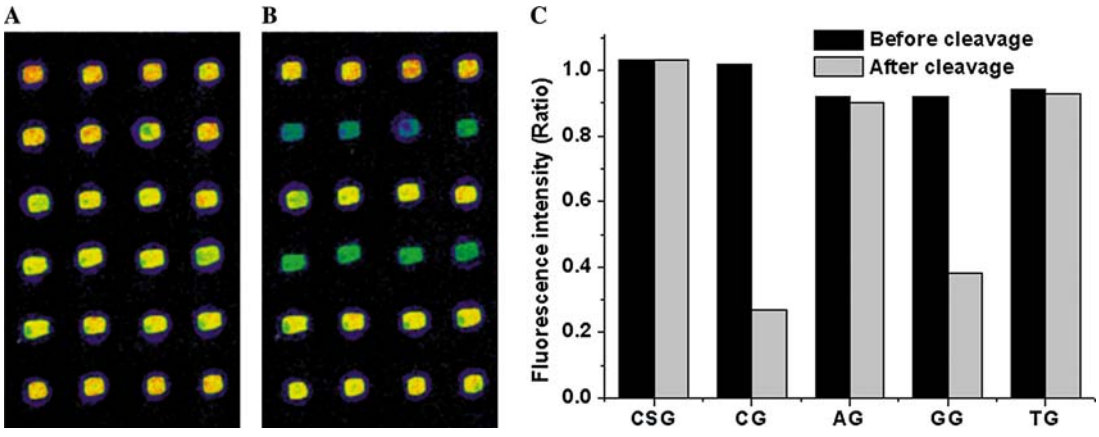

Fig. 3. The fluorescence intensities of ds DNA microarrays with unmethylated target before and after *Hpa*II cleavage. (A) Fluorescence image before cleavage; (B) fluorescence image after cleavage. From top to down, dots are CSG, CG, AG, GG, TG, and C, respectively. The plot of (C) is the fluorescence intensity ratio before and after cleavage.

GG, and TG), and a negative control not containing *Hpa*II or *Msp*I recognition sequences (Fig. 1). The aim of negative control was to compare the signal change between before and after cleavage.

Cleavage experiments on the ds DNA microarray. The same slides were incubated with 30 U *Hpa*II or *Msp*I in 20 μl of 10 mM Tris–HCl, pH 8.5; 10 mM $MgCl_2$; 100 mM KCl; and 0.1 g/L BSA for 6–12 h at 37 °C, respectively. After cleavage, the slide was rinsed and washed at room temperature with 2× SSC–0.1% SDS and 0.1× SSC–0.1% SDS for a total of 15 min, respectively, and then dried by centrifugation at 600 rpm for 5 min. The cleaved slides were scanned and the fluorescence intensities were computed for each spot. The fluorescence intensity ratios were calculated by comparing the fluorescence signals before and after cleavage.

Image scanning and data processing. The slides were scanned with a ScanArray Lite Microarray Analysis Systems (A Packard BioScience Company, USA) before and after cleavage. The images acquired by the scanner were analyzed with software Genepix Pro 3.0. Each spot was defined by the positioning of a grid of circles over the array image. For each fluorescent image, the average pixel intensity within each circle was determined and a local background using mean pixel intensity was computed for each spot. The net signal was determined by subtraction of this local background from the mean average intensity for each spot.

Results and discussion

Hybridization of unmethylated target T1 to DNA microarray

The fluorescence signals are shown in Figs. 2A and B, reflecting the fluorescence intensity of each spot. The average intensities of CSG, CG, AG, GG, TG, and C were calculated from the four replicate spots, respectively. Then the intensity ratios of CSG/C, CG/C, AG/C, GG/C, and TG/C were calculated in order to compare the signal change before and after cleavage.

Figs. 2A and B display that there were no significant differences for ratios of CSG/C, CG/C, AG/C, GG/C, and TG/C before and after incubation when *Hpa*II or *Msp*I was not included in the reaction buffer. The fluorescence images were not shown. The results indicated that the duplexes in Fig. 1 were stable under the experimental condition. Fig. 3 displays that there were significant differences for ratios of CG/C and GG/C before and after *Hpa*II cleavage. The ratios of CG/C and GG/C before cleavage were 3.8 and 2.4 times higher than those after cleavage, respectively. There were no significant differences for ratios of CSG/C, AG/C, and TG/C. The results showed that *Hpa*II could partially cleave the sequence containing one GG mismatch in the corresponding recognition site. In contrast, the digestion could not be obtained for the sequences containing other base mismatches in its recognition site. ^{1}H NMR studies show also that the mismatch GG was well stacked in the helix and the bases remain in an intrahelix orientation [15–18]. The overall order of base-pair lifetimes in the sequence context of the base pair was GC > GG > AA > CC> AT > TT. The mismatch GG had a longer base-pair lifetime relative to other base mismatches. These indicated that mismatch GG had less effect on the conformation of the recognition sequence than other base mismatches. Fig. 3 indicated also that *Hpa*II could not cleave the duplex containing the semi-methylated recognition site. The result indicated that *Hpa*II was not only sensitive to methylation, but also sensitive to semi-methylation. Fig. 4 displays that there were significant differences for ratios of CSG/C and CG/C before and after *Msp*I cleavage. The ratios of CSG/C and CG/C before cleavage were 1.5 and 1.6 times higher than those after cleavage, respectively. There were no significant differences for ratios of AG/C, GG/C, and TG/C. The results indicated that the semi-methylated recognition site had less effect on the cleavage ratio of *Msp*I. However, the cleavage could not be found for the

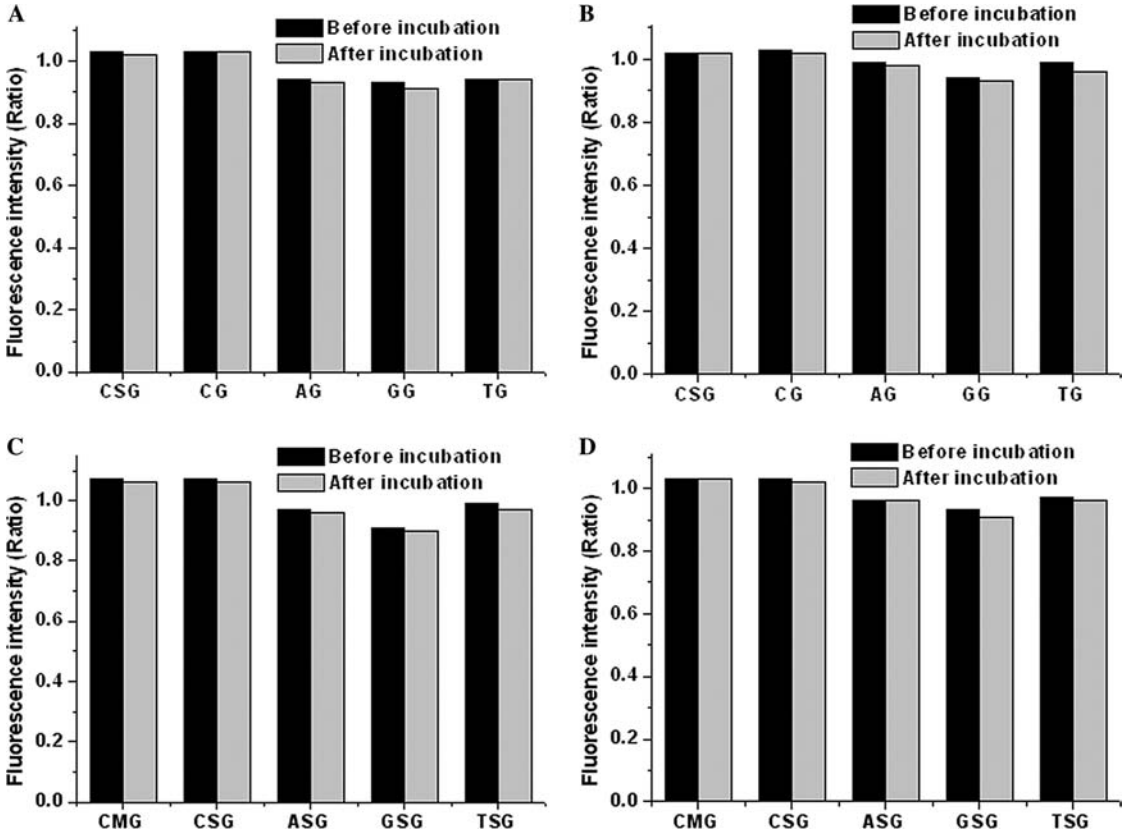

Fig. 2. The fluorescence intensities of ds DNA microarrays with methylated and unmethylated targets before and after enzyme-free incubation. The plots of (A,B) are the fluorescence intensity ratios for the methylated target before and after incubation, respectively. The plots of (C,D) are the fluorescence intensity ratios for the unmethylated target before and after incubation, respectively.

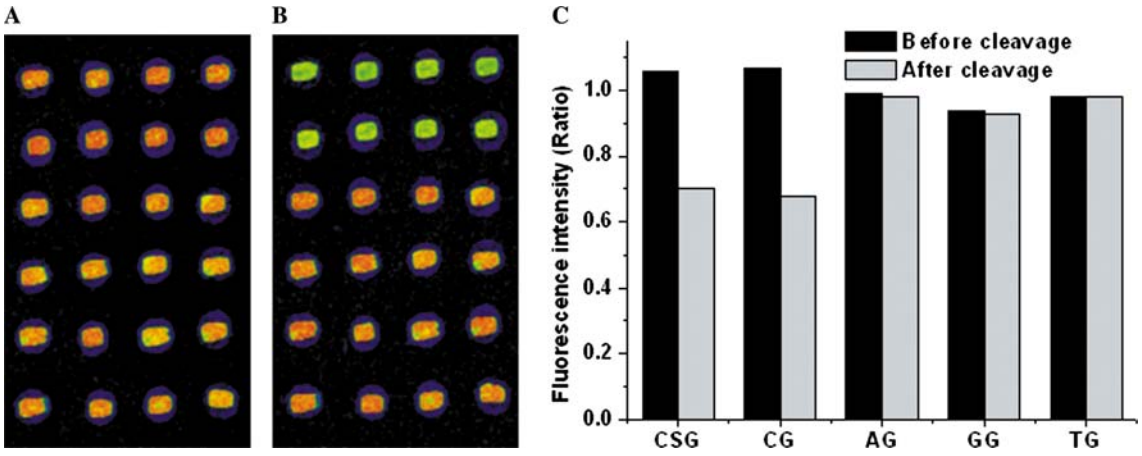

Fig. 4. The fluorescence intensities of ds DNA microarrays with unmethylated target before and after *Msp*I cleavage. (A) Fluorescence image before cleavage; (B) fluorescence image after cleavage. From top to down, dots are CSG, CG, AG, GG, TG, and C, respectively. The plot of (C) is the fluorescence intensity ratio before and after cleavage.

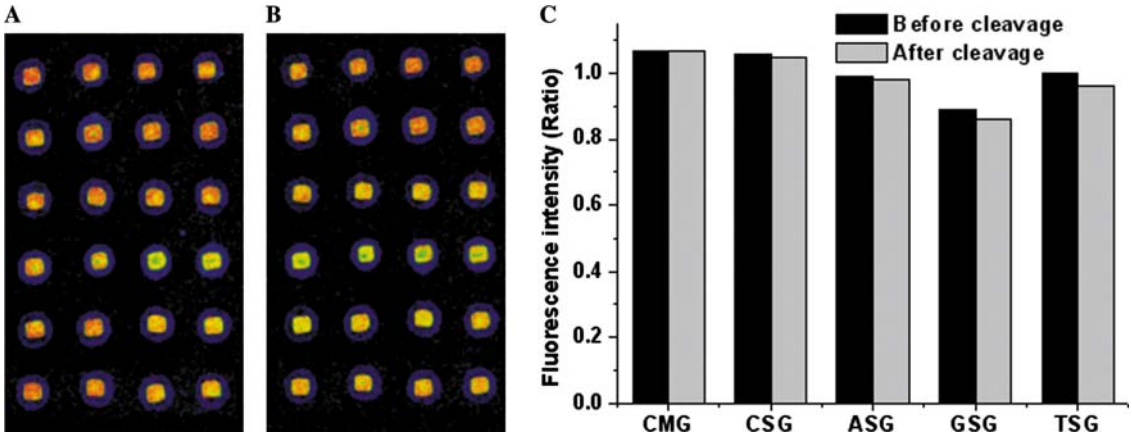

Fig. 5. The fluorescence intensities of ds DNA microarrays with methylated target before and after *Hpa*II cleavage. (A) Fluorescence image before cleavage; (B) fluorescence image after cleavage. From top to down, dots are CMG, CSG, ASG, GSG, TSG, and C, respectively. The plot of (C) is the fluorescence intensity ratio before and after cleavage.

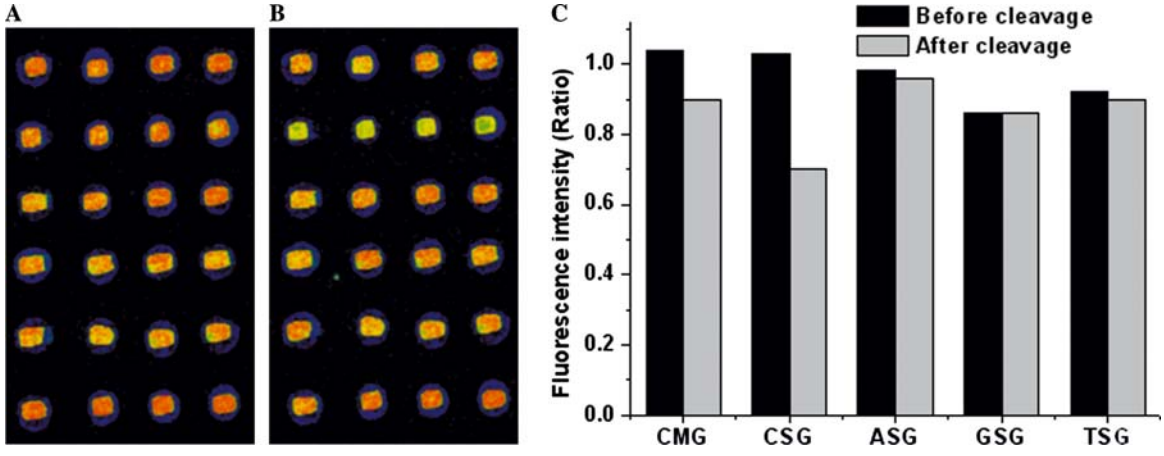

Fig. 6. The fluorescence intensities of ds DNA microarrays with methylated target before and after *Msp*I cleavage. (A) Fluorescence image before cleavage; (B) fluorescence image after cleavage. From top to down, dots are CMG, CSG, ASG, GSG, TSG, and C, respectively. The plot of (C) is the fluorescence intensity ratio before and after cleavage.

sequences containing the base mismatches in its recognition site. *Hpa*II and *Msp*I are a pair of isoschizomers, which have the same recognition sequence. However, they display the differential sensitivity to cytosine methylation. Furthermore, there was significant difference for recognizing base mismatches within the recognition sequence between *Hpa*II and *Msp*I. The former could well recognize the mismatch GG. However, the latter could not recognize any base mismatches.

Hybridization of methylated target T2 to DNA microarray

The fluorescence signals are shown in Figs. 2C and D, reflecting the fluorescence intensity of each spot. The average intensities of CMG, CSG, ASG, GSG, TSG, and C were calculated from the four replicate spots, respectively. Then the intensity ratios of CMG/C, CSG/C, ASG/C, GSG/C, and TSG/C were calculated in order to compare the signal change before and after cleavage. Fig. 5

Figs. 2C and D display that there were no significant differences for ratios of CMG/C, CSG/C, ASG/C, GSG/C, and TSG/C before and after incubation when *Hpa*II or *Msp*I was not included in the reaction buffer. The fluorescence images were not shown. The results indicated that the duplexes in Fig. 1 were stable under the experimental condition. Fig. 3 shows that there were no significant differences for CMG/C, CSG/C, ASG/C, GSG/C, and TSG/C before and after *Hpa*II cleavage. The results indicated that the methylation had remarkably affected the cleavage ratio of *Hpa*II. *Hpa*II could not cleave the duplexes, even though an oligonucleotide was only methylated. It was closely associated with the fact that *Hpa*II was methylation-sensitive enzyme. Fig. 6 displays that there were significant differences for ratios of CMG/C and CSG/C before and after *Msp*I cleavage. However, there were no significant differences for ASG/C, GSG/C, and TSG/C. The ratios of CMG/C and CSG/C were before cleavage 1.2 and 1.5 times higher than those after cleavage, respectively. The results indicated that *Msp*I could cleave the methylated and semi-methylated canonical recognition sequences, but could not cleave the semi-methylated mispairs within the recognition sequences. Moreover, the cleavage ratio of the CMG was lower than that of the CSG. The results indicated that the cleavage ratio of *Msp*I could be partially blocked by the methylation in the recognition site. *Msp*I could not cleave the semi-methylated mispairs within the recognition sequences, which indicates that the base mismatches had large effect on the conformation of the recognition sequence.

Conclusion

The above results are summarized in Table 2. *Hpa*II and *Msp*I are a pair of isoschizomers, whereas they display the differential sensitivity to cytosine methylation and single base mismatches. Among all mismatches, GG and TG are the most stable mismatches [19]. However, there were significant differences for being recognized by *Hpa*II. According to these results, it could be concluded that the presence of one mismatch GG in the recognition site might have little effect on the conformation of either phosphodiester or deoxyribose residues.

The base mismatches of DNA sequence will perturb its double-strand conformation. If we judge from DNA structure alone, it would appear unlikely that the DNA backbone is a major determinant in the recognition process. However, recent reports provided some evidence that the phosphodiester backbone might play more than just a passive role, and its conformation and flexibility may be an important component in protein–DNA interaction [20–22].

The formation of stable mismatched base pairs can occur as a consequence of errors in genetics recombination and replication. If these mispairs are not corrected by the repair enzyme systems, they can lead to mutations, and thus the correction of the mismatched base pairs is necessity to maintain the integrity of the genetic information. Nevertheless, all mismatches are not repaired with the same efficiency: purine–purine mismatches are among the most efficiently repaired whereas the pyrimidine–pyrimidine pairs are not well repaired [23,24]. However, GG mismatches are generally better repaired than AG mismatches. This may be because AG mismatches can adopt conformations that are not recognized by the repair systems.

Our results indicated that two out of the three hydrogen bonds of the CG base pair in the canonical *Hpa*II recognition sequence that characterizes the protein–DNA interaction can still be formed for GG mismatched recognition site. For the duplex containing the GG mismatch, Faibis et al. [15] proposed for the major

Table 2
The result summary of methylation and single base mismatches for the accuracy effect of restriction endonucleases *Hpa*II and *Msp*I

	CMG	CSG	CG	ASG	AG	GSG	GG	TSG	TG
*Hpa*II	–	–	++++	–	–	–	+++	–	–
*Msp*I	+	++	++	–	–	–	–	–	–

(–) indicates the restriction enzyme cannot cleave this duplex; (+) indicates the restriction enzyme can cleave this duplex. The more number of (+) indicates the high level of cleavage.

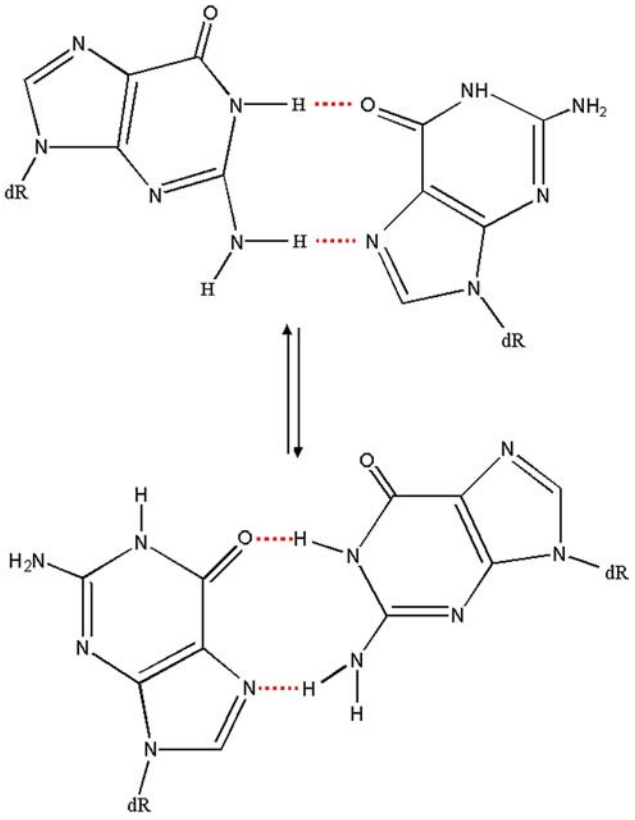

Fig. 7. Structure of the GG mismatch as described in the literature [17,25–27]. Two conformations, with the same donor and acceptor pattern, can coexist, one is obtained from the order by a 180° rotation about the pseudodyadic axis.

species, a type of pairing involving one hydrogen bond between the imino group of one central guanine and the carbonyl group of the opposite guanine. Both bases are in an anti-conformation. Two conformations, with the same donor and acceptor pattern, can coexist, one is obtained from the order by a 180° rotation about the pseudodyadic axis (Fig. 7). Exchange between the two forms is observed from NMR at low temperature. A minor species involving hydrogen bonding between the guanine amino group and the carbonyl group of the guanine on the opposite strand may also exist as shown by the molecular dynamics calculations.

In a word, our experiments successfully demonstrated that the ds DNA microarray could be used to analyze effect of the different mismatches and methylation within recognition site on the accuracy of *Hpa*II and *Msp*I. It has also been shown that the microarray can be used as a powerful and high-throughout tool to identify important interactions between protein and DNA.

Acknowledgments

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