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Report

The Addition of Large Volume Bisulfite-Treated Heterogeneous DNA Promotes the Amplification of the Targeted Region in Bisulfite-Treated DNA by PCR

Rie Shinoe, Itsuki Sakamoto, Marie Ueki, Reina Izumi, Aoi Tamura, Honoka Yamanobe, Ryoya Wada, and Masahiko Shiraishi*

Division of Genomics and Molecular Biology, Department of Pharmaceutical Sciences, International University of Health and Welfare, 2600-1 Kitakanemaru, Otawara, Tochigi 324-8501, Japan
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Bisulfite modification of cytosine residues is a widely used method for analyzing genomic DNA methylation. Despite its robustness, degradation of DNA during modification has hampered the application to the analysis of small amounts of DNA. We show that the addition of large volume bisulfite-treated heterogeneous DNA promotes the amplification of the targeted region in bisulfite-treated DNA by PCR. The addition of untreated DNA did not promote the amplification. The addition of large volume bisulfite-treated heterogeneous DNA neither promoted the amplification when untreated DNA was used as template. PCR products were detected when a ten thousandth of aliquot of bisulfite-treated 1 µg of human genomic DNA (0.1 ng) was used as template. This figure is equivalent to that of human genomic DNA derived from as little as 15 cells. This procedure permits the analysis of genomic DNA methylation when only limited numbers of cells are available.

Key words DNA methylation, bisulfite modification, epigenetics

INTRODUCTION

The analysis of methylation status of genomic DNA is important in understanding the integrity of epigenetic gene expression. The most widely used method to determine methylation status of a specific cytosine residue is bisulfite modification of DNA.[1] The treatment of DNA with bisulfite results in the conversion of cytosine to uracil, while 5-methylcytosine remains unmodified. Subsequent PCR and nucleotide sequence analysis permits unambiguous discrimination of cytosine and 5-methylsitosine.[2]

There are two major disadvantages of bisulfite modification. The first is that it is time consuming. One of the current authors has overcome this problem by developing a high concentration of bisulfite solution, permitting the conversion in 20 minutes.[3] The second disadvantage is the marked degradation of DNA during bisulfite treatment.[4] Although we have not yet overcome this issue, we show in this paper that the addition of large volume bisulfite-treated heterogeneous DNA (bth-DNA) promotes the amplification of the targeted region in the bisulfite-treated DNA by PCR.

MATERIALS AND METHODS

Source of Reagents Plasmid vectors pGEM-5Zf(+) (Promega), pUC19 (TaKaRa), and λ phage DNA (New England BioLabs) were obtained commercially. Restriction endonucleases were obtained commercially (*Hin*d III from TOYOBO, Osaka, Japan, and others from New England BioLabs). pUC-Myc65 is a plasmid containing positions 1–8078 of accession no. X00364 cloned in *Eco*RI/*Hin*dIII sites of the cloning vector pUC19. High-molecular-weight DNA of human cancer cell line MCF-7 was prepared by the method of Blin and Stafford.[5]

Treatment of DNA with Bisulfite This was performed as described[4] with slight modifications. Briefly, an appropriate amount of DNA was treated in a 25 µL of 0.3 N NaOH solution for 30 min at 37°C. To the alkaline-treated solution, 250 µL of 10 M bisulfite solution was added and incubated for 40 min at 70°C. The desalting was performed using GeneClean kit (funakoshi, Tokyo, Japan) following manufacturer's recommendation with slight modifications. The DNA was released from the matrix by the addition of 90 µL of H_2O. To the DNA solution, 11 µL of 2 N NaOH was added and incubated for 5 min at 37°C. After the addition of 4 M ammonium acetate and 10 µg of yeast tRNA, 750 µL of ethanol was added and maintained at –80°C for at least 30 min. the DNA was recovered by centrifugation and dissolved in an appropriate volume of TE (10 mM Tris·Cl (pH 7.5), 1 mM EDTA (pH8.0)). All alkaline solutions were prepared at the time of use.

PCR Analysis of Bisulfite-Treated High Molecular Weight DNA PCR experiments were performed as described[3] using

*To whom correspondence should be addressed. e-mail: mshirais@iuhw.ac.jp

TaKaRa Ex Taq (TaKaRa, Kusatsu, Japan). Primers were identical to those reported.[3] It should be emphasized that, in all experiments described in this paper, the amounts of template DNA treated with bisulfite are denoted under the assumption that no degradation occurred. In the experiments shown in Figs. 1 and 2, a 354-base pair (bp) region corresponding to positions 6188–6541 (positions 6188–6208, TGGTGAGAGGAGTAAGGGTGG, and 6523–6541, TTTGGGGAGATAATTTTGT, are covered by primers) of accession no. X00364 was amplified.[3] Since both primers have an additional 8-bp 5'-flanking adaptor sequence, the length of the PCR product is 370-bp. After incubation for 3 min at 95°C, 30 cycles of amplification (30 sec at 95°C and 1 min at 60°C) was applied.

Details of the analyzed region and PCR primes used in experiments shown in Fig. 3 are as described.[3] In the experiments shown in Figs. 3a and b, nested PCR was adopted. The initial amplification was as follows: After incubation for 3 min at 95°C, 40 cycles of amplification (30 sec at 95°C; 30 sec at 55°C; 30 sec at 72°C) was applied in a volume of 50 µL solution. A two µL aliquot of the first amplification was subjected to the second amplification similarly as in the initial amplification. In the experiment shown in Fig. 3c, heminested PCR was adopted. The initial amplification was as follows: After incubation for 3 min at 95°C, 25 cycles of amplification (30 sec at 95°C; 30 sec at 64°C; 30 sec at 72°C) was applied in a volume of 50 µL solution. A two µL aliquot of the first amplification was subjected to the second amplification as in the initial amplification except that 35 cycles of amplification was applied.

Nucleotide sequence analysis of the PCR fragments shown in Figs 3b and 3c were performed as described.[3] PCR products were analyzed by 3% NuSieve GTG (Lonza) - 1% SeaKem GTG (Rikaken, Tokyo, Japan) agarose gel electrophoresis. Real-time PCR was performed using 7500 Real Time PCR System (Applied Biosystems) and QuantiNova® SYBR® Green PCR kit (QIAGEN) following manufacturers' recommendations. PCR primers were as described above.

All bisulfite-modification and PCR experiments were repeated at least three times to confirm reproducibility.

RESULTS

Conversion of Cytosine to Uracil with Bisulfite in the Presence of Heterogeneous Double-Strand DNA We investigated whether the addition of heterogeneous DNA could suppress degradation as reported.[4] In the absence (Fig. 1, lanes 3–5) or presence (Fig. 1, lanes 6–8) of 1 µg of *Pst*I digests of pGEM-5Zf(+), 10 ng of *Eco*RI digests of pUCMyc65 was treated with bisulfite. Aliquots of recovered DNA fragments were serially diluted and subjected to PCR. In lane 4, PCR products were not detected. In lane 7, in contrast, PCR products of an expected length (370 bp) were detected. In lanes 4 and 7, the same amount of DNA was used as template if there occurred no degradation. Similar results were obtained when *Eco*RI digests of the plasmid pUC19 or *Hin*dIII digests of λ DNA was used instead of *Pst*I digests of pGEM-5Zf(+) (data not shown).

We then examined whether the increased volume of PCR products could be attributed to suppression of degradation. In all bisulfite-treatment experiments shown in Fig. 2, 10-fold excess of corresponding DNAs were subjected to the modification, and a tenth of recovered aliquots, whose volumes are shown below each image, were used as template. When *Eco*RI digests of pUCMyc65 and *Pst*I digests of pGEM-5Zf(+) were treated with bisulfite in the same reaction tube and used as template, PCR fragments were detected (Fig. 2a, lane 4). Under the same PCR conditions, PCR fragments were detected when these DNAs were separately treated with bisulfite and mixed in the same PCR tube prior to the amplification (Fig. 2a, lane 5), but not when PCR was performed without *Pst*I digests of pGEM-5Zf(+) treated with bisulfite (Fig. 2a, lane 3). The amount of *Eco*RI digests of pUCMyc65 is identical in lanes 2 and 4. It is highly suggested that the increased volume of PCR

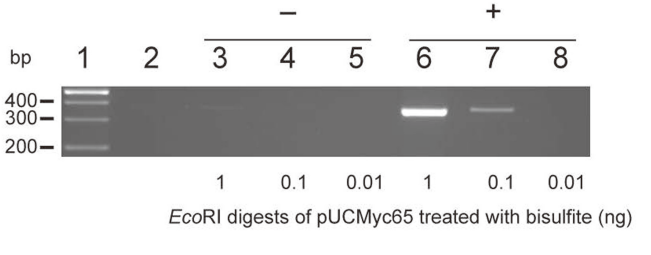

Fig. 1. Increased Volume of PCR Products with Template DNAs Treated with Bisulfite in the Presence of a Heterogeneous Double-Strand DNA

Lane 1, size markers, lane 2, no template DNA. In lanes 3–5 are the results for the analysis of 10 ng of *Eco*RI digests of pUCMyc65 without *Pst*I digests of pGEM-5Zf(+) (denoted as –). In lanes 6–8 are those with 1 µg of *Pst*I digests of pGEM-5Zf(+) (denoted as +). Amounts of template for PCR are shown below the image. All figures are denoted under the assumption that no degradation occurred.

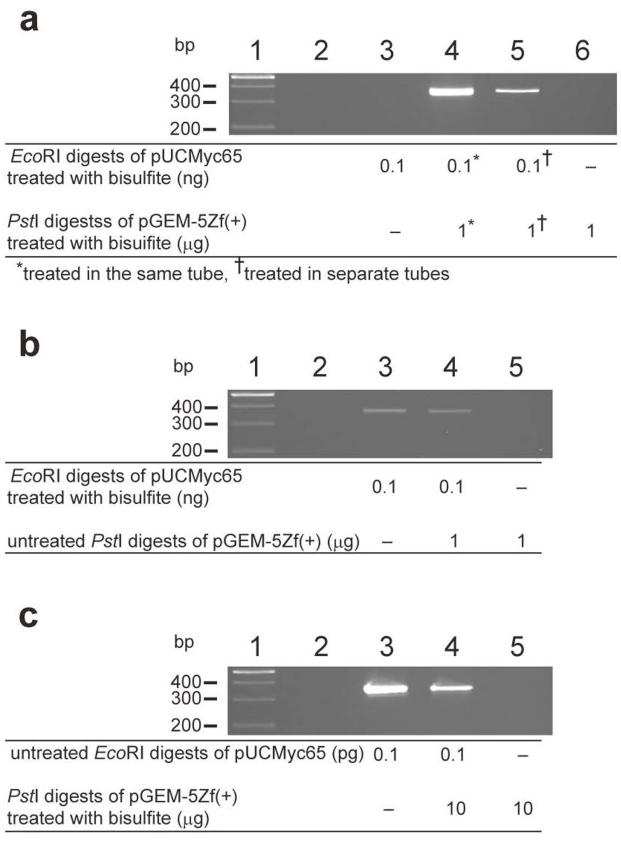

Fig. 2. The Necessity of Bisulfite Treatment Both for Target DNA and for bth-DNA

Lane 1, size markers, lane 2, no template DNA. Details of the templates are shown below each image.

products could be attributed not to suppression of degradation but to the promotion of the amplification when bth-DNA was added.

There is a *Sau*3AI site (GATC) in the analyzed region. PCR fragments shown in lane 4 were resistant to *Sau*3AI digestion, indicating that the conversion of cytosine to uracil was not affected in the presence of large volume of *Pst*I digests of pGEM-5Zf(+) (data not shown).

We then examined the combination of *Eco*RI digests of pUCMyc65 and *Pst*I digests of pGEM-5Zf(+) in terms of the presence or absence of bisulfite treatment. When PCR was performed using bisulfite-treated pUCMyc65 mixed with large amount of non-treated *Pst*I digests of pGEM-5Zf(+), the promotion of the amplification did not occur (Fig. 2b, lanes 3 and 4). Neither did the promotion of the amplification occur when PCR was performed using non-treated pUCMyc65 mixed with large amount of *Pst*I digests of pGEM-5Zf(+) (Fig. 2c, lanes 3 and 4). It was shown that the addition of large amount of bth-DNA promotes the amplification of the targeted region in the bisulfite-treated DNA by PCR.

The Application to Human Chromosomal DNA One of our authors previously reported the analysis of the methylation status of a part of two genes in a human cancer cell line MCF-7 using high concentration of bisulfite solution,[3] whose results were identical to those obtained using a conventional bisulfite reagent.[6,7] Experiments were performed to investigate the minimal amount of DNA that could be detected by the current method.

We applied nested PCR to detect small amounts of DNA. In all bisulfite-treatment experiments shown in Fig. 3, 1 μg of MCF-7 DNAs was subjected to the modification, and appropriate volume of diluted aliquots, whose volumes are shown below each image or in the legend to the figure, were used as template. We first examined at which stage the large amount of bth-DNA should be added to promote PCR. PCR fragments were detected when the large amount of *Pst*I digests of pGEM-5Zf(+) treated with bisulfite (bth-DNA) was added at the first amplification, regardless of its presence (Fig. 3a, lane 5) or absence (Fig. 3a, lane 6) in the second amplification. PCR fragments were not detected when the large amount of bth-DNA was added only at the second amplification (Fig. 3a, lane 7). In the following experiments, the large amount of bth-DNA was added only at the first amplification.

A 329-bp DNA region containing a part of the exon 1 of the *CDH1* gene[3] was intended to amplify by nested PCR using bisulfite-treated DNA under various conditions as template (Fig. 3b). When 0.1 ng of bisulfite-treated MCF-7 DNA was subjected to PCR in the presence of bth-DNA, PCR products (344 bp) were detected (Fig. 3b, lane 6). PCR products were not detected in the absence of bth-DNA (Fig. 3b, lane 4).

A 205-bp DNA region containing a part of the exon 1 of the *RASSF1A* gene[3] was intended to amplify by heminested PCR using bisulfite-treated DNA under various conditions as template (Fig. 3c). When 0.1 ng of bisulfite-treated MCF-7 DNA was subjected to PCR in the presence of bth-DNA, PCR products (219 bp) were detected (Fig. 3c, lane 6). PCR products were not detected in the absence of bth-DNA (Fig. 3c, lane 4). In the experiments shown in Fig. 3c, we used *Hin*dIII digests of λ DNA treated with bisulfite as bth-DNA because PCR fragment of a similar size to the expected fragments was detected when *Pst*I digests of pGEM-5Zf(+) treated with bisulfite was used as template (data not shown).

We performed nucleotide sequence analysis of the PCR fragments shown in Fig. 3b lane 5 and Fig. 3c lane 5. All cytosine residues that were expected to unmethylated were converted to uracil, and the results were identical to those previously reported[3] (data not shown). It has been shown that the addition of a large amount of bth-DNA does not result in biased representation of specific sequences.

The weight of chromosomal DNA derived from 6 x 10^{23} (Avogadro constant) human cells is 2 x 330 x 2 x 3 x 10^9 g, based on premises that the cells are diploid, the average molecular weight of four nucleotides is 330, and the number of nucleotides comprising a human chromosomal DNA is 2 x 3 x 10^9. Thus, proportionally, 0.1 ng of human chromosomal DNA is derived from approximately 15 cells.

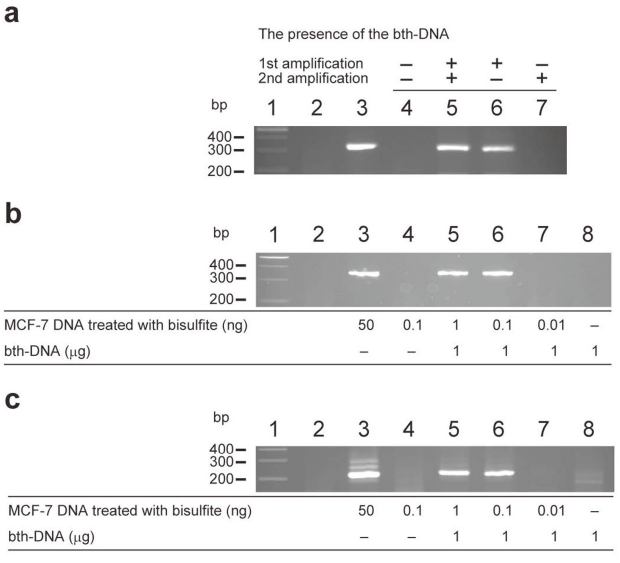

Fig. 3. **a)** The amplification of DNA containing a part of the exon 1 of the *CDH1* gene to determine at which stage of the amplification bth-DNA works. Lane 1, size markers; lane 2, no template DNA; lane 3, 50 ng of MCF-7 DNA treated with bisulfite (positive control); lanes 4–7, 0.1 ng of MCF-7 DNA treated with bisulfite. The presence (+) or absence (–) of *Pst*I digests of pGEM-5Zf(+) treated with bisulfite are shown below the image. **b)** The amplification of DNA containing a part of the exon 1 of the *CDH1* gene using serially diluted MCF-7 DNA treated with bisulfite. Lane 1, size markers, lane 2, no template DNA. Details of templates for lanes 3–8 are shown below the image. Here the bth-DNA is *Pst*I digests of pGEM-5Zf(+) treated with bisulfite. **c)** The amplification of DNA containing a part of the exon 1α of the *RASSF1A* gene. Lane 1, size markers, lane 2, no template DNA. Details of templates for lanes 3–8 are shown below the image. Here the bth-DNA is *Hin*dIII digests of λ DNA treated with bisulfite.

DISCUSSION

We have shown that the addition of bth-DNA markedly promotes the amplification of bisulfite-treated DNA during PCR. This observation may be useful for the analysis of the methylation status of a specific nucleotide sequence where there is subnanogram quantity of DNA.

Our preliminary experiments show that DNA regions containing a few cytosine residues are relatively resistant to degradation compared to those enriched with cytosine residues and the addition of bth-DNA does not promote the amplification of such sequences by PCR (data not shown). The efficiency of degradation of DNA and the effect of the addition of bth-DNA could depend on the target region.

Methods based on post-bisulfite adaptor ligation have been developed to permit single-cell methylome analysis.[8–10] The

method we describe is relatively simple and robust when the target sequence is specified. Our method is also applicable to the post-bisulfite adaptor ligation method to amplify adaptor-ligated DNA.

These are some issues to be resolved. Firstly, it is unclear why the addition of bth-DNA promotes amplification. It is also unclear why the addition of bth-DNA is effective only in the first amplification in nested PCR. The addition of bth-DNA could promote the amplification when the amount of template DNA is very low. Secondly, quantitation of the template DNA after bisulfite treatment is important. We performed real-time PCR experiments to quantitate DNA used in experiments shown in Fig. 1. Preliminary data shows that the absence or presence of bth-DNA does not affect degradation (data not shown). Considering that the addition of bth-DNA could promote amplification, however, the addition of bth-DNA in real-time PCR experiments could also affect amplification, especially if the first issue is the case. Thirdly, although PCR products were detected when a ten thousandth of aliquot of bisulfite-treated 1 μg of human genomic DNA (0.1 ng) was used as template, we have not tested whether PCR products can be detected when 0.1 ng of MCF-7 DNA was treated directly with bisulfite and the entire recovered DNA was used as template. Studies to elucidate these points are in progress.

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Conflict of interest The authors declare no conflict of interest.

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