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A Bisulfite Method of 5-Methylcytosine Mapping That Minimizes Template Degradation

Anthony M. Raizis, Frédéric Schmitt, and Jean-Pierre Jost[1]
Friedrich Miescher Institut, P.O. Box 2543, CH-4002 Basel, Switzerland

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The bisulfite method is a highly sensitive approach to 5-methylcytosine mapping that utilizes the capability of the polymerase chain reaction to exponentially amplify DNA. We have observed that the bisulfite reaction results in a significant level of template degradation due to DNA depurination. Furthermore, our data suggest that the DNA fragmentation which occurs limits the sensitivity of the method. We describe a simple solution to limit degradation of the DNA template. © 1995 Academic Press, Inc.

DNA methylation has been shown to be an essential phenomenon required for normal embryonic development (1) and imprinting (2). The detection and mapping of DNA methylation are essential steps toward understanding the molecular signals which indicate whether a given sequence is methylated. The bisulfite method (3) is a vast improvement over previous techniques (4,5), as much less genomic DNA is required for the detection of cytosine methylation in DNA.

The bisulfite method of mapping 5-methylcytosine exploits the fact that sodium bisulfite ($NaHSO_3$) reacts readily with the 5,6-double bond of cytosine (6), but poorly with methylated cytosine (7). Cytosine reacts with the bisulfite ion to form a sulfonated cytosine reaction intermediate which is susceptible to deamination, giving rise to a sulfonated uracil. The sulfonate group can be removed under alkaline conditions, resulting in the formation of uracil (6). Uracil is recognized as a thymine by *Taq* polymerase and hence upon PCR the resultant product will contain cytosine only at the position where 5-methylcytosine occurs in the starting template DNA (3).

Several reaction conditions have been described (8,9). One set of reaction conditions involves treating DNA with 3.5 M sodium bisulfite (pH 5.0) at 0°C for 24 h (8). Another set of reaction conditions involves treatment of the DNA with 3.1 M sodium bisulfite (pH 5.0) at 55°C for 16 h (9). In the latter example the sensitivity was dramatically improved by the use of nested primers. Although nested primers indeed overcome many of the problems encountered with the bisulfite method, a difficulty remains in that the design of primers requires stringent criteria; i.e., (a) the primers should be designed to avoid CpG's; (b) primers should be 25 to 30 bases long; (c) the primer pairs should be of approximately equal annealing temperatures; (d) primers should not have internal homology or be biased for one particular nucleotide; (e) the primers should at the 3′ end contain at least one but preferably two GC pairs which remain GC after bisulfite treatment (10). These criteria are often difficult to achieve and therefore it would be an advantage in certain situations if only one pair of primers were sufficient.

We observe that prolonged bisulfite treatment causes the template DNA to become significantly degraded, mostly as a result of DNA depurination. The excessive depurination limits the sensitivity of the PCR reaction presumably by reducing the number of full-length molecules that are able to be amplified. We describe a simple solution to this problem which dramatically increases the sensitivity of the method.

MATERIALS AND METHODS

Derivation of Plasmid Construct and Primer Sequences

The plasmid used for experiments was the recombinant plasmid pGL2proneo. This plasmid consists of the luciferase-expressing vector pGL2-promoter (Promega) and a 1.14-kb *Xho*I–*Bam*HI fragment corresponding to the neomycin resistance gene (neo^r) derived from PMC1neo (11). The neo^r gene was cloned into the *Sal*I–*Bam*HI site between nucleotides 2930 and 2936. The neomycin gene contains a thymidine kinase promoter along with a polyoma enhancer. The luciferase gene is driven by the SV40 promoter (Fig. 1) to which primers selective for bisulfite-modified DNA were designed as described in Ref. (3). The primer sequences used

[1] To whom correspondence should be addressed. Fax: 0041-61-721-4091.

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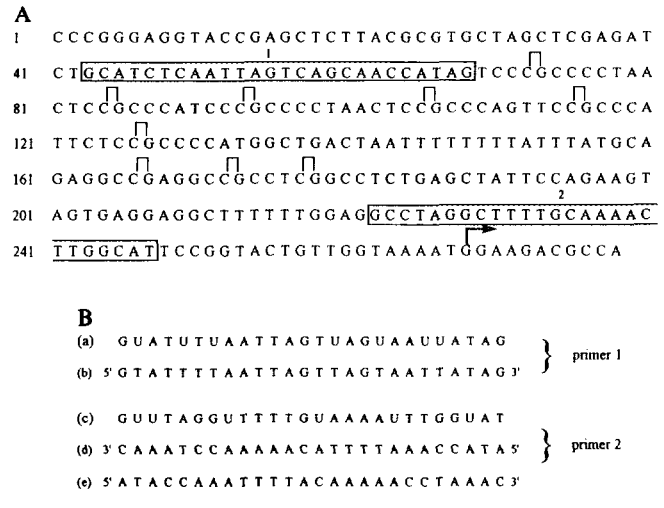

FIG. 1. Design of selective primers to the SV40 promoter sequence. Primers specific for the top strand of the bisulfite-modified SV40 promoter DNA sequence were designed as described in Ref. (3). (a) Sequence 1, corresponding to upper boxed region in (A), after cytosine conversion to uracil; (b) the DNA sequence of primer 1; (c) sequence 2, corresponding to lower boxed region after cytosine conversion to uracil; (d) the DNA sequence of primer 2 in the antisense orientation; (e) the DNA sequence of primer 2. A total of nine CpG's are contained between primers and these are indicated with open squares in (A). The transcription start site is indicated by an arrow.

were GTATTTTAATTAGTTAGTAATTATAG, corresponding to primer 1, and ATACCAAATTTTACAAAAACCTAAAC, which corresponds to primer 2.

In Vitro Methylation of Plasmid DNA and Stable Transfection

Plasmid DNA was linearized with the restriction enzyme *ScaI* (Biofinex). The linearized plasmid was methylated *in vitro* with *SSSI* methylase (New England Biolabs) in the presence of 160 μM *S*-adenosylmethionine according to manufacturer's specifications. The plasmid was used directly either for the bisulfite technique or for stable integration in HeLa cells. Stable integration of the *SSSI* methylated plasmid DNA was achieved by transfecting 10 μg of pGL2proneo using the transfection reagent DOTAP (Boehringer) according to manufacturer's specifications. Cells were grown in DMEM and 10% fetal calf serum (GibcoBRL). Neomycin-resistant colonies were selected in the presence of geneticin–sulfate (Gibco BRL) at a concentration of 800 μg/ml.

Bisulfite Reaction

To obtain a 5 M bisulfite solution, solid sodium metabisulfite ($Na_2S_2O_5$) must be used. Sodium metabisulfite dissolves to form two HSO_3^- ions such that 2.5 M metabisulfite is equivalent to 5 M bisulfite. The bisulfite solution of 2.5 M sodium metabisulfite (pH 5.0) and 100 mM hydroquinone was prepared fresh by first adding 1.9 g of sodium metabisulfite (Merck) to 2.5 ml of H_2O and the solution was vortexed. The solid sodium metabisulfite did not completely dissolve until 0.7 ml of 2 M NaOH and 0.5 ml of 1 M hydroquinone were added. The solution was again vortexed until all the solid material was in solution. The pH was then brought to 5.0 with NaOH and the final volume was adjusted to 4 ml with H_2O. The template DNA (1–50 pg of plasmid mixed with up to 2 μg of intact HeLa genomic DNA as carrier) was diluted with H_2O to give a final volume of 10–30 μl. The template was then denatured at room temperature by the addition of 1/10 vol of 2 M NaOH for 10 min. The bisulfite solution (200 μl/μg DNA) was then added to the template mixture and the tube was quickly vortexed before incubation at 50°C. After 4 h the bisulfite was removed as follows: the solution was cooled on ice and 0.5 vol of sodium acetate (pH 7.0) was added followed by 2.3 vol of H_2O and 400 μg of Dextran T70 carrier (Pharmacia) which was added last. From this solution the DNA was precipitated at room temperature by the addition of an equal volume of isopropanol. After thorough mixing the DNA was immediately pelleted by microfuging for 10 min. The supernatant was removed and the pellet was redissolved in 300 μl of H_2O followed by 30 μl of 3 M sodium acetate (pH 7.0) and then reprecipitated with 2 vol of ethanol. The precipitate was pelleted by microfuging for 5 min. The pellet was redissolved in 200 μl of 0.2 M NaOH and allowed to stand at room temperature for 15 min. The DNA was then precipitated by addition of 0.5 vol of 7.5 M ammonium acetate followed by 2 vol of ethanol. After microfuging for 10 min, the DNA was redissolved in 60 μl of H_2O and analyzed by denaturing alkaline agarose gel electrophoresis as described in Ref. (12) or immediately used for PCR.

PCR

The PCR cocktail was assembled as follows: 100 ng of each primer, 10 ng of primer end-labeled with [γ-^{32}P]-ATP (3000 Ci/mmol) to a specific activity of 0.5–1 $\times$ 10^8 cpm/μg, 10 μl of 10$\times$ PCR buffer (100 mM Tris, pH 8.3, 15 mM $MgCl_2$, 500 mM KCl, 1% NP40, and 1% gelatin), bisulfite-modified DNA, 2.5 units of *Taq* polymerase (Boehringer), and H_2O to a final volume of 100 μl. Paraffin oil was used to overlay the mixture. The PCR reaction was performed by denaturing at 94°C for 30 s and annealing in the range of 10–14°C below the theoretical primer melting temperature for 45 s. The extension step was at 72°C for 2 min. After 35 cycles the PCR product was examined on a denaturing 6% acrylamide gel followed by autoradiography on Fuji X-ray film.

DNA Sequencing

After PCR amplification, gel electrophoresis, and autoradiography, the PCR product was gel purified and redissolved in H_2O to a final concentration of approximately 200 cpm/μl. The dissolved sample (10 μl per

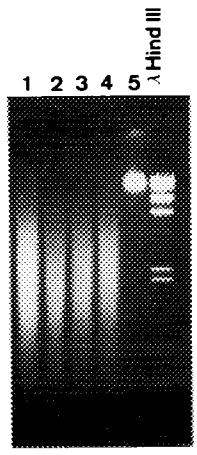

FIG. 2. Degradation of high-molecular-weight DNA treated with sodium bisulfite. High-molecular-weight HeLa genomic DNA (2 μg) was denatured with 0.2 M NaOH for 10 min and then samples were treated with either 3 M sodium bisulfite (pH 5.0) (lanes 2–4) or sodium acetate (pH 5.0) (lane 1) at 50°C for 16 h. The hydroquinone concentration was varied: 0 mM (lane 2), 10 mM (lane 3), and 100 mM (lane 4). Untreated DNA is shown in lane 5. After overnight incubation the DNA samples were treated with 0.2 M NaOH for 15 min, ethanol precipitated, and redissolved in alkaline gel electrophoresis loading buffer. DNA samples were separated by electrophoresis through an alkaline denaturing 1% agarose gel and then stained with ethidium bromide.

sequencing reaction) was subjected directly to Maxam and Gilbert DNA chemical cleavage reactions. The reactions used were formic acid (cleaves adenine and guanine), dimethyl sulfate (cleaves guanine), hydrazine (cleaves cytosine and thymine residues), and hydroxylamine (cleaves cytosine). The formic acid, dimethyl sulfate hydrazine, and piperidine reactions are described in Ref. (12) along with a detailed description of their handling and disposal. The hydroxylamine reaction was performed as described in Ref. (13). A complete list of alternative Maxam and Gilbert sequencing reactions is documented in Ref. (14).

RESULTS

The DNA degradation that occurs upon sodium bisulfite treatment at pH 5.0 may be due to one of at least two possibilities: (a) depurination as a consequence of an acidic pH, (b) a nonspecific side reaction due to the strong oxidative properties of sodium bisulfite, or perhaps both. To address this question we compared the extent of degradation of high-molecular-weight DNA incubated with sodium acetate (pH 5.0) (Fig. 2, lane 1) and sodium bisulfite (pH 5.0) in the presence and absence of hydroquinone, a powerful antioxidant (Fig. 2, lanes 2–4). The data show that sodium acetate at pH 5.0 gives a smear over a molecular weight range similar to that of sodium bisulfite at the same pH. Increasing the hydroquinone concentration from 0 to 100 mM gave only a slight shift of the DNA smear toward a higher molecular weight. These data suggest that the DNA fragmentation that occurs during bisulfite treatment is due mostly to the acidic pH.

The problem of template degradation can be potentially overcome through the manipulation of three variables: pH, temperature, and time of reaction. We have tried the bisulfite reaction at increased pH and at lower temperatures but have found that altering these variables results in considerably reduced efficiency of deamination (data not shown). Given that template degradation occurs as a consequence of depurination as opposed to a nonspecific oxidation reaction, we examined the possibility of increasing reaction kinetics. By increasing the bisulfite concentration it might be possible to increase the kinetics of deamination and therefore reduce the time over which depurination occurs. The maximum concentration at which sodium bisulfite is soluble at pH 5.0 is 5 M with respect to the HSO_3^- ion which is equivalent to 2.5 M $Na_2S_2O_5$. At this bisulfite concentration we examined the kinetics of deamination indirectly by PCR. Primers selective for modified DNA (Fig. 1) were used in the PCR assay and the yield of product was monitored (Fig. 3). We observed that after only 4 h of incubation, a maximum yield of PCR product was obtained (Fig. 3, lane 2) with no significant increase after 6 h (Fig.

FIG. 3. Kinetics of cytosine to uracil conversion in DNA treated with 2.5 M sodium metabisulfite. Plasmid DNA (50 pg) mixed with high-molecular-weight HeLa genomic DNA (2 μg) was treated with 2.5 M sodium metabisulfite for varying times: 2 h (lane 1), 4 h (lane 2), 6 h (lane 3), 12 h (lane 4), 16 h (lane 5). The DNA was desulfonylated by alkali as described under Materials and Methods. The bisulfite-treated plasmid DNA was then amplified by PCR with primers selective for the SV40 promoter sequence. Included in the PCR mixture was 10 ng of a primer end-labeled to a specific activity of 0.5–1 × 10^8 cpm/μg with [γ-^{32}P]ATP 3000 Ci/mmol using polynucleotide kinase enzyme. The labeled PCR product (top arrow) was separated from the unincorporated oligonucleotides (bottom arrow) by electrophoresis through a denaturing 6% acrylamide gel. The gel was autoradiographed for 15 min at room temperature on Fuji X-ray film.

3, lane 3). However, after longer incubation times from 12 to 16 h (Fig. 3, lanes 4 and 5, respectively) a significant decrease in the yield of reaction product was found. This result was in contrast to that found in Ref. (9), where the optimal yield of PCR product using 3.1 M bisulfite required 16 h of incubation. High-molecular-weight DNA treated with 5 M bisulfite for 4 h was subsequently examined on a 1% denaturing agarose gel and found to be in the range 9–23-kb (Fig. 4, lane 3). However, overnight treatment with 3.1 M bisulfite resulted in considerably more DNA fragmentation (Fig. 4, lane 2). We conclude (a) that the bisulfite ion at 5 M concentration significantly increases the reaction kinetics of deamination in comparison to 3.1 M bisulfite and (b) treatment of DNA for only 4 h with 5 M bisulfite results in relatively little DNA fragmentation.

We next examined the template requirement for obtaining a sufficient yield of PCR product for DNA sequencing. Under conditions of 5 M treatment for 4 h, as little as 1 pg of target plasmid DNA was required to obtain a PCR product (Fig. 5, lane 6). This result was in contrast to that obtained using two other procedures, i.e., 3.1 M bisulfite for 16 h (Fig. 5, lane 1) (9) and 0°C for 24 h (Fig. 5, lane 2) (8). Under these conditions we were unable to obtain a comparable level of PCR product even with 50 pg of plasmid template DNA using these methods. We conclude that 5 M treatment of DNA for 4 h results in considerably reduced template requirement for obtaining a detectable PCR product.

Having established a set of conditions that improves the efficiency of the bisulfite method, we next determined the completeness of the reaction by DNA sequencing of the PCR product directly. In order to check the completeness of the reaction, plasmid DNA (50 pg) was methylated *in vitro* with *SSSI* methylase which methylates all CpG's. The DNA was then subjected to 5 M bisulfite treatment for 4 h at 50°C. Sequence analysis of the gel-purified end-labeled PCR products was done using the Maxam and Gilbert chemical degradation technique. We found that sequencing the upper strand of the PCR product resulted in complete conversion of cytosine to uracil except at methylated CpG's (Fig. 6A, lane C). Nonmethylated C's, however, were converted to T's (Fig. 6A, lane C + T). On overexposure of the gel, faint bands were found in the C track (not shown); however, these were not T specific and therefore more likely due to slight nonspecific degradation of the end-labeled PCR product. In the upper section of the gel there appears to be an additional nonspecific band (Fig. 6A, lane C), which is indicated by a small arrow. To resolve this segment we sequenced the opposite strand (Fig. 6B, lane G) and found that the corresponding region consists of nonspecific bands (indicated by small arrows). These bands were of much weaker intensity than the bands corresponding to methylated CpG's and were found only at four cytosine residues. Since only a small subset of non-CpG cytosine residues was not fully converted, the result suggests that they were most likely a consequence of weak *SSSI* methylating activity at these sites. We

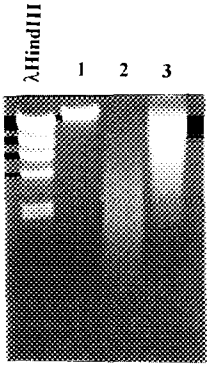

FIG. 4. High-molecular-weight DNA degradation comparing two methods of bisulfite treatment. High-molecular-weight HeLa genomic DNA (2 μg) was alkali denatured and then treated with 2.5 M sodium bisulfite at 50°C for 4 h (lane 3) or 1.5 M sodium metabisulfite at 50°C for 16 h (lane 2) or untreated (lane 1). The DNA was desulfonated and analyzed by alkaline denaturing electrophoresis as described under Materials and Methods.

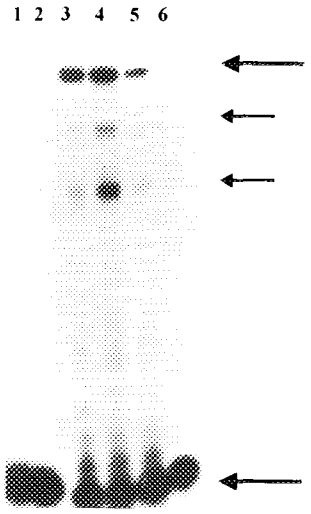

FIG. 5. Comparison of several bisulfite methods. Several bisulfite methods were compared. In three separate bisulfite reactions identical plasmid samples (50 pg), each mixed with high-molecular-weight HeLa genomic DNA (2 μg), were alkali denatured and then bisulfite treated with 3.1 M sodium bisulfite (pH 5.0) at 55°C for 16 h as described in Ref. (9) (lane 1), 3.5 M sodium bisulfite at 0°C for 24 h as described in Ref. (8) (lane 2), or 5 M sodium bisulfite at 50°C for 4 h (lanes 3–6). After deamination the DNA was desulfonylated as described under Materials and Methods. The modified DNA was then amplified by PCR with the inclusion of labeled oligonucleotide as described under Materials and Methods. The amount of modified plasmid DNA used in the amplification step was 50 pg (lanes 1 and 2), 25 pg (lane 3), 10 pg (lane 4), 5 pg (lane 5), and 1 pg (lane 6). The PCR product (top arrow) was separated from unincorporated primers (bottom arrow) and nonspecific bands (middle arrows) by electrophoresis through a denaturing 6% polyacrylamide gel. Autoradiography was for 15 min at room temperature on Fuji X-ray film.

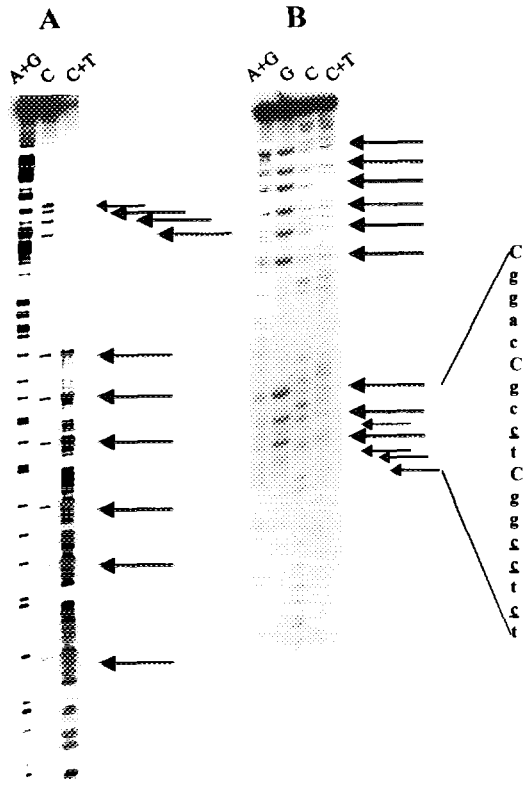

FIG. 6. Mapping of 5-methylcytosine residues in plasmid DNA using a modified bisulfite protocol. Plasmid DNA (50 pg) was methylated *in vitro* with *SSSI* methylase as described under Materials and Methods. The DNA was denatured, bisulfite treated (2.5 M sodium metabisulfite) for 4 h at 50°C, desulfonated with alkali, and amplified by PCR incorporating a labeled primer as described in the legend to Fig. 2. DNA sequencing was done by the Maxam and Gilbert chemical sequencing technique as described under Materials and Methods. (A) DNA sequence obtained by end labeling primer 1 (see Fig. 1), while primer 2 was end labeled for the sequence in (B). Major bands corresponding to methylated C residues are indicated by large arrows and bold capital C's. Minor nonspecific bands are indicated with small arrows and underlined c's.

conclude from these data that the bisulfite reaction under our conditions results in sufficient cytosine to uracil conversion and does not affect 5-methylcytosine.

We next examined whether our conditions of bisulfite treatment also work with high-molecular-weight genomic DNA. It has been previously suggested that shearing the DNA with restriction enzyme might be an important step preceding the bisulfite reaction (3). In order to examine our procedure on high-molecular-weight DNA the vector pGL2proneo was methylated *in vitro* with *SSSI* methylase and transfected into HeLa cells. Stable transformants were obtained after 14 days at approximately 5–10% less frequency than the nonmethylated clone. DNA from 2×10^6 cells containing the integrated plasmid construct pGL2proneo was subsequently isolated from each clone. The bisulfite method under our conditions was applied to high-molecular-weight DNA derived from 10 individual clones and the resultant PCR products were sequenced directly. All clones were found to be methylated. The sequence of one such clone is shown in Fig. 7. To rule out the possibility that contaminating free plasmid was no longer present in these clones, the Hirt supernatant was isolated and the presence of the SV40 promoter was checked by PCR. No contaminating free plasmid was observed (data not shown). We conclude that our conditions for the bisulfite reaction work also for high-molecular-weight genomic DNA. This result further indicates that shearing DNA with restriction enzyme, prior to bisulfite treatment, is an unnecessary step.

DISCUSSION

We have found that the major cause of DNA fragmentation during bisulfite treatment occurs as a consequence of DNA depurination. To overcome this problem we have attempted to reduce the time required to achieve completion of the bisulfite reaction. In order to achieve faster reaction kinetics we have increased the bisulfite

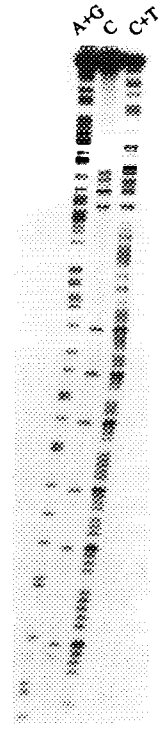

FIG. 7. Mapping of 5-methylcytosine residues in plasmid DNA stably integrated into the HeLa genome. Plasmid DNA (10 μg) was methylated *in vitro* with *SSSI* methylase as described under Materials and Methods. The DNA was transfected into HeLa cells and the cells were grown in neomycin-selective medium. After 14 days one of the neomycin-resistant colonies was picked and further grown until approximately 2×10^6 cells were obtained and DNA was extracted. The genomic DNA (2 μg) was bisulfite treated and sequenced as described in the legend to Fig. 6. The labeled oligonucleotide used in this experiment corresponds to primer 1 (see Fig. 1).

concentration to 5 M, which results in a significant reduction in time required for complete deamination. Under these conditions the optimal yield of PCR product occurs after only 4 h of bisulfite treatment during which only minimal DNA fragmentation occurs. We further find that the minimum amount of template DNA necessary to obtain a detectable PCR product is as little as 1 pg without the use of nested primers. Using these conditions we are able to detect DNA methylation in small quantities of *in vitro*-methylated plasmid DNA and also high-molecular-weight genomic DNA. In contrast, we have been unable to achieve the same level of sensitivity using previously described methods (8,9). In one case (9), our data suggest that although deamination may be complete, the DNA appears highly degraded. In the second case (8), we find that DNA is relatively intact after overnight bisulfite treatment at 0°C. However, in our hands the deamination reaction may not be complete since we are unable to obtain a significant PCR product with plasmid (Fig. 5) or genomic DNA (data not shown).

We should emphasize that although our bisulfite conditions are able to yield a PCR product with as little as 1 pg of pGL2proneo plasmid DNA, the same does not necessarily apply to all DNA sequences. As has been previously reported (9), we find that the bisulfite technique does not work in certain situations for reasons that remain unclear. However, our conditions have been successfully tried on several plant DNA sequences by independent investigators (Schmitt and Mittelsten Scheid, personal communication).

In our approach to the bisulfite method we find radiolabeled primers for the purpose of visualizing the PCR product to be highly advantageous. In many situations we often have difficulties in obtaining a visible PCR product on agarose gels with ethidium bromide staining. However, with the use of radiolabeled primers, we find optimizing PCR and bisulfite reaction conditions to be considerably easier. Once optimized, the PCR product can then be cloned or sequenced directly by the Maxam and Gilbert chemical cleavage technique. For DNA end labeling we usually supplement the PCR reaction mix with only 10 ng of labeled primer. This amount of label is usually sufficient to obtain good incorporation even in the presence of 100 ng of the corresponding unlabeled primer. However, in exceptional cases where the product is extremely faint we have sometimes used 100 ng of the labeled oligonucleotide to boost sensitivity.

Using the Maxam and Gilbert DNA sequencing strategy we find that relatively little template is required for sequencing. We routinely use 5 ng of the labeled PCR product per track, although we have also been successful with as little as 1 ng (data not shown). The only drawback in using smaller quantities of the PCR product is that the sequencing gels are required to be exposed longer, i.e., for up to 7 days at −80°C, to obtain photographic quality autoradiographs.

In summary, we report an alternative bisulfite method that limits fragmentation of the template DNA and boosts sensitivity. Our bisulfite method may be more useful for certain DNA sequences where design of nested primers presents a major problem.

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