5058–5059 *Nucleic Acids Research, 1996, Vol. 24, No. 24*

Rapid analysis of DNA methylation using new restriction enzyme sites created by bisulfite modification

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Received August 27, 1996; Revised and Accepted November 6, 1996

ABSTRACT

Bisulfite converts non-methylated cytosine in DNA to uracil leaving 5-methylcytosine unaltered. Here, predicted changes in restriction enzyme sites following reaction of genomic DNA with bisulfite and amplification of the product by the polymerase chain reaction (PCR) were used to assess the methylation of CpG sites. This procedure differs from conventional DNA methylation analysis by methylation-sensitive restriction enzymes because it does not rely on an absence of cleavage to detect methylated sites, the two strands of DNA produce different restriction enzyme sites and may be differentially analyzed, and closely related sequences may be separately analyzed by using specific PCR primers.

Methylation of CpG sites in DNA is an important control mechanism in development, differentiation and aging. The bisulfite reaction, which converts non-methylated cytosine residues in DNA to uracil, while leaving 5-methylcytosine unaltered, has greatly enhanced the analysis of methylated cytosine in genomic DNA. Here we demonstrate an adaptation of the bisulfite modification method in which changes in restriction enzyme sites resulting from the action of bisulfite on methylated and non-methylated CpG sites are predicted. The restriction enzyme digestion of PCR products of bisulfite-reacted DNA allows rapid analysis of patterns of regional methylation or demethylation of genomic DNA where an analysis of the methylation status of every CpG in the sequence is not required.

We wished to investigate the demethylation of the CYP17A2 gene in primary cultures of bovine adrenocortical cells (1). To provide a system for studying factors affecting this demethylation event, cells were transfected with a fully methylated fragment of the CYP17A2 gene attached to a selectable plasmid. As previously described (1), a bovine genomic library was screened using CYP17 cDNA. The ~11 kb *Sal*I–*Eco*RI insert of a hybridizing λ clone, containing the entire coding region and 2.5 kb of 5′ flanking region of CYP17A2, was subcloned into pBluescript II KS⁻. Primers designed to amplify part of the plasmid (Fig. 1, including part of the flanking region of the gene that undergoes demethylation) were used to synthesize a fully methylated DNA fragment by PCR in which ^{methyl5}-dCTP (Amersham Corp., Arlington Heights, IL) completely replaced dCTP (2). The use of an in-sample temperature probe (MJ Research Inc., Watertown, MA) ensured that the temperature required for proper denaturation of ^{m5}dC-substituted DNA (95°C) was maintained for at least 20 s.

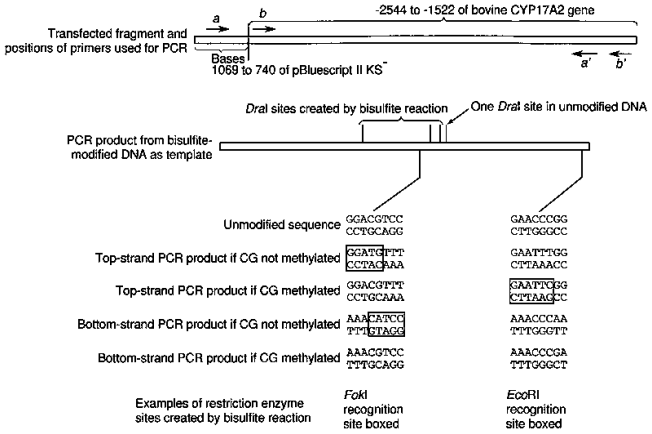

Figure 1. Examples of changes in restriction enzyme sites after reaction with bisulfite. The fully-methylated fragment from pBluescript-CYP17A2 transfected into bovine adrenocortical cells is shown, together with the positions of primers (arrows) used to amplify genomic DNA following reaction with bisulfite. The positions of the primers used to amplify the top and bottom strands differ slightly and are not shown separately. The primer pairs with positions a, a′ were used to amplify the transfected construct and the primer pairs with positions b, b′ were used to amplify the endogenous genes. A top-strand PCR product (primers b, b′) is shown together with the positions of *Dra*I sites formed by the action of bisulfite, which may be used to monitor the completeness of the bisulfite reaction. Below are two examples of the creation of new restriction enzyme sites, dependent respectively on the non-methylation (both top and bottom strands) or methylation (top strand only) of two CpG sites in the sequence.

For transfection, bovine adrenocortical cells were prepared and grown as previously described (3). The methylated DNA fragment was prepared for transfection by cleavage of the ends with *Ssp*I, for which sites were incorporated into the 5′ ends of the primers. Plasmid pSV2neo was cleaved with *Sca*I. The two blunt-ended fragments were then ligated to each other to form concatemers (in a 3:1 ratio of CYP17A2 fragment to pSV2neo) using T4 DNA ligase. The resultant high molecular weight DNA was transfected by Lipofectin (Life Technologies) as previously described (4) using

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20 µg DNA per 10 cm dish. Pooled transfectant clones were selected in medium containing 200 µg/ml G418 for 14 days.

DNA isolated from transfected cells was reacted with bisulfite using the procedure described by Frommer *et al.* (5). The modifications described by Feil *et al.* (6) were followed, except that DNA was prepared by needle shearing and the incubation with bisulfite was performed at 50°C, as in the original method. Primers designed to be complementary to the sequence predicted to be formed in the presence of bisulfite were used to amplify the modified sequence by PCR (Fig. 1). Separate sets of primers were designed to amplify the top and bottom strands of the transfected sequence and the endogenous genes. Figure 1 shows examples of restriction enzyme sites predicted to be formed by the action of bisulfite on the transfected sequence. Restriction enzyme sites in the modified sequence were predicted using nucleic acid analysis software (Vector NTI, Informax Corp.). Prediction of new sites was performed separately on the upper and lower DNA strands and the effect of methylation on each CpG site was assessed. Using all possible combinations of bases that surround CpG sites, we estimate that ~25% are amenable to analysis by this method, i.e. 25% of CpGs give rise to restriction enzyme sites that differ after reaction with bisulfite dependent on the methylation status of the site.

Amplification of the transfected fragment using bisulfite-treated genomic DNA as template was accomplished using a primer complementary to the plasmid sequence together with a gene-specific primer (Fig. 1). These primers did not amplify the genomic DNA sequence. As shown in Figure 2b and c, restriction enzyme digestion of the PCR product indicates that the fully methylated DNA fragment undergoes demethylation after transfection into these cells. The corresponding sequence of the endogenous gene is also non-methylated, as shown here (Fig. 2a) confirming previous analysis using methylation sensitive restriction enzymes and Southern blotting (1). The completion of the bisulfite reaction and the amplification only of bisulfite-modified DNA was assessed by cleavage with enzymes such as *Dra*I that recognize sequences containing only adenine and thymine, which are created by the action of bisulfite in non-CpG sequences (Fig. 2a–d). Lack of bisulfite modification of the fully methylated fragment prior to transfection was also demonstrated by the pattern of restriction enzyme digestion (Fig. 2d).

This method enables the rapid assessment of the methylation status of populations of DNA molecules unbiased by selection of individual PCR products that may occur during subcloning into plasmids. It allows the analysis of the percentage methylation of a CpG site. In this respect it differs from conventional DNA methylation analysis by methylation-sensitive restriction enzymes and Southern blotting in that: (i) it does not rely on an absence of cleavage to detect methylated sites; dependent on the site, a positive result (cleavage) can indicate either methylation or non-methylation; (ii) since the two strands of DNA produce different restriction enzyme sites, they can be differentially analyzed; (iii) closely related sequences, e.g. transfected and endogenous genes, as shown here, may be separately analyzed by using specific PCR primers.

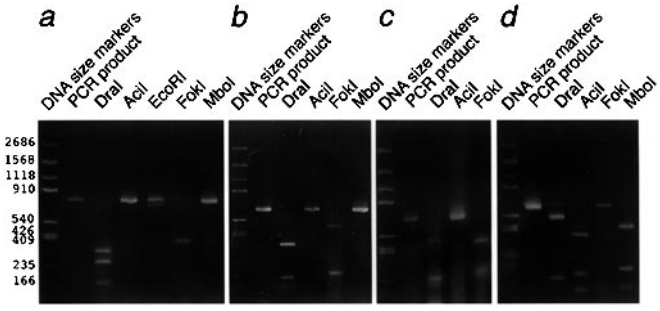

Figure 2. Demonstration of creation and loss of restriction enzyme sites by reaction with bisulfite. (**a**) Top-strand PCR product; bisulfite-treated control (non-transfected) genomic DNA as template. The 760-bp product was amplified from the endogenous CYP17 genes after reaction of genomic DNA with bisulfite. The pattern of digestion of the PCR product with *Dra*I indicates that only bisulfite-modified DNA was amplified. The digestion with *Fok*I shows that the CpG site shown in Figure 1 is non-methylated and the absence of digestion with *Aci*I and *Mbo*I shows that eight other CpG sites in the sequence are non-methylated. The presence of a shorter band below the full-length PCR product in the *Eco*RI lane indicates that the site shown in Figure 1 is partially methylated in these cells. Control digestions of other DNA with *Eco*RI and longer digestions of the PCR product with *Eco*RI showed that the result shown is not caused by a partial digestion by the enzyme. (**b**) Top-strand PCR product; bisulfite-treated genomic DNA from cells transfected with methylated DNA as template. The use of one plasmid-specific primer and one gene-specific primer ensured that only the transfected construct and not the endogenous CYP17 genes was amplified. As in (a), the pattern of digestion of the 549 bp product with *Dra*I indicates that only bisulfite-modified DNA was amplified. Digestion with *Fok*I and the absence of digestion with *Aci*I or *Mbo*I show that the originally fully methylated fragment has become demethylated in the cultured cells. (**c**) Bottom-strand PCR product; bisulfite-treated genomic DNA from cells transfected with methylated DNA as template. Digestion of the 655 bp product with *Fok*I shows that this CpG site has undergone demethylation on the bottom strand as well as the top strand in the transfected fragment. (**d**) Top-strand PCR product; methylated DNA fragment before transfection as template. Although the primers were designed to be complementary to the sequence following bisulfite modification, as shown here they also amplify the fully methylated fragment. This sequence remains unmodified in the presence of bisulfite, demonstrated by complete digestion with *Aci*I and *Mbo*I and single-site digestion with *Dra*I.

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