



A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands

(genomic sequencing/DNA methylation/bisulfite modification/PCR/kininogen gene)

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ABSTRACT The modulation of DNA–protein interactions by methylation of protein-binding sites in DNA and the occurrence in genomic imprinting, X chromosome inactivation, and fragile X syndrome of different methylation patterns in DNA of different chromosomal origin have underlined the need to establish methylation patterns in individual strands of particular genomic sequences. We report a genomic sequencing method that provides positive identification of 5-methylcytosine residues and yields strand-specific sequences of individual molecules in genomic DNA. The method utilizes bisulfite-induced modification of genomic DNA, under conditions whereby cytosine is converted to uracil, but 5-methylcytosine remains nonreactive. The sequence under investigation is then amplified by PCR with two sets of strand-specific primers to yield a pair of fragments, one from each strand, in which all uracil and thymine residues have been amplified as thymine and only 5-methylcytosine residues have been amplified as cytosine. The PCR products can be sequenced directly to provide a strand-specific average sequence for the population of molecules or can be cloned and sequenced to provide methylation maps of single DNA molecules. We tested the method by defining the methylation status within single DNA strands of two closely spaced CpG dinucleotides in the promoter of the human kininogen gene. During the analysis, we encountered in sperm DNA an unusual methylation pattern, which suggests that the high methylation level of single-copy sequences in sperm may be locally modulated by binding of protein factors in germ-line cells.

Cytosine methylation has long been recognized as an important factor in the silencing of genes in mammalian cells. Recent studies have shown that cytosine methylation at single CpG dinucleotides within the recognition sites of a number of transcription factors is sufficient to block binding of the factors to DNA (1–6) and to inhibit transcription (3–5). Therefore, to determine the role of cytosine methylation in specific regulatory mechanisms *in vivo*, it has become important to know the methylation status of individual CpG dinucleotides in genomic DNA. Genomic sequencing protocols, which have been developed to ascertain the methylation status of selected regions within genes, utilize the Maxam and Gilbert chemical cleavage reactions carried out on genomic DNA (7) with various additional procedures to enhance the signal from the sequence under investigation (8, 9). These protocols are versatile in that they can be adapted for identification of protein-binding sites on genomic DNA *in vivo* (8, 10) but have two major drawbacks with respect to the identification of 5-methylcytosine residues. First, 5-methylcytosine is identified by the lack of a band in all tracks of a

sequencing gel; any background cleavage ladder or close spacing of bands can result in difficulties of interpretation. Second, the sequence obtained represents a population average for many DNA molecules, so that the protocols cannot be adapted for sequencing small or mixed DNA samples. To address these problems, we have developed a genomic sequencing method that provides positive identification and localization of 5-methylcytosine in genomic DNA. The method is based on sodium bisulfite-mediated conversion of cytosine to uracil in single-stranded DNA (11–15), followed by PCR amplification of the resultant modified DNA. Exact methylation maps of single DNA strands from individual genomic DNA molecules can readily be established, where the position of each 5-methylcytosine is given by a clear positive band on a sequencing gel.

MATERIALS AND METHODS

DNA. Human DNA from HeLa cells, placenta, liver, and sperm was prepared by standard procedures (16, 17). A plasmid, pHKG2.0, containing the promoter and first exon of the human kininogen gene was constructed by subcloning into Bluescribe pBS+ (Stratagene) a 1.9-kilobase (kb) fragment (*Sca*I/*Eco*RI) from the plasmid pHKG5.2, a kind gift of N. Kitamura (18). To avoid possible PCR artefacts arising from plasmid contamination of human genomic DNA samples, the only cloned kininogen DNA permitted into the genomic sequencing laboratory had been previously methylated with *Alu*I methylase and tested for complete methylation by comparing the relative amounts of linear, relaxed, and supercoiled DNA before and after extensive *Alu*I digestion.

Sodium Bisulfite Modification. The bisulfite reaction was carried out on 10 μ g of DNA, consisting of plasmid DNA with a known methylation pattern, or of human genomic DNA containing added plasmid DNA at single-copy frequency, or of 2 μ g of human genomic DNA plus 8 μ g of carrier Bluescript plasmid. DNA was linearized with the restriction enzyme *Eco*RI or sheared through a fine needle, alkali denatured, neutralized, and precipitated. Denatured DNA was incubated in a total vol of 1.2 ml with freshly prepared 3.1 M sodium bisulfite/0.5 mM hydroquinone, pH 5.0, at 50°C for 16 or 40 hr under mineral oil, followed by successive dialyses at 4°C in large volumes of (i) 5 mM sodium acetate/0.5 mM hydroquinone, pH 5.2, (ii) 0.5 mM sodium acetate (pH 5.2), and (iii) deionized water, to remove unreacted bisulfite. The dialyzed solution was dried under vacuum and resuspended in 100 μ l of 10 mM Tris-HCl/0.1 mM EDTA, pH 7.5. NaOH was added to a final concentration of 0.3 M at room temperature for 10 min, followed by ammonium acetate (pH 7) to a

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final concentration of 3 M. The bisulfite-reacted DNA was precipitated, resuspended in 100 μ l of 10 mM Tris-HCl/0.1 mM EDTA, pH 7.5, and stored at -20°C for up to 2 months.

PCR Primers. Primers for Bluescript plasmid DNA were as follows: For the upper strand,

pBl a1: 5'-AACTGCGAGCCAACTTACTTCTAACAAC-3';

pBl a2: 5'-GTTTTAGAAATCTTAGTAATAAATTAGTTA-3'.

For the lower strand,

pBl b1: 5'-CTCCGAATTCATCAACAATAAACCAACCA-3';

pBl b2: 5'-ATAACTGCGAGTTAATTTATTTTGTATTA-3'.

These were used to amplify a 320-base-pair (bp) segment of bisulfite-reacted pBluescript KS+, from positions 2125–2443 (Stratagene). Primers for the kininogen promoter region in bisulfite-reacted human DNA were as listed in Fig. 1B.

PCR Amplification, Cloning, and Sequencing. The sequence of interest in the bisulfite-reacted DNA was amplified by PCR in two separate reaction mixtures, each containing one pair of strand-specific primers. Each amplification reaction was normally carried out on 1–5 μ l of DNA for 25–30 cycles using standard conditions, with denaturation at 94°C , annealing at 50°C – 55°C , and extension at 72°C . The amplified fragments were digested with the appropriate restriction enzymes, for which a site had been included in each strand-specific primer. The amplified fragments were gel purified and cloned into M13mp19. Individual clones were sequenced by the dideoxynucleotide chain-termination method.

RESULTS

Positive Identification of 5-Methylcytosine Following Treatment with Sodium Bisulfite. It has been demonstrated that, in single-stranded DNA, sodium bisulfite preferentially deaminates cytosine residues to uracil, compared with a very slow rate of deamination of 5-methylcytosine to thymine (13–15). To use this difference in bisulfite reactivity for genomic sequencing of 5-methylcytosine residues, total genomic DNA is fully denatured and treated with sodium bisulfite under conditions such that cytosine is converted stoichiometrically to uracil, but 5-methylcytosine remains nonreactive. We have used either mechanically sheared or restriction enzyme-cleaved DNA, which is then denatured by alkali prior to treatment with bisulfite. The second part of the procedure involves PCR amplification of any region of interest in the bisulfite-reacted DNA to yield a fragment in which all uracil (formerly cytosine) and thymine residues have been amplified as thymine and only 5-methylcytosine residues have been amplified as cytosine. Because the bisulfite reaction yields products in which opposite DNA strands are no longer complementary, it is possible to design two pairs of PCR primers such that each pair is specific for only one of the bisulfite-reacted DNA strands. The design of primers used to sequence the promoter region of the human kininogen gene is shown in Fig. 1. It is shown that the primers for each strand will differ in every position where there is a C or G in the original sequence.

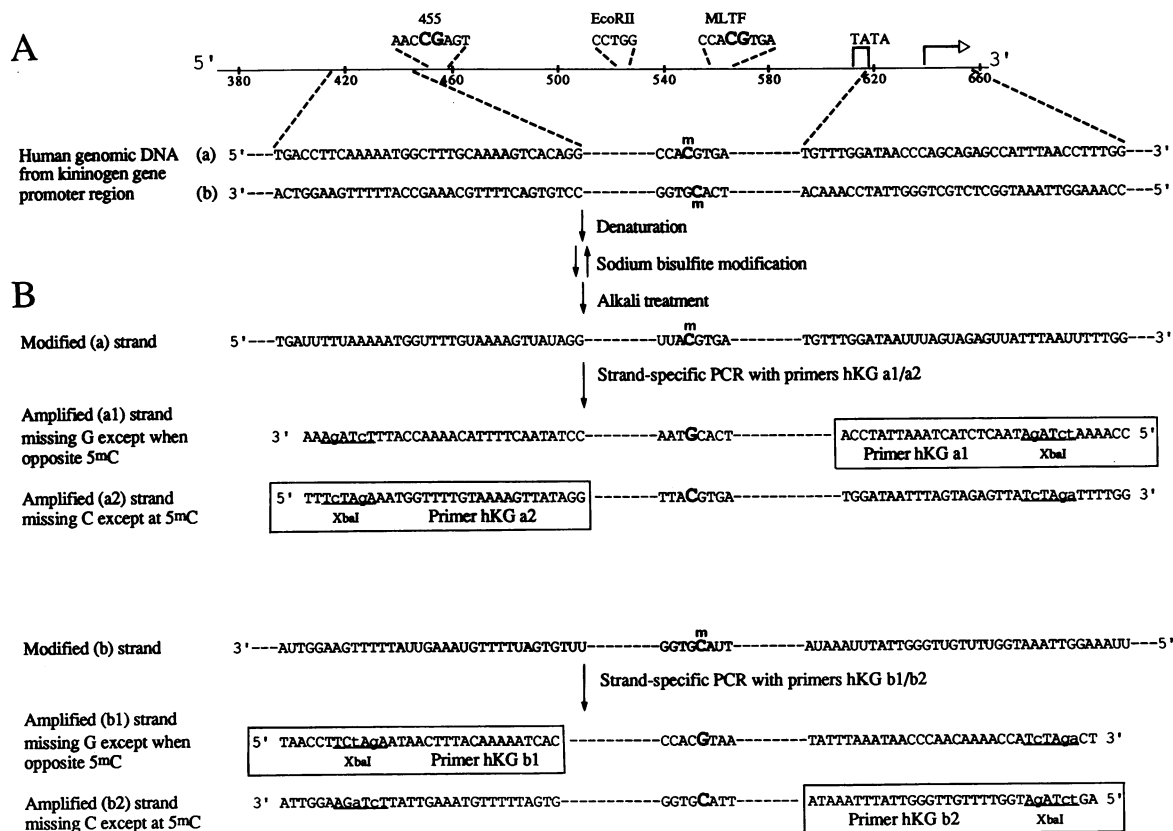


FIG. 1. Amplification of a sequence in the promoter region of the human kininogen gene after bisulfite treatment of genomic DNA. (A) Kininogen promoter region before bisulfite treatment. Numbers below the line represent base pairs, corresponding to EMBL and GenBank data base entries HSKIN01 and HUMKIN01. Locations are shown for the TATA box, the two CpG dinucleotides, and one CpTpG trinucleotide, which forms part of an EcoRII site. The most 5' transcription start site is marked with an arrow. Nucleotide sequences used to design strand-specific primers are given for both the upper (a) and lower (b) strands of genomic DNA. The sequence of a hypothetically methylated MLTF/USF site is shown between the primer sites. (B) Strand-specific primers for the bisulfite-reacted DNA. Sequences in the fully reacted (a) and (b) strands used to design the two pairs of strand-specific primers are shown. Sequences of the MLTF/USF site show that the location of 5-methylcytosine residues in the original genomic DNA are given by the location of any cytosine residues remaining on one strand and guanine residues remaining on the complementary strand of each PCR fragment.

The procedure yields a sequence and methylation pattern specific for each strand of the original genomic DNA. If the PCR products from the bisulfite-treated DNA are cloned and individual clones are sequenced, the sequences will provide methylation maps of single DNA strands from individual DNA molecules in the original genomic DNA sample. Alternatively, the PCR products can be directly sequenced to obtain a population average for each strand. In both instances, the position of each 5-methylcytosine will be given by a positive band on a sequencing gel.

Test Analyses of DNA Sequences with Known Methylation Patterns. The method was initially tested on Bluescript plasmid DNA, including an unmethylated sample and a sample in which at least 95% of all *Alu* I sites had been methylated. A 320-bp segment of the plasmid with three *Alu* I sites was selected as the region of interest and two pairs of primers were designed, one pair for each strand of the plasmid DNA. Initially, all combinations of the following bisulfite treatment conditions were assayed, each on 10 μ g of denatured plasmid DNA: sodium bisulfite at 3.1 and 3.9 M, pH 5.0 and 5.9, reacted at 37°C and 50°C for 48 and 96 hr. All samples were amplified with both sets of primers. The 96-hr reactions did not consistently yield PCR products, so these were discontinued. Of the 48-hr reactions, PCR products with both pairs of primers were more abundant for the reactions carried out in 3.1 M than in 3.9 M sodium bisulfite. The PCR products from the four sets of reaction conditions (pH 5.0 and 5.9; 37°C and 50°C) using 3.1 M sodium bisulfite were cloned into M13, and six representative clones for each pair of primers from each set of reaction conditions were sequenced. The optimal conditions (3.1 M sodium bisulfite; pH 5.0; 50°C) were next tested by adding Bluescript plasmid at single-copy frequency (10 pg) to 10 μ g of total genomic DNA. It was found that the bisulfite reaction could be carried out for 16, 40, or 48 hr, with no difference in the PCR end products. Representative sequencing gels are shown in Fig. 2.

Distribution of 5-Methylcytosine in a Segment of Mammalian Genomic DNA. To test its applicability to the analysis of single-copy sequences in genomic DNA, the bisulfite sequencing procedure was used to determine the methylation pattern of a 180-bp region located just upstream of the major transcription start site of the human kininogen gene (18). The kininogen promoter region contains two CpG dinucleotides, which we have referred to as CpG/455 and CpG/MLTF (Fig. 1A). CpG/MLTF is located within a strong binding site (unpublished observations) for the methylation-sensitive transcription factor MLTF/USF (3, 19–21). After bisulfite treatment of the genomic DNA, a fragment from the promoter region of the kininogen gene was amplified by PCR in separate reaction mixtures containing either primers hKG a1 + a2 or hKG b1 + b2, as shown in Fig. 1. The position of each 5-methylcytosine residue in the original DNA could then clearly be identified by the presence of a band in the C or G track, depending on the direction of sequencing of the strand-specific PCR product (Fig. 1B). The reactivity of unmethylated CpG dinucleotides at sites CpG/455 and CpG/MLTF was confirmed by separate bisulfite reactions using the plasmid pKMG2.0 methylated at *Alu* I sites to ensure that it could be differentiated from genomic DNA in case of contamination problems. To determine the relationship between methylation at the two CpG dinucleotides in single molecules, we cloned the PCR products in M13 and sequenced individual clones.

The methylation pattern of the kininogen promoter region was established for a number of cell types: liver, HeLa cells, placenta, and sperm (Fig. 3; Table 1). CpG/MLTF was deaminated by bisulfite in essentially all sequenced molecules from liver, an expressing tissue, and from nonexpressing HeLa cells. CpG/455, on the other hand, was nonreactive in 50/67 bisulfite-treated HeLa molecules compared to only

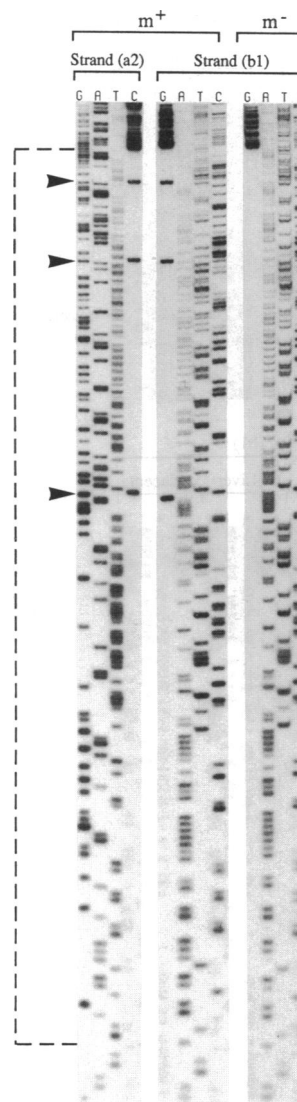


FIG. 2. Methylation maps of single DNA strands of a plasmid sequence with known methylation pattern. Cloned M13 inserts of PCR products, derived from bisulfite-treated Bluescript plasmid DNA using either the pBl a1 + a2 or pBl b1 + b2 primer pairs, were sequenced. The direction of sequencing was through the *Eco*RI site in the a2 or b1 primer. Thus, for the bisulfite-reacted (a) strand, the sequence reads as the original DNA, in which all cytosine residues have been converted to thymine except at three methylated *Alu* I sites. For the bisulfite-reacted (b) strand, the sequence of its complement is being read so that guanine residues have been converted to adenine except when opposite 5-methylcytosine sites on the original (b) strand. The extent of the Bluescript insert is marked by a dashed line. The locations of the three *Alu* I sites in the original Bluescript sequence are marked by arrowheads.

9/51 liver molecules (Table 1). We cannot say whether this tissue-specific difference in the extent of methylation at CpG/455 relates to transcriptional activity or whether it relates to the relatively high level of *de novo* methylation that occurs in the HeLa cell line (22). Interestingly, only the HeLa sequences showed any sign of strand-specific differences in methylation levels (Table 1), with a considerably higher level of nonreactive CpG/455 sites on the lower strand.

Placenta is known to be a tissue with low levels of total methylation (ref. 23 and references cited therein). In 19 sequenced strands from bisulfite-reacted placenta DNA, we identified only one m²CpG in the kininogen sequence (Table 1). With the exception of CpG islands, single-copy sequences in sperm are heavily methylated at CpG dinucleotides (23–26). Therefore, we were surprised to find that a major proportion (21/31) of the CpG/MLTF sites in sperm were deaminated by bisulfite—i.e., were unmethylated. The proportion of reactive CpG/455 sites in sperm, on the other hand, was lower (11/31). Bisulfite reactivity at the two sites did not occur at random in individual molecules; DNA strands that contained a nonreactive site at CpG/MLTF nearly always also contained a nonreactive site at CpG/455, indicating that the pattern did not result from a higher bisulfite reactivity of CpG/MLTF compared to CpG/455 (Table 1). Controls of plasmid DNA methylated at defined restriction sites and added at single-copy concentration to the bisulfite reaction mixtures did not manifest any differences in

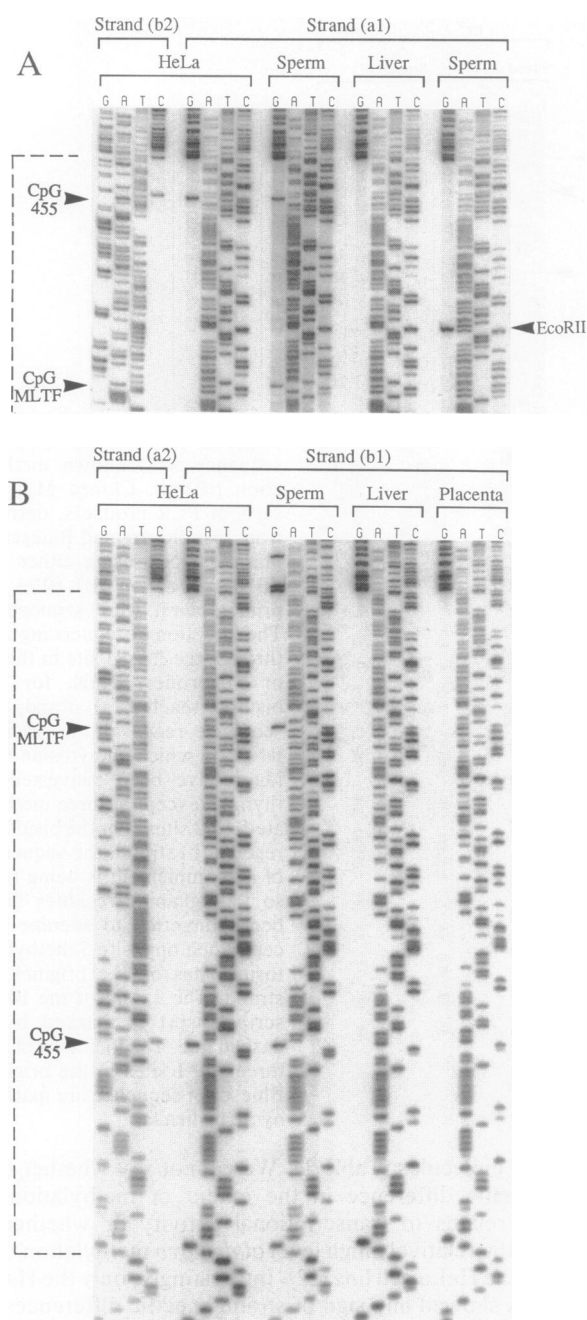


FIG. 3. Characteristic strand-specific sequences from the kininogen promoter region. (A) Strands (b2) and (a1) sequenced in the direction primer-CpG/MLTF-CpG/455-primer. (B) Strands (a2) and (b1) sequenced in the direction primer-CpG/455-CpG/MLTF-primer. The sequenced DNAs used for the bisulfite reactions and the DNA strands sequenced are shown above each set of sequencing products. The extent of the kininogen gene insert is marked by a dashed line. The location on the sequencing gels of CpG/455 and CpG/MLTF and the location of the CpNpG trinucleotide within an *EcoRII* site are marked by arrowheads.

reactivity of particular 5-methylcytosine residues. We conclude that the MLTF/USF binding site in sperm DNA is methylated less frequently than a CpG dinucleotide located 100 bp further upstream.

Digestion with the methylation-sensitive restriction enzyme *Pml* I (CACGTG), which recognizes the core of the MLTF/USF binding site and does not digest either a fully methylated or a hemimethylated site, has confirmed this result. HeLa, placenta, and liver genomic DNA showed total digestion at the kininogen *Pml* I site on Southern blots,

whereas sperm DNA was partially digested to $\approx 50\%$. Methylation at CpG/455 could not be tested by restriction analysis.

We found no methylation at any non-CpG site in DNA from HeLa cells, liver, or placenta. However, in a total of 31 sequenced molecules from sperm DNA, we found 3 molecules that had not become deaminated by bisulfite at a single CpTpG/CpApG, which was located within an *EcoRII* site (Figs. 1A and 3). We do not believe that this rare CpTpG/CpApG methylation site in sperm represents a plasmid contaminant. The three nonreactive sites occurred in two separate amplification reactions. One sequence was an (a) strand, and two were (b) strands with different methylation patterns at CpG/455 and CpG/MLTF (m^+-m^- and m^--m^-). None of the three apparently *EcoRII*-methylated molecules from sperm was also methylated at an *Alu* I site within the 180-bp sequenced region.

DISCUSSION

Using both *in vitro* methylated plasmid DNA and mammalian genomic DNA, we have demonstrated the feasibility of identifying specific patterns of cytosine methylation by using the discrimination in deamination reactivity of cytosine and 5-methylcytosine provided by bisulfite. We have found no consistent lack of reactivity of any unmethylated cytosine residue in the test substrates. Therefore, false positives or overestimation of the extent of a partially methylated sequence does not present a problem in the analysis. It should be possible to use the bisulfite method to obtain a good estimate of exact methylation levels. A small proportion of 5-methylcytosine residues in single-stranded DNA may be deaminated to thymine by the bisulfite treatment (15), so that the exact proportion of 5-methylcytosine in the original DNA may be slightly greater than the measured proportion of nonreactive sites after bisulfite treatment and PCR. We have found no difference between the reactivities of the various methylated sites analyzed in plasmids of known methylation pattern. By carrying out concomitant bisulfite reactions with methylated cloned DNA including the region of interest and incorporating a methylated plasmid control into each genomic bisulfite reaction, the extent of 5-methylcytosine reactivity can be accurately determined for each sequencing experiment. For example, based on *Alu* I-methylated plasmid controls, we can estimate that 35/42 nonreactive HeLa (b) strand sequences (Table 1) indicate a level of methylation of CpG/455 in the (b) strand of close to 100%, and that the reactivity values of the other sites could be increased proportionately to yield exact methylation level.

Major advantages of the bisulfite method are (i) the positive display of 5-methylcytosine and (ii) the capacity to generate sequence data for individual DNA strands from single DNA molecules. In methods dependent on Maxam and Gilbert sequencing reactions, each cleaved DNA strand contributes to one band in the sequencing gel. By contrast, the methylation status of every cytosine in a single bisulfite-reacted molecule can be read in the cloned product. This can be of great advantage, for example, when analyzing the successive demethylation of sites along a DNA strand as in the chicken vitellogenin gene (27) and the E2A promoter of integrated adenovirus (28) or when the DNA being analyzed comes from a mixed population of cells. The data can be related to the parental origin of the DNA of interest (25, 29–32), the direction of transcription, or the binding characteristics of specific protein factors.

The minimum quantity of genomic DNA, analyzed in the experiments described here, was 2 μ g in a total reaction mixture of 1.2 ml. This is already 2- to 5-fold less than the quantity used in Maxam and Gilbert-based procedures. Smaller quantities of genomic DNA mixed with plasmid

Table 1. Methylation patterns of individually sequenced strands of the kininogen gene promoter region

Tissue/cell type	No. of strands with given methylation pattern CpG/455-CpG/MLTF				No. of independent amplifications	No. of bisulfite reactions
	m ⁺ -m ⁺	m ⁺ -m ⁻	m ⁻ -m ⁺	m ⁻ -m ⁻		
Liver	2	7	0	42	5	2
HeLa	2	48	0	17	7	2
Sperm	8	12	2	9	3	2
Placenta	0	1	0	18	3	1
HeLa upper strand (a)	1	14	0	10	3	2
HeLa lower strand (b)	1	34	0	7	4	2

carrier DNA were not assayed. It can be appreciated that, with no change in reaction conditions, the procedure could immediately be scaled down to permit sequencing of 200 ng or less of genomic DNA in a total reaction mixture of 120 μ l. Since each DNA strand is a source of complete methylation data, there is no theoretical limit to the sensitivity of the procedure. In practice, a certain amount of DNA degradation occurs, presumably as a result of depurination under the acid conditions required for the bisulfite reaction. We expect that, with careful optimization of conditions to maximize the cytosine to uracil deamination reaction while minimizing the extent of depurination, methylation analysis of DNA from a very small sample of cells should be achievable.

The bisulfite genomic sequencing method identified variable, tissue-specific levels of methylation at two CpG dinucleotides within the kininogen promoter fragment. The kininogen sequence also contained a number of CpNpG trinucleotides that have been proposed as methylation sites in vertebrate genomes (33). Isolated m⁵CpA and m⁵CpT dinucleotides have previously been identified in an integrated adenovirus type 2 genome (28). However, no consistently nonreactive cytosine residues were found in any other sequence elements, with the exception of one CpTpG/CpApG trinucleotide in sperm DNA. The significance of this result is uncertain, since only 10–15% of sperm molecules appear to be methylated at the CpTpG/CpApG site.

The binding site for a ubiquitous, methylation-sensitive transcription factor, MLTF/USF, was unmethylated in DNA from both expressing liver and nonexpressing HeLa cells, suggesting that sequence-specific methylation at the MLTF/USF site does not determine the transcriptional status of the kininogen gene. In sperm DNA, the MLTF/USF site was methylated much less frequently than a CpG dinucleotide located just 100 bp further upstream. This result indicates that the methylation pattern established in germ-line cells may be modulated by the binding characteristics of a ubiquitous transcription factor and, in general, may depend on local competitive interactions between the methylase enzyme and protein factors bound to DNA.

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