Reliable Detection of DNA CpG Methylation Profiles by the Isoschizomers MspI/HpaII Using Oligonucleotide Stimulators


The increasing interest in methylation patterns of mammalian DNA demands a simple and reliable method to clearly differentiate between cytosine (C) and 5-methylcytosine (5-MeC) within a specific DNA region. The simplest and most commonly used approach is the analysis with restriction endonucleases exhibiting different methylation sensitivities, like the MspI/HpaII pair of isoschizomeric enzymes (New England Biolabs, Beverly, MA, USA). Whereas MspI cleaves the recognition sequence 5′-CCGG independently of the methylation state of the internal C, cleavage by HpaII is blocked by the presence of 5-MeC at this site (3). The method is compromised by the fact that methylation can be detected only in those CpGs that are located within the tetranucleotide recognition sequence.

Another problem is that not all restriction endonucleases cleave their respective unmethylated recognition sequences completely. An incomplete digestion of unmethylated DNA is typically observed when DNA substrates with a low frequency of recognition sites are incubated with restriction endonucleases requiring at least two copies of the recognition site for cleavage activity (2). HpaII seems to belong to this group of restriction endonucleases, because genomes possessing only one or very few recognition sites [simian virus 40 (SV40) (Reference 4 and unpublished) and hamster polyoma virus (unpublished)] exhibit an intrinsic resistance towards this enzyme. This means that unmethylated DNA molecules are not (or are not completely) digested by the enzyme, mimicking a
DNA methylation which, however, does not exist. This would lead to false-positive results concerning the methylation state of the DNA under investigation. The aim of our study was to find a solution to this problem leading to an easy and reliable methodological approach for CpG methylation detection.

For our investigation, we used SV40 DNA, which contains a single MspI/HpaII site, as a substrate. Methylated and unmethylated viral DNAs were incubated with MspI or HpaII (Figure 1). Complete cleavage was only observed with MspI (lanes 4 and 8). HpaII failed to cleave the recognition site in the methylated (lane 2) as well as in the unmethylated genome (lane 6). As was previously demonstrated for EcoRII and several other restriction endonucleases that require the simultaneous interaction with two recognition sites for DNA cleavage, the intrinsic resistance of (unmethylated) DNA sites could be overcome by adding site-containing oligonucleotide duplexes (4–6,9). Lane 7 shows that cleavage of the unmethylated site by HpaII was driven to completion by the addition of site-containing oligonucleotide duplexes, whereas the specifically methylated site remained resistant under the same conditions (lane 3). We analyzed the dependence of HpaII cleavage on the concentration of added oligonucleotide duplexes and found that complete digestion required a molar excess within the 100-fold range of oligonucleotide duplexes over substrate sites. Furthermore, linearized, nonmethylated SV40 DNA was cut by HpaII to about 50%, and this reaction could also be stimulated by site-specific oligonucleotide duplexes (data not shown), as in the case of circular SV40 DNA.

Besides the oligonucleotide duplex described in the legend to Figure 1, we also used a self-complementary 14-mer oligonucleotide (5′-TATAGCCGGCTATA) that could stimulate the enzyme in an even broader concentration range from a 20–100-fold molar excess over substrate sites (data not shown). As shown previously for EcoRII, this can be explained by the fact that the efficiency of intermolecular cooperative interaction is inversely proportional to the length of the activator DNA (2).

Figure 1. MspI/HpaII cleavage of M.HpaII-methylated and unmethylated circular SV40 DNA. 180 ng (0.05 pmol) DNA were digested with 2 U of HpaII (lanes 2, 3, 6 and 7) or MspI (lanes 4 and 8) for 2 h under standard conditions (10 mM bis-Tris propane-HCl, 10 mM MgCl₂, 1 mM dithiothreitol [DTT]). Lanes 1 and 5 contain undigested DNA. Methylated SV40 DNA was prepared by incubating 10 µg DNA with 4 U HpaII methylase for 1 h at 37°C in reaction buffer (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 5 mM 2-mercaptoethanol) supplemented with 80 µM S-adenosylmethionine. 5 pmol of the specific oligonucleotide duplex (with terminal overhangs) 5′-GATCCGGAGCGGCCGCGCCGCGGCCTG, 5′-AATTCAGGCGCGGCCGCGCCGCTCCG (the HpaII recognition site is underlined) were included in the reaction mixtures loaded in lanes 3 and 7. The 1-kb ladder was used as a molecular weight marker (M).

Figure 2. DNA sequencing of unmethylated (A) and M.HpaII-methylated (B) SV40 DNA (nucleotides 334–361) after bisulphite treatment. 1.95 µg pBR322 DNA were added to 50 ng of linearized SV40 DNA and used in the bisulphite conversion reaction. 2 µL of the resulting 100 µL chemically modified DNA were used as template in a 50-µL PCR mixture. The reaction conditions were found to be optimal at 3 mM MgCl₂ for 1 min at 94°C for denaturation, 2 min at 72°C for annealing/extension for 25 cycles. For the amplification, AmpliTaq® DNA Polymerase (Perkin-Elmer, Norwalk, CT, USA) and the specific primers SV40F1 (5′-TTTGGTTGTTGATTAATTGAGATGTATGTTTTG) and SV40F2 (5′-CAGCTTCAATTACAACAAACACCTCTCGCAACAC) were used. The PCR products were resolved on a 1.2% agarose gel and purified using the QIAEX II Gel Extraction Kit (Qiagen, Chatsworth, CA, USA). After cloning, the purified recombinant plasmid DNA was cycle-sequenced with Thermo Sequenase™ (Amersham, Arlington Heights, IL, USA) and the sequence resolved on an ALF DNA Sequencer™ (Pharmacia Biotech, Piscataway, NJ, USA). The relevant HpaII recognition site is underlined.
Therefore, we would suggest using this type of oligonucleotide duplex in experiments with mammalian genomic DNA where it is more difficult to calculate the DNA and resulting substrate site concentration. To stimulate the cleavage of agarose-embedded DNA, we added a tenfold excess of oligonucleotide duplexes compared to that of the corresponding digest without agarose (data not shown).

Several other methods have been developed for determining the methylation state of all cytosines in each strand of a native DNA (8). We chose bisulphite genomic sequencing to confirm the methylation state of the MspI/HpaII site in the different SV40 DNA substrates and to compare both methods. When DNA is treated with sodium bisulphite, unmethylated cytosine residues are exclusively converted to uracil, whereas 5-MeC yields cytosine residues detectable in the sequencing reaction of the amplified material. Our test substrate, SV40 (M.HpaII-methylated or unmethylated), was subjected to the bisulphite treatment according to Clark et al. (1). The gel-purified polymerase chain reaction (PCR) products were cloned in a TA Cloning® Vector (Invitrogen, Carlsbad, CA, USA) and then sequenced. In Figure 2, the respective sequencing data are shown. When unmethylated template was used in the bisulphite reaction, the HpaII recognition site was converted to 5′-TTGG (representing 5′-CCGG in the original sequence; Figure 2A). The sequence 5′-TCGG appeared at the same position in the case of the M.HpaII-methylated template, representing the original sequence 5′-CmCGG (Figure 2B).

For a quantitative determination of the methylation state of a DNA population, a large number of individual clones has to be sequenced and the results statistically evaluated. On the other hand, densitometry of restriction digestion patterns yields the average degree of DNA methylation. Hence, the use of the recommended oligonucleotide duplex stimulation of HpaII, which ensures complete cleavage of intrinsically resistant unmethylated sites, is a faster way of analysis and does not require the establishment of more sophisticated methods in the laboratory.

We present an improved protocol for detecting CpG methylation by the isoschizomeric restriction endonucleases MspI/HpaII, generating highly reliable cleavage results. Since we have found that the principle of oligonucleotide duplex stimulation is also applicable to the cleavage of agarose-embedded DNA (data not shown), the protocol is suitable for the analysis of larger genomic DNA. Restriction analysis with the isoschizomeric restriction endonucleases MspI/HpaII can be considered a fast initial examination that should be
followed up by bisulphite genomic sequencing to determine the methylation state of every cytosine residue within a sequence region for which differences in the MspI/HpaII restriction pattern are observed.

Note that in some previously published investigations, sites partially resistant to HpaII digestion were interpreted as being partially methylated. Therefore, we would like to stress the importance of the intrinsic resistance of certain DNA sites to HpaII and its methodological consequences. It is of interest in this context that the detection of Dcm methylation at the recognition site 5’-CC(A/T)GG with the isoschizomeric enzymes BstNI/EcoRII can also be optimized by adding sequence-specific oligonucleotide duplexes (7).

REFERENCES


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