Sensitivity of *HincII* to CpG methylation

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Received December 2, 1992; Revised and Accepted February 22, 1993

Restriction Fragment Length Polymorphism (RFLP) analysis in human genetics can be confounded by sensitivity of restriction enzymes to the cytosine methylation that occurs at many cytosine–guanine (CpG) dinucleotides. Previous reports provided conflicting information on the sensitivity of *HincII* to C methylation (1–3). We identified alleles at a chromosome 4p16 locus that were inherited in a non-Mendelian manner due to inhibition of *HincII* cleavage by CpG methylation. We confirmed that cleavage by *HincII*, whose recognition sequence is 5' GTPyPuAC 3', is inhibited by the presence of 5-methylcytosine at the terminal base of its recognition site.

To assay for methylation sensitivity of *HincII*, we used CpG Methylase, which methylates DNA *de novo* at CpGs (4). We treated two plasmids of known sequence that contain *HincII* sites with 24 units of CpG Methylase/μg DNA in 140 μl overnight at 37°C. Then 0.5 μg of methylated or unmethylated plasmid DNA was digested overnight with 20 units of *HincII* in 35 μl. This level of overtreatment is many-fold greater than is commonly used for genomic DNA digestion. Plasmid 1 contains 5 *HincII* sites, none of which contain an internal CpG, but two of which are followed by a G, so that the terminal C of the site is part of a CpG. One *HincII* site in Plasmid 1 followed by a G was not cut, while the other site was cut partially. The other three sites, which do not contain or overlap with CpGs, were cut to completion (Figure 1). We also tested plasmid Clone B, which contains a *HincII* site of sequence 5' AGTTAACG 3' from the HOX7 gene (5) and a site in the vector that contains an internal CpG, but is not followed by a G, allowing us to test whether *HincII* digestion is inhibited by methylation of the two Cs in the internal CpG. Cleavage at only one of the two sites in Clone B was inhibited by methylation (Figure 1). Double digests indicated that methylation inhibited cleavage at the *HincII* site that is followed by a G, but not at the site with the internal CpG (data not shown).

These results suggest that digestion by *HincII* is inhibited by methylation of the terminal C of the *HincII* site, although the degree of inhibition or methylation varies at different sites. Furthermore, methylation of internal Cs in the CpG dyads found in some *HincII* sites does not inhibit digestion by the enzyme when extensive overtreatment is used. Our results agree with previous data suggesting that *HincII* is inhibited by complete C methylation (3), and further indicate that the inhibition is due to methylation of the terminal C of the site.

We suggest that caution be used when employing *HincII* for human genetic studies, because the enzyme's activity is affected by CpG methylation. CpG Methylase could be useful for mammalian geneticists because it allows the focused determination of restriction enzyme sensitivity to methylation at any CpG dinucleotide contained within, or overlapping, the site of the enzyme of interest, since it is less restricted by sequence specificity than are most methylases. It also allows the separation of the effects of CpG methylation from those of other forms of C methylation.

ACKNOWLEDGEMENTS

This work was supported by NIH Training Grant GM07810 (L.N.B.), grants from the N.I.H. and the Wills Foundation (R.M.M. and D.R.C.) and a grant from the Medical Research Council UK (J.E.H.).

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Figure 1. *HincII* digests of DNAs containing multiple *HincII* sites, with and without CpG Methylase pretreatment. Samples were digested with *HincII* without prior CpG Methylase treatment (–), after a mock reaction lacking CpG Methylase (m), or after CpG Methylase treatment (+). Filled arrows designate fragments produced by complete inhibition of digestion at two *HincII* sites, and by *HincII* cleavage when the DNA is unmethylated. Open arrows indicate fragments produced by partial inhibition of digestion at a *HincII* site, and the two corresponding fragments produced when cleavage is not inhibited.

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