Restriction enzyme **Hin**cII is sensitive to methylation of cytosine that occurs 5′ to the recognition sequence

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Received November 12, 1995; Revised and Accepted January 22, 1996

In this paper we demonstrate that the **Hin**cII restriction endonuclease, in addition to being sensitive to methylation of the 3′ A and C residues, is also sensitive to methylation of a cytosine immediately 5′ to the recognition sequence. Having encountered this property in one of the sites in the mouse c-fos gene, we confirmed the sensitivity of **Hin**cII to the 5′ cytosine methylation in *in vitro* methylated pUC12, pBR322 and p-fos-1 plasmids.

**Hin**cII is a six base-cutter which recognises the sequence GTPyPuAC, the cleavage site being between the Py/Pu. Its activity is known to be sensitive to the methylation of the A residue in the sequence (1). Recently, Bull et al. (2) used plasmid constructs having GTCGACG sequence for **Hin**cII digestion and showed that the presence of methylated cytosine flanked by a G at the 3′ end of recognition sequence inhibits **Hin**cII digestion. They also showed that methylation in the internal CpG did not affect the digestion. However, the **Hin**cII sensitivity to C methylation has not been universally accepted (see ref. 3). Here, we report that in the DNA motif CGTCGACC, **Hin**cII digestion is sensitive to methylation of the 5′C which is not a part of its recognition sequence. We also confirm that methylation of the internal C has no effect on the digestion by **Hin**cII.

While studying kinetics of methylation at individual CpGs in the c-fos gene during mouse development, one of the sites analysed was CGTCGACC, present at the 3′ end of the gene. Both SalI and **Hin**cII cleave this sequence, except that SalI is sensitive to the internal CpG methylation while **Hin**cII is not (2–4). However, not only SalI but also **Hin**cII showed differential sensitivity patterns between the fetal and adult liver as well as brain (unpublished observations). As seen in Figure 1, **Hin**cII digestion leads to two fragments (3.9 kb and expected 2.4 kb) in the fetal tissues (Fig. 1, lanes 1 and 3) but in the adult, liver shows only 1 fragment (3.9 kb; Fig. 1, lane 4), brain shows, in addition to the 3.9, a fainter 2.4 kb fragment (Fig. 1, lane 2). In order to test whether this novel **Hin**cII pattern was due to methylation of the C residues in this sequence the following experiment was done.

Plasmids pBR322 (4.36 kb), v-fos cloned in pBR322 (p-fos-1 clone, 5.6 kb) and pUC12 (2.68 kb), which are known to have CGTCGAC, were *in vitro* methylated with SssI methyltransferase (10 µg DNA with 12 U M.SssI at 37°C for 1 h in the presence of 160 µM S-adenosyl methionine in 50 µl reaction volume) and then digested overnight with excess amount of **Hin**cII (20 U/1 µg). The *in vitro* methylated pBR322, which has two

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enzyme, and since both the sites were resistant to the enzyme, it was most likely due to methylation of the cytosine upstream to the HindII site. The possibility of interference in the HindII digestion by the internal CpG dinucleotide was ruled out by digesting the M.SssI treated pUC12, which harbours one HindII site (5'‐AGTCGACC‐3'), with HindII. pUC12 was completely digested (Fig. 3, lane 4). Since the present investigation was initiated in the proto-oncogene, c-fos, which contains one HindII site 5'‐CGTCGACC‐3' immediately downstream of the stop codon site (5), HindII digestion was tested in a v-fos gene cloned in pBR322 (pfos-1 clone). In this construct, there were three HindII sites, one of v-fos and two of pBR322 and the sequence in v-fos gene was similar to the one at 651 bp in pBR322. Following M.SssI methylation the v-fos site showed complete resistance to the enzyme, as seen in the genomic DNA from adult tissues (Fig. 4, lane 7). The pBR322 sites showed partial cleavage as earlier observed with the pBR322 DNA. The efficiency of methylation by M.SssI in the above reactions was checked by performing digestions with MspI and HpaII (Fig. 4, lanes 3 and 4).

The above results provide strong evidence in favour of HindII sensitivity to the methylation of cytosine occurring 5' to its recognition sequence. Viewed together with the observation of Bull et al. (2), which shows HindII sensitivity to the methylation of terminal cytosine in the 5'-GTCGACC-3' sequence, it is clear that HindII is sensitive not only to the 3' flank CpG methylation, but also to the 5' flank CpG methylation. Therefore, care is warranted while using HindII restriction enzyme in methylation studies.

ACKNOWLEDGEMENTS
Authors are pleased to record their appreciation of Dr R.J. Roberts, NEB, USA for his interest and advice, and New England BioLabs, USA for the gift of SssI methyltransferase. Funding for the work was provided by the Department of Science & Technology, New Delhi (to R.R.). K.C. is grateful to the Council of Scientific & Industrial Research for the Senior Research Fellowship.

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