Nucleic Acids Research, 1996, Vol. 24, No. 6 **1045–1046**

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

Kanduri Chandrasekhar* and Rajiva Raman

Cytogenetics Laboratory, Department of Zoology, Banaras Hindu University, Varanasi 221005, India

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 **GTCGAC**G 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 C**GTCGACC**, *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 C**GTCGAC**C, present at the 3′ end of the gene. Both *Sal*I and *Hin*cII cleave this sequence, except that *Sal*I is sensitive to the internal CpG methylation while *Hin*cII is not (2–4). However, not only *Sal*I but also *Hin*cII showed differential sensitivity patterns between the fetal and adult liver as well as brain (unpublished observations).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 C**GTCGAC**, were *in vitro* methylated with *Sss*I methyltransferase (10 µg DNA with 12 U M.*Sss*I 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

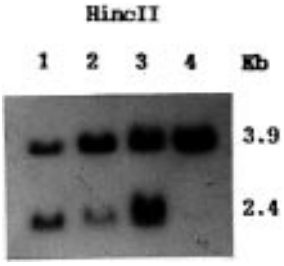

Figure 1. Southern hybridisation of *Hin*cII-digested genomic DNAs from fetal brain (lane 1), adult brain (lane 2), fetal liver (lane 3) and adult liver (lane 4) with 368 bp *Msp*I fragment from v-*fos*.

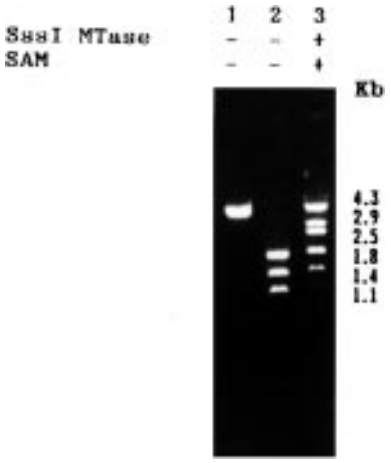

Figure 2. *Pvu*II-linearized pBR322 plasmid DNA (lane 1), digested with *Hin*cII (lane 2) prior to methylation, and after methylation (lane 3).

*Hin*cII sites (5′-C**GTCGAC**C-3′ at 651 bp, and 5′-C**GTCAAC**C-3′ at 3905 bp positions), yielded six fragments (Fig. 2, lane 3) as against the expected three fragments in the unmethylated, linearised plasmid (Fig. 2, lane 2). This result clearly indicated that when methylated the two *Hin*cII sites were only partially cleaved even at high concentration of the *Hin*cII

* To whom correspondence should be addressed

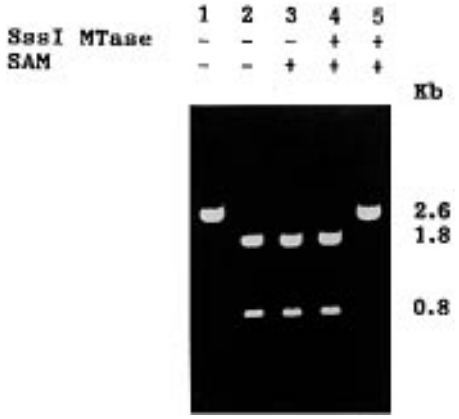

Figure 3. Digestion of *Xmn*I-linearized pUC12 plasmid DNA (lane 1), with *Hin*cII (lane 2), in the presence of *S*-adenosyl methionine alone (lane 3), and after methylation with M.*Sss*I (lane 4). Lane 5 contains M.*Sss*I treated *Sal*I digested pUC12 DNA.

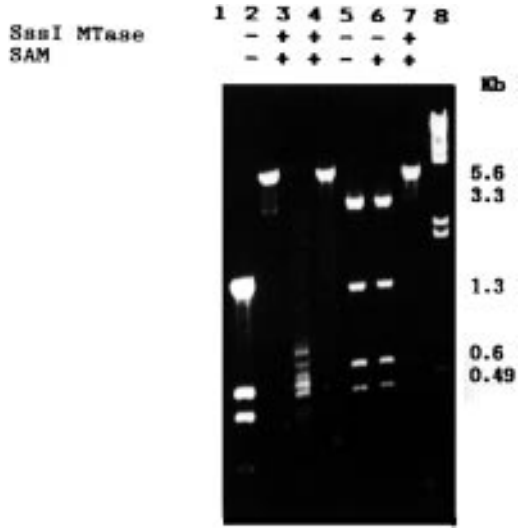

Figure 4. *Hin*cII digestion pattern of the p*fos*-1 clone (*Bgl*II–*Pvu*II fragment of v-*fos* gene cloned in pBR322). Lanes 1 and 8 contain reference molecular weights consisting of *Hinf*I-digested pUC13 plasmid DNA (1419, 517, 396, 214, 75 and 65 bp) and *Hin*dIII-digested lambda DNA, respectively. Lane 2, *Hin*dIII-linearized p*fos*-1 clone; lane 3, *Msp*I-digested M.*Sss*I-treated p*fos*-1 clone; lane 4, *Hpa*II-digested M.*Sss*I-treated p*fos*-1 clone; lane 5, *Hin*cII digested unmethylated p*fos*-1 clone; lane 6, *Hin*cII digestion of p*fos*-1 clone in the presence of *S*-adenosyl methionine alone and lane 7, *Hin*cII-digested p*fos*-1 clone after treatment with M.*Sss*I.

enzyme, and since both the sites were resistant to the enzyme, it was most likely due to methylation of the cytosine upstream to the *Hin*cII site. The possibility of interference in the *Hin*cII digestion by the internal CpG dinucleotide was ruled out by digesting the M.*Sss*I treated pUC12, which harbours one *Hin*cII site (5′-A**GTCGAC**C-3′), with *Hin*cII. pUC12 was completely digested (Fig. 3, lane 4). Since the present investigation was initiated in the proto-oncogene, c-*fos*, which contains one *Hin*cII site 5′-C**GTCGAC**C-3′ immediately downstream of the stop codon site (5), *Hin*cII digestion was tested in a v-*fos* gene cloned in pBR322 (p*fos*-1 clone). In this construct, there were three *Hin*cII 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.*Sss*I 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.*Sss*I in the above reactions was checked by performing digestions with *Msp*I and *Hpa*II (Fig. 4, lanes 3 and 4).

The above results provide strong evidence in favour of *Hin*cII sensitivity to the methylation of cytosine occurring 5′ to its recognition sequence. Viewed together with the observation of Bull *et al.* (2), which shows *Hin*cII sensitivity to the methylation of terminal cytosine in the 5′-**GTCGAC**G-3′ sequence, it is clear that *Hin*cII is sensitive not only to the 3′ flank CpG methylation, but also to the 5′ flank CpG methylation. Therefore, care is warranted while using *Hin*cII 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 *Sss*I 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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