Detecting differences in 5-methylcytosine using restriction enzyme isoschizomers: an endogenous control for complete digestion

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Received May 31, 1995; Revised and Accepted October 9, 1995

Growing research effort in recent years has focused on the role of 5-methylcytosine (5-MeC) in gene expression. 5-MeC has been implicated in such phenomena as genomic imprinting, maintenance of heterochromatin, and the direct control of gene expression through proteins which bind methylated DNA (1). As interest in 5-MeC has intensified, several new techniques have been developed to find and study genes under the influence of 5-MeC (2-4). Despite these new techniques, Southern blot analysis of genomic DNA cut with methylation-sensitive isoschizomers like *MspI/HpaII* has remained the simplest technique for detecting 5-MeC differences between cell populations. Incomplete digestion by the methylation-sensitive isoschizomer, however, can cause both false positives and false negatives; thus a simple control is desirable to ensure that the enzymatic digestions are complete.

We have determined that the mitochondrial genome, which is devoid of 5-MeC in mammals, can be used as an endogenous control for complete digestion by *HpaII* or any other methylation-sensitive restriction enzyme (5). Previous efforts to control for complete enzymatic digestion have involved the addition of foreign unmethylated DNA (such as a plasmid) to the digestion reaction or the addition of a digestion reaction aliquot to the foreign DNA (6,7). The mitochondrial genome makes a more powerful control because it is co-isolated with genomic DNA, thus controlling for every step of the DNA isolation and Southern analysis. In addition, using the endogenous mitochondrial genome eliminates the additional steps involved with previous controls.

To demonstrate the use of mitochondrial DNA in detecting incomplete digestion and the false negatives that can result a blot was prepared with DNA digested with *MspI*, *HpaII* or *HpaII* partially inactivated by heat (2.5 or 5 min at 75°C) or 1% sodium dodecyl sulfate (SDS) in the reaction. Genomic DNA isolation and blot preparation followed standard procedures (8). The blot was first probed with a portion of thymidine kinase (TK), a gene that displays methylation differences between the human multiple myeloma cell lines RPMI 8226 (8226/S) and 8226/V (9). *MspI*, which is insensitive to the methylation state of the internal cytosine in its recognition sequence 5'-CCGG-3', produces three gene fragments which were recognized by the TK probe (Fig. 1, left, lanes 1 and 2). The *MspI* fragments demonstrate the limit digest of TK in 8226/S and 8226/V and confirm that TK has remained intact in the drug selected 8226/V cell line. In contrast, digestion of 8226/S and 8226/V DNA with *HpaII*, which does not cut if the internal

Figure 1. Southern blot analysis of the thymidine kinase gene and the mitochondrial genome. S, 8226/S. V, 8226/V. DNA loaded in lanes 5-10 was cut with *HpaII* that had been inactivated as indicated. All restriction digests were carried out overnight at 37°C. Molecular weight markers were derived from *HindIII*-digested λ DNA. Numbers on the left indicate size in kilobases.

cytosine in the recognition sequence is methylated (5'-CmCGG-3'), did not produce identical TK gene fragments (Fig. 1, left, lanes 3 and 4). The changes in the TK *HpaII* fragment pattern of 8226/V as compared with 8226/S indicate that there has been an overall increase in cytosine methylation of the TK gene as a result of drug selection. Finally, partial digestion with inactivated *HpaII* (Fig. 1, left, lanes 5-10) shows large TK fragments with no apparent 5-MeC differences between 8226/S and 8226/V. These results, produced by incomplete *HpaII* digestion, erroneously suggest that almost all potential sites of methylation within the TK gene region of the two cell lines contain a 5-MeC.

After removal of the TK probe, the blot was re-probed with a portion of the mitochondrial genome (nucleotides 10244-11921) which recognizes two mitochondrial fragments of 0.43 and 2.3 kb in complete *MspI/HpaII* digests (Fig. 1, right, lanes 1-4) (10).

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The appearance of only the 0.43 and 2.3 kb fragments confirms that the restriction digests in the first four lanes were complete. Multiple mitochondrial DNA fragments were produced, however, by the incomplete *HpaII* digestion seen in lanes 5–10 (Fig. 1, right). Based on these results, an investigator could conclude that the different fragment patterns seen in *HpaII*-digested genomic DNA hybridized with TK (Fig. 1, left, lanes 3 and 4) were the result of real differences in 5-MeC levels within the cell lines' TK gene. Similarly, it can be concluded that the results of the incomplete digestions, which suggested that both cell lines' TK genes were highly methylated, are incorrect.

In addition to eliminating the additional steps required with exogenous controls, using the mitochondrial genome has two additional benefits inherent to its high copy number: strong signal and high sensitivity. In conclusion, the mitochondrial genome provides a flexible and facile endogenous control for restriction digests of nuclear DNA in the detection and analysis of 5-methylcytosine differences between cell populations.

ACKNOWLEDGEMENTS

This work was supported in part by grant CA49049 to W. S. Dalton. The authors wish to thank Dr L. C. Erickson of Loyola University of Chicago and Dr L. I. Grossman of Wayne State

University for providing the plasmids containing TK and mitochondrial sequences for use as probes.

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