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# Effects of Cytosine Methylation at Restriction Sites on Deoxyribonucleic Acid (DNA) Typing

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**ABSTRACT:** The effects of endogenous 5-methylcytosines on deoxyribonucleic acid (DNA) fingerprints were studied. Analysis with methylation-sensitive restriction endonuclease *Sau*3AI and its methylation-insensitive isoschizomer *Mbo*I showed some differences in the patterns generated as a result of 5-methylcytosines at the recognition sites. Moreover, a few bands of sperm DNA did not match those of blood DNA from the same individual, a phenomenon only observed in the digests of methylation-sensitive endonucleases. These findings indicate the unsuitability of methylation-sensitive restriction endonucleases for DNA fingerprinting and other forms of DNA typing, because of the tissue-specific status of the methylation.

**KEYWORDS:** forensic science, deoxyribonucleic acid (DNA), genetic typing

Recent recombinant deoxyribonucleic acid (DNA) technology has provided some novel and powerful methods for forensic science application [*1–3*]. Human genomic DNA can be analyzed directly for individual identification and paternity testing on the basis of variations in its sequence.

Restriction endonucleases derived from bacteria can cleave human DNA at sites of specific sequences. Small changes in the DNA sequences sometimes result in gains or losses of these restriction sites [*4*]. Alternatively, DNA regions consisting of tandem repeats of short sequences frequently show hypervariability because of the extensive variation in the number of repeats [*5,6*]. DNA cleavage with restriction endonucleases and subsequent Southern hybridization of these DNA regions can be used to show restriction fragment length polymorphism (RFLP), thus providing informative genetic markers for forensic science application, as well as linkage analysis and gene mapping [*7,8*].

Jeffreys and his colleagues have developed the "DNA fingerprinting" system, using probes capable of detecting a number of hypervariable DNA regions simultaneously [*9*].

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The Southern hybridization profiles obtained with these probes consist of multiple hypervariable restriction fragments, which are specific to individuals. Since these fragments are stably inherited and segregate in a Mendelian fashion, DNA fingerprinting has become a powerful method for paternity testing and individual identification [*10,11*]. So far, a number of probes for DNA fingerprinting have been described [*12,13*].

Approximately 3 to 4% of all cytosine residues (C) in human DNA are modified to give 5-methylcytosines, which are found only at 5′-CG-3′ sequences [*14*]. The status of methylation is established during development, and the preexisting patterns are inherited in the DNA replication process. Some restriction endonucleases are affected by these 5-methylcytosines, becoming unable to cleave DNA when the cytosine residues are methylated at their recognition sites. Since the status of methylation is specific to various tissues [*15*], methylation-sensitive endonucleases sometimes yield differences in Southern hybridization patterns between tissues in some DNA regions [*16*]. In the present study, the authors determined the effects of methylation on DNA fingerprinting, which can detect multiple DNA regions simultaneously. Tandem repeats of a 28-base-pair (bp) sequence downstream from the c-Ha-*ras*-1 gene was used as a probe [*12*]. DNA fingerprints from peripheral blood samples were obtained using the methylation-sensitive endonuclease *Sau*3AI and were compared with those obtained using its isoschizomer *Mbo*I, which is not sensitive to methylation in human DNA. The different patterns obtained with these endonucleases indicated that DNA cleavage at some sites was prevented by the 5-methylcytosines. Moreover, we analyzed the Southern hybridization patterns obtained from sperm DNA and blood DNA of the same individual. Inconsistency in the banding patterns between these samples was only observed with the methylation-sensitive endonuclease, showing the unsuitability of such endonucleases for DNA typing, including DNA fingerprinting, because of the effects of tissue-specific methylated cytosines.

## Materials and Methods

### *DNA Isolation from Blood and Sperm*

Fresh peripheral blood and semen samples were obtained from two individuals. High-molecular-weight (HMW) DNAs from blood samples were isolated as described by Bell et al. [*17*]. Those from sperm were prepared as described by Bahnak et al. [*18*]. Briefly, semen was centrifuged, and the collected sperm cells were washed twice in 0.15*M* sodium chloride (NaCl), 2m*M* ethylenediaminetetraacetic acid (EDTA). They were lysed completely in solution containing a strong chaotropic agent (6*M* guanidinium thiocyanate, 25m*M* sodium citrate, 0.5% Sarkosyl, 0.1*M* 2-mercaptoethanol) overnight at 37°C. One gram of cesium chloride (CsCl) was added to the lysate, which was then layered on 5.7*M* CsCl, 0.1*M* EDTA, pH 7.0, and centrifuged for 20 h at 30 000 rpm. The banded HMW DNA was recovered and dialyzed against TE buffer (10m*M* Tris/hydrochloric acid (HCl), 1m*M* EDTA, pH 8.0) for 24 h.

### *Probe DNA*

Plasmid DNA (pEJ) containing the human c-Ha-*ras*-1 oncogene and its flanking regions [*6,19*] was digested with restriction endonucleases *Sph*I and *Cla*I. An 860-bp fragment, consisting of tandem repeats of a 28-bp sequence, was purified by 5% polyacrylamide gel electrophoresis and labeled with [$\alpha$-$^{32}$P] dCTP by nick-translation [*20*].

### *DNA Digestion with Restriction Endonucleases*

Some four-base and five-base restriction endonucleases were used. *Sau*3AI and *Hin*fI are sensitive to 5-methylcytosines included in their recognition sequences, whereas *Mbo*I

and *Hae*III are insensitive [*21*]. Three micrograms of each DNA was digested with six units of each endonuclease under the conditions recommended by the manufacturers. The DNA digests were precipitated with two volumes of ethanol at −20°C and rinsed in 70% ethanol. The vacuum-dried DNAs were dissolved in 1 × TE.

*Southern Blot Hybridization*

The DNA solution was electrophoresed through 0.6% agarose gel in a cold room at 4°C with circulation of the running buffer (50m*M* Tris-HCl, 20m*M* sodium acetate, 2m*M* EDTA, 18m*M* NaCl, pH 8.0). All DNA fragments less than 4 kpb in size were allowed to run off the gel. The DNA in the gel was then transferred to nitrocellulose filters. Hybridization was carried out in 1*M* NaCl at 65°C, as described by Honjo et al. [*22*]. The filters were washed twice (30 min each) in 1 × SSC (0.15*M* NaCl, 0.015*M* sodium citrate) and 0.1% sodium dodecyl sulfate (SDS) at 65°C and were then autoradiographed using X-RP (Kodak) for 3 to 5 days at −70°C using a DuPont Lightening Plus intensifying screen.

## Results

*DNA Fingerprinting Using a Pair of Isoschizomers*

DNAs were isolated from peripheral blood samples and digested with restriction endonucleases. Endonuclease *Sau*3AI and its isoschizomer *Mbo*I both recognize and cleave DNA at the same 5′-GATC-3′ sequence, but only *Mbo*I can cleave DNA when the cytosine residue is methylated. The DNA digests from the same individual were electrophoresed through agarose gel, blotted onto the nitrocellulose filter and probed with the 28-bp repeats downstream of the c-Ha-*ras*-1 gene, as described previously [*12*]. Sixteen fragments larger than 4 kb were resolved with either *Sau*3AI or its isoschizomer *Mbo*I (Fig. 1). However, the banding patterns produced with each were not identical. Some fragments observed in the *Mbo*I digest were not shown at the corresponding size positions in the *Sau*3AI digest. This indicated that some cytosine residues at the 5′-GATC-3′ sites were methylated, allowing only *Mbo*I to cleave the DNA and provide the characteristic fragments for the Southern hybridization patterns. Since *Sau*3AI cannot cleave DNA at these methylated sites, its generated fragments are larger than those produced by *Mbo*I. The 22-kb and 5.5-kb fragments in the *Sau*3AI digest are considered to originate from the 20-kb and 5-kb fragments in the *Mbo*I digest, respectively, and the 9.5-kb and 9.2-kb *Sau*3AI-fragments are from the 8.5-kb or 8.0-kb *Mbo*I fragments, based on the similar intensities of these bands.

*DNA Fingerprinting from Different Tissues*

The methylation status of cytosine residues is not consistent among tissues. To study the effects of methylated cytosines in different tissues, we compared the Southern hybridization patterns from blood and sperm DNAs using methylation-sensitive restriction endonucleases. Peripheral blood and semen samples were prepared from two individuals, and DNA fingerprints were obtained as described above. One individual showed inconsistency between blood and sperm in the banding patterns with methylation-sensitive endonucleases (Fig. 2). In the methylation-sensitive *Sau*3AI digests, the 12-kb and 9-kb bands in the sperm sample were extremely faint in comparison with those of the blood sample, and the 9.7-kb and 7.2-kb bands were absent from the blood DNA. These differences in the observed fragments were not artifacts due to a small sample size or DNA degradation since the amounts of both DNAs were confirmed to be sufficient in the high-molecular-weight fraction by ethidium bromide staining before endonuclease

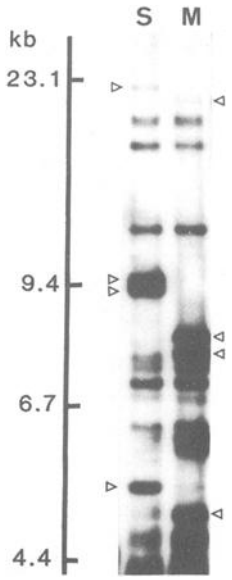


FIG. 1—*Effects of cytosine methylation on DNA fingerprints from blood DNA. The* clear arrowheads *indicate the mismatched fragments between the digest of* Sau*3AI* (S) *and that of its isoschizomer* Mbo*I* (M) *from the same individual. The molecular weight markers (*Hind*III-digested bacteriophage lambda DNA) are indicated in kilobase (kb) size.*

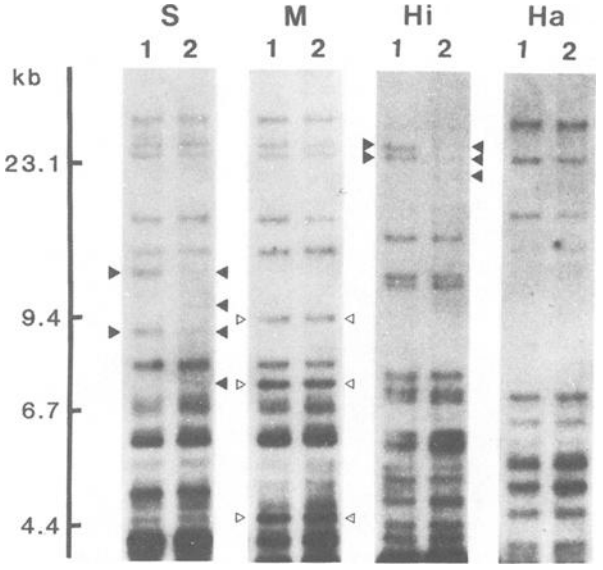


FIG. 2—*Inconsistency in DNA fingerprints between blood DNA and sperm DNA. Blood DNA* (Lanes 1) *and sperm DNA* (Lanes 2) *from the same individual were digested with* Sau*3AI* (S), Mbo*I* (M), Hin*fI* (Hi), *and* Hae*III* (Ha), *respectively. The* solid arrowheads *indicate the fragments that are inconsistent between the blood and sperm DNAs in the digests of methylation-sensitive endonucleases. The* clear arrowheads *indicate the* Mbo*I-digested fragments which differ from the* Sau*3AI-digested fragments. The molecular weight markers (*Hind*III-digested bacteriophage lambda DNA) are indicated in kilobase (kb) size.*

digestion. On the other hand, the inconsistent bands were not generated by incomplete digestion of DNA, caused by excess salts or protein included in the solution, because they amounted to only a small proportion of the banding patterns, and the other bands were identical, both in size and intensity, for the blood and sperm DNAs. Duplicate experiments showed similar results. In contrast, the two DNAs exhibited identical patterns in the digests of *Mbo*I, an isoschizomer of *Sau*3AI, which is not sensitive to methylation. Therefore, the inconsistent bands observed in the *Sau*3AI digests indicated a difference in methylated sites between blood and sperm DNAs. Some fragments in the *Mbo*I digests were different from those in the *Sau*3AI digests of both samples. Since *Sau*3AI digestion generates larger fragments than *Mbo*I digestion at methylated sites, the bands at 9.5 kb and 7.2 kb in the *Mbo*I digests could correspond to the 12-kb and 9-kb bands, respectively, in the *Sau*3AI-digested blood DNA, and to the 9.7-kb and 7.2-kb bands, in addition to the 12-kb and 9-kb bands, in the sperm DNA. We also analyzed the same samples using other endonucleases frequently used for DNA fingerprinting (Fig. 2). *Hin*fI is shown to cleave DNA more slowly when the cytosine residue at the 3′ end of the recognition sequence 5′-GA(G/A/T/C)TC-3′ is methylated [*21,23,24*]. We also observed inconsistency between blood DNA and sperm DNA in the bands with this endonuclease. On the other hand, *Hae*III, which is not sensitive to methylated cytosine in human DNA, produced identical patterns.

## Discussion

Human DNA contains methylated cytosine residues. The status of methylation is specific to each body tissue and has been reported to be implicated in the control of gene expression [*15*]. DNA cleavage with some restriction endonucleases is hindered by methylated cytosine residues at their recognition sites. However, methylation-sensitive endonucleases are frequently used for DNA typing, including DNA fingerprinting. In this study, we showed the effects of endogenous methylated cytosine residues on DNA fingerprinting. The Southern hybridization patterns obtained from peripheral blood showed differences between the digest of methylation-sensitive *Sau*3AI and that of its methylation-insensitive isoschizomer *Mbo*I, indicating the existence of 5-methylcytosines at some of their recognition sites. Since methylation of cytosine residues is restricted to the 5′-CG-3′ sequence in human DNA, only a few of the recognition sites (5′-GATC-3′) can be methylated. On the other hand, the recognition site of *Hap*II and *Msp*I, 5′-CCGG-3′, contains a potential methylation site. As expected, the difference between the patterns produced by methylation-sensitive *Hap*II and those by methylation-insensitive *Msp*I was much greater than between those of *Sau*3AI and *Mbo*I. Most of the *Hap*II-generated fragments remained in the high-molecular-weight fraction (data not shown).

The status of methylation is specific to certain tissues. Therefore, we compared the Southern hybridization patterns of blood and sperm DNAs using methylation-sensitive endonucleases. We observed a few inconsistencies in banding patterns between them both in the *Sau*3AI and *Hin*fI digests, whereas the digests of methylation-insensitive *Mbo*I and *Hae*III showed identical patterns. These results indicate differences between blood and sperm DNA in the status of methylation. Since probes for DNA fingerprinting hybridize with multiple DNA regions simultaneously, it is difficult to detect the allelic fragments originating from the same locus in their Southern hybridization patterns. However, two fragments in the *Mbo*I digest could correspond to the two fragments in *Sau*3AI-digested blood DNA and four fragments in *Sau*3AI-digested sperm DNA, since *Sau*3AI generates larger fragments. The additional fragments in the sperm DNA suggest partial methylation of these sites.

Recently, methylation of cytosines has been described with regard not only to tissue specificity but also to the specificity of individual alleles and their origin (that is, whether the allele is of paternal or maternal origin) [*16,25,26*]. However, the restriction endo-

nucleases frequently used for DNA fingerprinting and other forms of DNA typing are methylation-sensitive. Therefore, it is desirable to choose restriction endonucleases which are insensitive to methylation in order to avoid false-negative results.

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