

# Methylation of a middle repetitive DNA sequence class during differentiation in Friend erythroleukemia cells

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We have examined the methylation patterns within middle repetitive sequences in Friend erythroleukemia cells. Mouse-interspersed-family-1 (MIF-1) and a group characterized by a 1350-bp *Eco-Bam* fragment cloned into pBR322 as plasmid pFS-13, are both less modified in Friend cell DNA than in normal tissue DNA. The pattern of methylation present in pFS-13 homologous sequences was found to be stable during cell division, i.e., somatically inherited, and stable during differentiation induced by HMBA.

*DNA methylation      Friend erythroleukemia cell      Hypomethylation of DNA      Repetitive DNA sequence  
Induction of differentiation*

## 1. INTRODUCTION

Oncogenic transformation and normal development have much in common at the cellular level in eukaryotes [1], and it has been suggested that DNA methylation patterns may play a role in both processes [2-4]. Many tumors and transformed cell lines possess altered levels of 5-MCyt [5-8]. DNAs from normal mammalian tissues have comparable overall levels of 5-MCyt in the adult, while DNA from trophoblast contains less 5-MCyt than embryonic or somatic tissues [9-12]. Recently, methylation patterns within repeated sequences have been shown to reflect differences in overall levels at 5'CG [7,11]. We have studied the methylation patterns in moderately repetitive DNA sequences in Friend erythroleukemia cells before and after induction of differentiation.

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**Abbreviations:** APT-cellulose, aminophenylthioether cellulose; DPT-cellulose, diazophenyl-thioether cellulose; HMBA, hexamethylene-bis-acetamide; HPLC, high-performance liquid chromatography; bp, base pairs; 5-MCyt, 5-methylcytosine.

## 2. MATERIALS AND METHODS

DBA/2 mice were obtained from the Jackson Laboratory. Subclone DS-19 of the Friend erythroleukemia cell line was grown in AutoPow MEM (Flow Labs) + 0.05 mg/ml gentamicin (Gibco) and 10% fetal calf serum. In this medium, the cells had a doubling time of about 17 h when subcultured at  $5 \times 10^5$  cells/ml. Cultures were induced to differentiate by the addition of HMBA to a final concentration of 4 mM. Differentiation was monitored by benzidine staining for hemoglobin production. Viable cells were scored by trypan blue exclusion.

Genomic DNA was isolated as described in [13]. MIF-1 DNA was isolated as described in [14]. DNA from clone pFS-13 containing a 1350-bp *Eco-Bam* insert of mouse repetitive DNA [15] was isolated from pFS-13 bearing *Escherichia coli* 294 [16]. The conditions used for restriction enzyme digestions, and the controls performed for the completeness of digestions, were as previously described [17]. Restriction digests were separated on 1.6% agarose gels in 50 mM Tris, 5 mM disodium EDTA brought to pH 7.8 with acetic acid.

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APT cellulose was synthesized and activated as described in [18]. DNA, fragmented by depurinization, was transferred to DPT cellulose or nitrocellulose and probed with cloned or gel-purified DNA labelled by nick translation [19]. HPLC was performed on genomic DNA samples after ribonuclease digestion and formic acid hydrolysis [20].

### 3. RESULTS

Table 1 shows the 5-MCyt content of DNA from normal spleen and exponentially-growing Friend cells as determined by HPLC analysis. Friend-cell DNA was found to contain about 60% of the 5-MCyt found in DNA of normal tissue. This value is consistent with the 2-fold increase in *Hpa* II cleavage previously reported [7] and suggests that methylation at the 5'CCGG site is, in fact, representative of 5'CG methylation in mouse tissues and cell lines.

Many repeated-sequence classes are less modified in Friend cell DNA than they are in normal tissue DNA [7]. However, repeated sequences resolved by the 2-dimensional display technique used in those experiments were not well defined. We have recently studied an interspersed-repeated-sequence group from the DNA of mouse. This family is characterized by a 1350-bp fragment cloned into pBR322. Cloned representatives of the fragment, like pFS-13, share homology with about 2% of the mouse genome. Based on the results of Southern blotting, this group does not share homology with mouse-interspersed-family-1 (MIF-1), nor with the major AT-rich satellite sequences of mouse [15]. We are currently determining the sequence of the 1350-bp insert in pFS-13 to uncover

any homology between these and other previously characterized repeated-sequence groups that might go undetected in Southern blotting experiments.

To study the methylation in and around middle-repetitive sequences, DNA was digested with *Hpa* II or *Msp* I, and Southern Blots were probed with either MIF-1 or pFS-13 sequences. To ensure completeness of digestion, control digests were per-

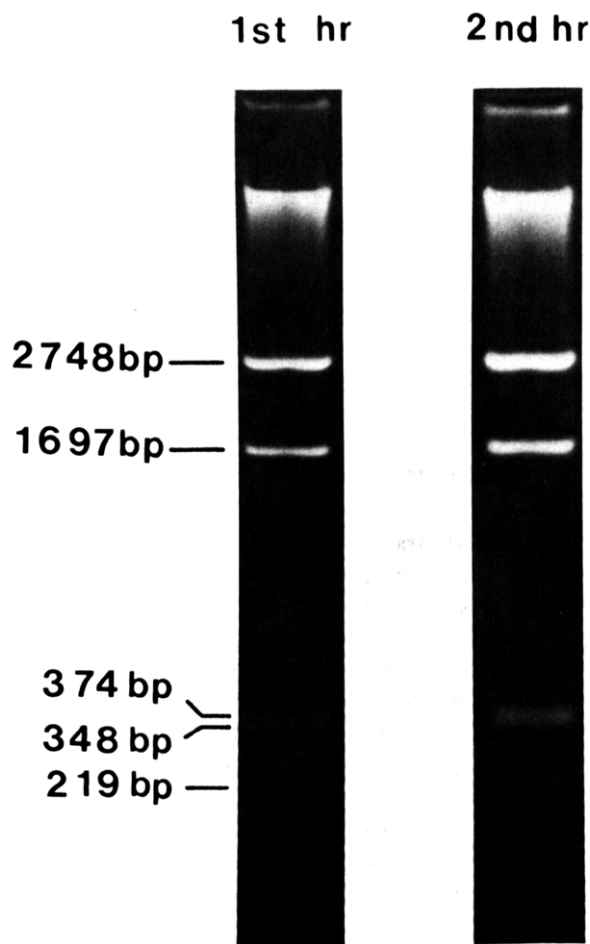


Fig. 1. Test for completion of digestion by *Msp* I or *Hpa* II. DNA digests were tested for completion by withdrawing a 20- $\mu$ l aliquot of an initiated restriction digest of mouse DNA and mixing it with 0.5  $\mu$ g of  $\phi$ X174 RF DNA. After 1 h of incubation, the test reaction was stopped and stored with EDTA on ice. At this time, a second test reaction was initiated and also incubated for 1 h. An ethidium bromide-stained agarose gel of the product is shown. When complete digestion of the  $\phi$ X174 DNA was obtained in both 1-h intervals, it was concluded that the mouse DNA was completely digested.

Table 1

5-MCyt content of Friend cell DNA and the DNA of normal mouse tissue.

DNA source	mol% 5-MCyt
Normal spleen tissue (strain DBA/2)	3.33 $\pm$ 0.30
Friend cell DNA (clone DS-19)	2.00 $\pm$ 0.46

DNA from normal mouse spleen and the Friend cell line was subjected to analysis by HPLC as described in section 2.

formed as shown in fig. 1. Digests shown in fig. 2 and 3 were complete by this criterion. Fig. 2A shows undigested, *Hpa* II-digested, and *Msp* I-digested DNA from normal tissue and the Friend cell line, probed with gel-purified MIF-1 DNA. In *Msp* I digests, two strong bands at about 5500 bp and 4000 bp are observed with this probe [21]. *Msp* I digests show identical patterns with this probe. On the other hand, the patterns seen in *Hpa* II digests are clearly different, with *Hpa* II producing more extensive cleavage of sequences homologous to MIF-1 in Friend cell DNA than in DNA from spleen. Four bands could be distinguished in the original autoradiographs in the *Hpa* II digests of Friend cell DNA (lane 4). Both of the bands at 4000 bp and 5500 bp seen in *Msp* I digests were present in *Hpa* II digests of Friend cell DNA in diminished amounts, and two new bands of hybridization were observed at about 4400 and 6300 bp. A smear of hybridization at high molecular length was observed in *Hpa* II digests of DNA from normal tissue with no visible banding pattern. The pattern observed in *Hpa* II digests of Friend cell DNA probed with MIF-1 is identical to that reported in [11] for mouse trophoblast DNA. The pattern observed in spleen DNA (lane 3) is identical to that reported for normal liver DNA and DNAs from other somatic and embryonic tissues [11].

A similar result was obtained with the interspersed-repeated-sequence family represented on plasmid pFS-13 (fig. 2B). Again, *Msp* I digests were indistinguishable, with a pattern consisting of bands at 750, 690, 600, 550, and 230 bp above a broad smear of hybridization. A sixth band of homology at about 150 bp was also observed under

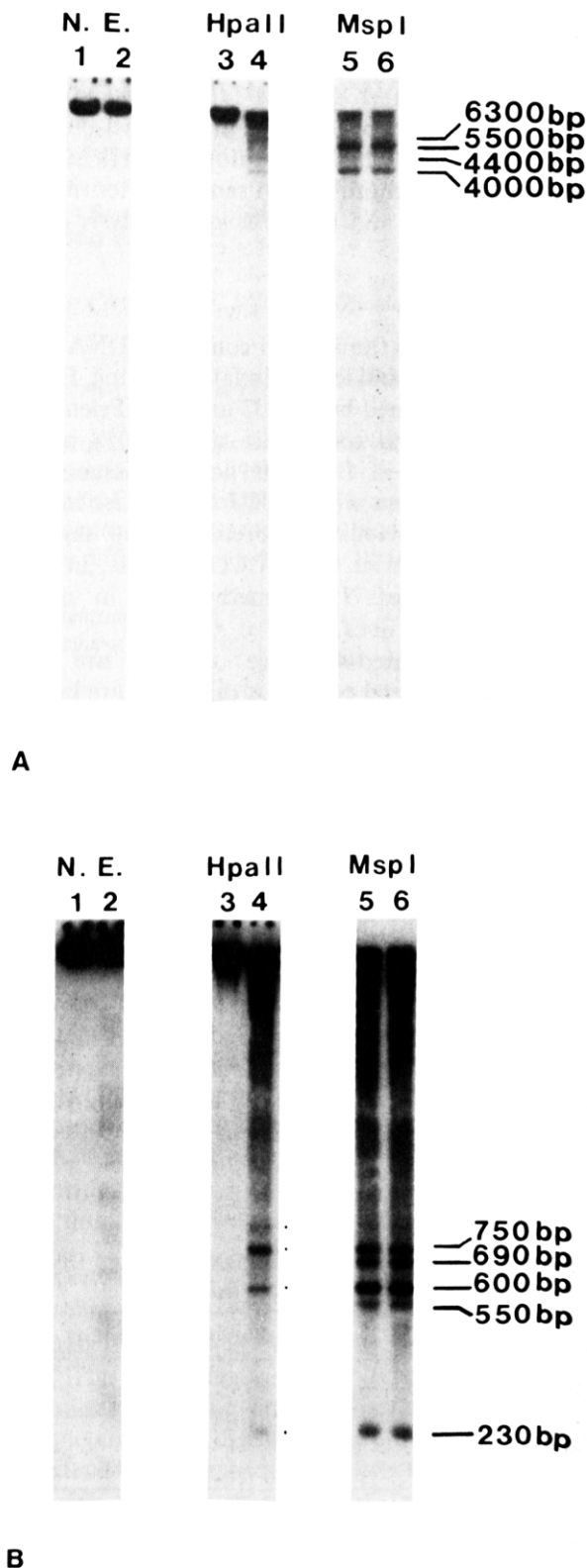


Fig. 2. Methylation of two interspersed repetitive DNA sequences in normal tissue and the Friend cell line. DNA from normal DBA/2 mouse-spleen tissue and the Friend cell line was digested to completion with *Hpa* II or *Msp* I, or incubated with no enzyme (N.E.) in mock digestions. 1- $\mu$ g aliquots of each digest were separated by electrophoresis and blotted to DPT paper. Lanes 1, 3, and 5: spleen DNA. Lanes 2, 4 and 6: Friend cell DNA. A, autoradiograph of blot probed with labelled MIF-1 DNA. B, autoradiograph of this same blot probed with pFS-13 plasmid DNA.

different electrophoretic conditions. The *Hpa* II digest of normal spleen DNA gave hybridization at high molecular length with no low-molecular-length banding pattern, showing that the sequences homologous to pFS-13 are heavily methylated in normal tissue. The pattern found in *Hpa* II digests of Friend cell DNA contained bands at each molecular length seen *Msp* I digests except those at 690 and 550 bp, along with a new band at about 850 bp. Densitometric scans of these autoradiographs have been analyzed and show that the banding pattern in *Msp* I digests probed with pFS-13 accounts for about 0.2% of the genome. The absence of sequences at 690 and 550 bp suggests that the sites flanking these DNA fragments selectively retain modification in Friend cell DNA in a concerted fashion [7]. This pattern was found to be stable for

at least 50 cell doublings.

The experiment shown in fig. 3 was performed to test the possibility that the pattern of methylation in the pFS-13 group might be altered by the process of differentiation. Friend cells were induced to differentiate with HMBA, and scored for viability and globin production. HMBA-induced cultures were more than 90% benzidine positive, and about 90% viable at the time of harvest. Uninduced cultures were about 85% viable and contained no benzidine-positive cells at the time of harvest. DNA was isolated, cleaved with *Hpa* II or *Msp* I, separated by gel electrophoresis, blotted, and probed with pFS-13 DNA. Uninduced and induced Friend cell DNAs showed banding patterns in *Hpa* II digests that were identical to one another, but different from normal tissue and from *Msp* I digests. The additional 150-bp band is present in *Hpa* II digests of uninduced and induced Friend cell DNA and *Msp* I digests of all types of DNA (not shown).

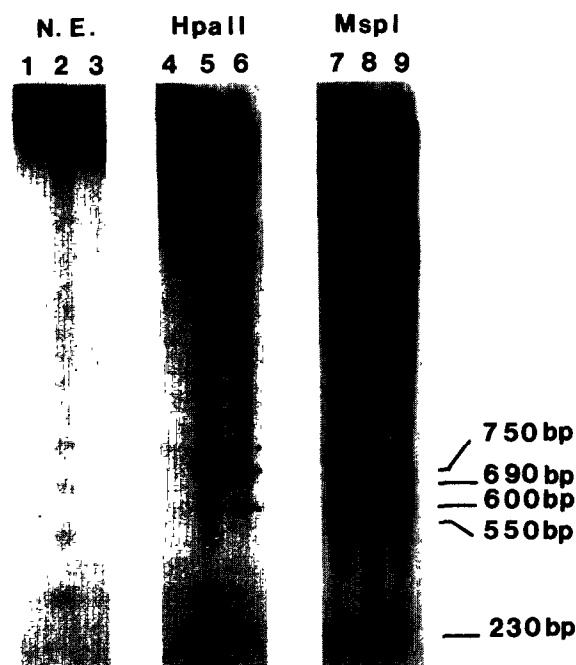


Fig. 3. Methylation of the pFS-13 repetitive sequence group before and after induction of differentiation in Friend cells. DNA from normal DBA/2 mouse spleen tissue and from induced and uninduced Friend cells was digested to completion with *Hpa* II or *Msp* I, or incubated with no enzyme (N.E.) in mock digestions, separated by electrophoresis and blotted to nitrocellulose paper. The blot was probed with labelled pFS-13 plasmid DNA. Lanes 1, 4, and 7: spleen DNA. Lanes 2, 5, and 8: Friend cell DNA. Lanes 3, 6, and 9: Friend cell DNA after differentiation.

#### 4. DISCUSSION

In a previous report [7], we demonstrated that the general level of methylation at the 5'CCGG site in the Friend cell line was substantially lower than in normal mouse spleen DNA. The initial observation of reduced methylation at 5'CCGG sites [7] is extended by the results of HPLC analyses given in table 1 to include the general level of methylation at cytosine. Authors in [12] have shown that somatic tissues of the mouse possess equivalent overall levels of 5-MCyt. On the other hand, DNA from tissues of the trophoblast are significantly less modified than somatic tissues in rabbit [11], mouse [12], and humans [10]. Thus the general level of methylation at cytosine and at 5'CCGG sites in Friend cell DNA as well as the specific pattern of methylation found in the MIF-1 group resemble that seen in trophoblast (fig. 2A). It is interesting that this normal tissue is capable of controlled invasiveness in both uterine and non-uterine tissues.

Authors in [22] have shown that Friend cell differentiation is accompanied by the expression of as many as 3800 new gene products. An alteration of this magnitude in gene expression would be expected to be reflected as a change in bulk modification of less than 0.1%. Authors in [23] obtained

results suggesting that a 1.6% change in bulk modification occurs during differentiation in Friend cells. The results in fig. 2B and 3 show that the repeated-sequence group homologous to the pFS-13 insert has a methylation pattern in Friend cells that is very different from that of normal tissue, but is not significantly altered by the induction of differentiation. This is consistent with previous findings using scans of restriction digests stained with ethidium bromide [7] and Southern blotting with globin-specific probes [24].

Perhaps each of these probes has failed to detect a small change in methylation that occurs during induction of differentiation. However, it is possible that the stably inherited hypomethylated state that is found in the DNA of the Friend cell line has left critical DNA sequences unmethylated, rendering a large group of genes inducible. This point of view is consistent with current data correlating DNA methylation patterns and gene expression [25], and with the observation that 5-azacytidine (a potent inhibitor of DNA methylation) is only a weak inducer of Friend cell differentiation [26].

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