Hypomethylation of DNA during Differentiation of Friend Erythroleukemia Cells

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ABSTRACT   DNA from mammalian cells has been shown to contain significant amounts of 5-methyl cytosine resulting from enzymatic transfer of methyl groups from s-adenosylmethionine to cytosine residues in the DNA polymer. The function of this modification is not known. We have found that DNA synthesized during chemically induced differentiation of Friend erythroleukemia cells is hypomethylated, as measured by its ability to accept methyl groups transferred by homologous DNA methyltransferases in vitro. The extent of hypomethylation detected by this sensitive method is small, a decrease of < 1.6% in 5-methylcytosine content.

Hypomethylated DNA can be isolated from Friend erythroleukemia cells grown in the presence of dimethyl sulfoxide, butyrate, hexamethylene-*bis*-acetamide, pentamethylene-*bis* acetamide, and ethionine. However, hypomethylated DNA is found only under conditions where differentiation is actually induced. DNA isolated from cells of a dimethyl sulfoxide-resistant subclone grown in the presence of that agent is not hypomethylated, although DNA of these cells becomes hypomethylated after growth in the presence of inducers that can trigger their differentiation. We also find that the DNA of Friend erythroleukemia cells does not become hypomethylated when the cells are exposed to inducing agents in the presence of substances that inhibit differentiation. These results suggest a close link between genome modification by methylation and differentiation of Friend erythroleukemia cells.

A significant proportion of cytosine residues in eukaryotic DNA are methylated in the 5 position (6, 31). Although there is no experimental evidence regarding the function of this enzymatic modification in higher organisms, early reports of tissue and cell specific variation in 5-methylcytosine (5MeC) content of DNA led to speculation that postreplicative methylation of the genome might regulate gene expression during differentiation (12, 28, 30).

The availability of cell lines that undergo differentiation in tissue culture allows direct examination of this hypothesis.

Using mouse erythroleukemia cells that undergo erythroid differentiation after exposure to chemical inducing agents, we have been able to demonstrate the following correlations between DNA methylation and the differentiation process (5): (a) Differentiation is induced in Friend erythroleukemia cells (FL cells) by exposure to L-ethionine at concentrations sufficient to inhibit in vivo methylation of DNA and tRNA. (b) DNA isolated from FL cells undergoing differentiation in response to other agents unrelated to ethionine is hypomethylated, as judged by its ability to act as a methyl acceptor in vitro. Unlike L-ethionine, these agents do not detectably inhibit methylation of tRNA in vivo.

If it is assumed that the changes we have observed are more than coincidentally linked with differentiation and instead reflect a change in the overall pattern of genome modification involved in the regulation of gene expression, several predictions can be made regarding the synthesis of hypomethylated DNA in FL cells: (a) Hypomethylation of DNA should occur soon after the addition of inducing agents, before synthesis of gene products characteristic of the differentiated state. (b) Variant clones of FL cells selected for insensitivity to specific inducing agents should synthesize hypomethylated DNA only in the presence of agents that actually cause their differentiation. (c) Agents that inhibit FL cell differentiation should prevent the synthesis of hypomethylated DNA in the presence of inducers. (d) Because FL cell differentiation involves a change in the expression of a limited number of genes (21), only a small proportion of those sites that are methylated in untreated FL cells should be affected by the differentiation process. Presumably these unmethylated cytosine residues will occur at specific locations in the genome.

We have previously demonstrated that hypomethylation of FL cell DNA can be detected as early as 24 h after the cells are exposed to inducing agents (5). In this report we present

evidence indicating that chemical inducers affect methylation of only a minor proportion of cytosine residues in FL cell DNA, causing a net loss of < 1.6% of the normal complement of 5MeC residues, and show that this loss does not occur when differentiation is blocked.

MATERIALS AND METHODS

FL Cells

Cells of strain 745A and its two independently derived subclones, 5-86 (25) and DR 10 (19), were graciously provided by Dr. C. Friend, Dr. W. Scher, and Dr. M. Terada. The cells were maintained in minimal Eagle's medium supplemented with 10% fetal bovine serum, 250 U/ml penicillin and 0.2 mg/ml streptomycin. The medium for routine culture of DR-10 cells also contained dimethyl sulfoxide (Me$_2$SO) at a final concentration of 240 mM. Inducing agents were added to the culture medium immediately before seeding with FL cells at 10^5/ml. Unsterilized Me$_2$SO or butyric acid was added directly to indicated final concentrations. All other compounds were dissolved in medium and filter sterilized. Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO$_2$. The percentage of hemoglobin-containing cells was determined by benzidine staining of cell suspensions (20). All determinations were performed in duplicate and at least 200 cells were counted.

Base Analysis of FL Cell DNA by High Performance Liquid Chromatography (HPLC)

Purified DNA from 2 × 10^8 cells was hydrolyzed in 250 µl of 70% perchloric acid by heating at 100°C for 1 h. The perchloric acid was neutralized with 5 N KOH, and KClO$_4$ was removed by centrifugation. An appropriate aliquot of the supernate (see Fig. 1 and Table I) was diluted to 100 µl with distilled water for loading into a 0.4 × 25 cm Partisil SCX column (Whatman Inc., Clifton, N.J.) preequilibrated with 0.02 M NH$_4$PO$_4$, pH 2.3. Isocratic elution at 500 psi and ambient temperature (23°C) with a flow rate of 1.25 ml/min gave the following elution times: thymine (T), 6.5 min; guanine (G), 11.2 min; cytosine (C), 14.5 min; adenine (A), 19 min, and 5-methylcytosine (5MeC), 23 min. For determination of the 5MeC content of FL cell DNA, 0.2 µCi/ml [6-^{3}H]uridine (sp act, 24.2 Ci/mmol) was added to the culture medium of FL cells in the logarithmic stage of growth. For radiolabeling the DNA of cultures treated with Me$_2$SO for
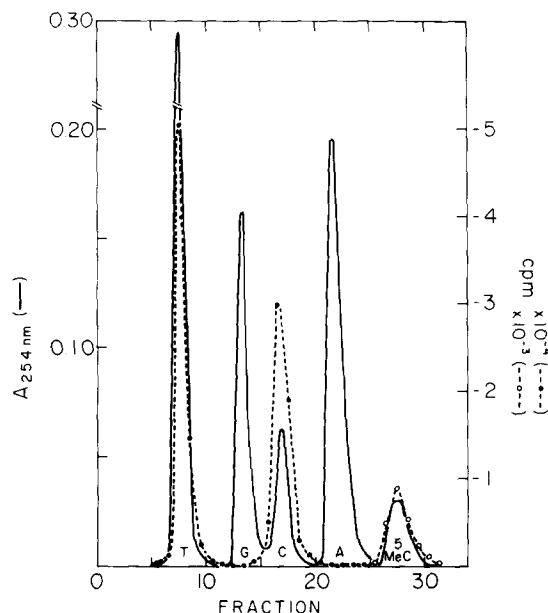

FIGURE 1 Determination of the 5-methylcytosine content of FL cell DNA. An aliquot of an acid hydrolysate of [6-^{3}H]uridine-labeled DNA from untreated FL cells containing ~135,000 cpm was supplemented with 250 pmol 5MeC before loading on a Partisil SCX column (see Materials and Methods). The distribution of recovered radiolabel was: T, 68,760 cpm; C, 58,750 cpm; 5MeC, 2,245 cpm. To emphasize the 5MeC peak, the scale for radiolabel per fraction is changed from cpm × 10^{-4} to cpm × 10^{-3} at fraction 25. A, absorbance.

| | DNA Source | | | |
| | Strain 745 A | | Subclone 5-86 | |
Treatment of cells before DNA isolation	pmol of CH$_3$/100 µg of DNA	% B+	pmol of CH$_3$/100 µg of DNA	% B+
None	10.8	1-2	12.3	>1
Me$_2$SO (240 mM)	41	78	40.6	80
L-Ethionine (4 mM)	54.6	40	45	35
Butyrate (2 mM)	36.2	82	ND	ND
HMBA (4 mM)	30.7	95	33.5	93
PMBA (4 mM)	31.8	90	ND	ND

Assays for methyl transfer to DNA were as described in Materials and Methods. All values represent the average incorporation for three to five assays on a minimum of three separate DNA preparations from cells grown in the presence of the indicated inducing agent for 3-4 d. Variation from these values did not exceed ±5 pmol. The differences in extent of methylation of DNA shown here could be detected as differences in rate of methylation during shorter incubations (10-25 min; data not given). HPLC analysis of acid hydrolysates of the DNA reisolated after in vitro methylation (see Materials and Methods) indicated that all recovered radiolabel from [^{3}H]methyl-5-adenosylmethionine was present in 5MeC. (In a typical experiment with 4,300 cpm loaded onto a Partisil SCX column, 4,170 cpm coeluted with marker 5MeC. No other peak of radiolabeled material was observed.) ND, not determined. B+, benzidine-positive cells were scored in cultures exposed to the inducing agents for 5 d. HMBA, hexamethylene-bis-acetamide; PMBA, pentamethylene-bis-acetamide.

periods >48 h, cells were diluted with fresh medium containing Me$_2$SO at 56 h to maintain logarithmic growth rates. 18 h after addition of radiolabel the cells were harvested and their DNA isolated by the method of Blin and Stafford (3). Acid hydrolysates of such DNA containing ~120,000 cpm were supplemented with 250 pmol of 5MeC before chromatography. 1-ml fractions of column effluent were collected and assayed for radiolabel in a liquid scintillation counter using a water-accepting fluor. An ISCO model UA-5 detector (Instrumentation Specialities Co., Lincoln, Nebr.) with a low-volume flow cell was used to monitor column effluent at 254 nm.

Methylation of FL Cell DNA In Vitro

FL cells seeded at 10^5/ml in either the presence or absence of inducing agents were grown for 72-96 h at 37°C in slowly rotated roller bottles flushed with 5% CO$_2$. When necessary to maintain a logarithmic rate of growth, the cultures were diluted at 48-56 h with fresh medium containing the same concentration of inducing agent. DNA was prepared from the isolated nuclei of these cells by the Marmur procedure (16). Extraction of DNA methyltransferase from the nuclei of untreated FL cells and conditions of assay have been described (5). Incubation mixtures contained 16 µM [^{3}H]methyl-5-adenosylmethionine (3-10 Ci/mmol), 25 µg/ml DNA, and an excess of DNA methyltransferase, i.e., an amount of enzyme that gave maximal methyl incorporation into DNA isolated from FL cells grown for 4 d in the presence of 4 mM L-ethionine and a linear response to added DNA in the concentration range 10-60 µg/ml. Methyl incorporation reaches plateau levels within 30-40 min under these conditions. For all experiments reported here, the reaction was terminated at 1 h by addition of sarkosyl to 0.6%. The incubation mixtures were treated with RNase (20 µg/ml, 30 min, 37°C) before isolation of DNA from the mixture by banding in a CsCl/Cs$_2$SO$_4$ gradient (see ref. 27; NH$_4$SCN omitted).

Acid-precipitable radiolabel and optical density at 260 nm were determined for this purified DNA allowing extent of methylation to be reported as [^{3}H]-methyl incorporated/100 µg of reisolated DNA. Less than 100 cpm of acid-precipitable material was found at the density of DNA in gradients loaded with a similarly treated incubation mixture to which no DNA had been added.

RESULTS

We have determined the extent to which C residues in the DNA of FL cells are methylated in vivo, by using [6-^{3}H]uridine

to radiolabel newly synthesized DNA. Since uridine is a common precursor for both d(deoxy)C and d5MeC and methylation of dC occurs only after it has been incorporated into DNA, measurement of the ratio of radiolabeled 5MeC/C in DNA allows an estimate of the extent of C methylation. This measurement of 5MeC content should not be affected by changes in the rate of uridine transport or by changes in nucleotide or nucleoside pool sizes that might occur where the cells are exposed to chemical inducers. A typical chromatographic analysis of an acid hydrolysate of radiolabeled DNA from untreated FL cells is shown in Fig. 1. Duplicate analysis of eight different preparations of DNA from untreated FL cells gave an average value of 3.6 ± 0.13 5MeC residues/100 C residues. The extent of DNA methylation, as measured by this technique, was unaffected by growing the cells in the presence of a potent inducing agent, Me₂SO. The 5MeC content of DNA from cells grown in the presence of 240 mM Me₂SO for 24, 48, 72, and 96 h did not differ significantly from that of untreated cells. Duplicate analyses of six different DNA preparations from cells exposed for 96 h gave an average of 3.6 ± 0.2 5MeC/100 C residues.

These results contrast with those obtained in vitro, which indicate that the DNA from differentiating FL cells are hypomethylated. DNA methyltransferase isolated from FL cell chromatin transfers 10–15 pmol of methyl group from s-adenosylmethionine to 100 μg of DNA isolated from untreated FL cells in the logarithmic stage of growth. As shown in Table I, the same enzyme preparation transfers 40 pmol of methyl groups per 100 μg to DNA isolated from FL cells grown in the presence of Me₂SO for 4 d and 30–55 pmol of methyl groups per 100 μg to DNA from FL cells differentiating in response to a variety of other chemical inducers (data shown are for strain 745A and a subclone of this strain, 5-86). Thus, these DNA accept one methyl group per 6,000–10,000 bases or 0.03–0.06 more methyl groups per 100 C residues than DNA from untreated FL cells. This means that during differentiation, the methylation of <1.6% of those C residues that would be methylated in the DNA of nondifferentiating cells is affected. The HPLC method used to determine the extent of methylation in vivo is not sensitive enough to detect a change of this magnitude.

As we have previously reported (5), this small change in extent of methylation cannot be correlated with an effect of inducing agents on the rate of growth of FL cells.[1] It is, however, closely linked to the differentiation process. As shown in Table II, FL cell DNA does not become hypomethylated when the cells are grown in the presence of an inducing agent that fails to cause their differentiation. DR-10 cells, a subclone of 745A, are not induced to differentiate by Me₂SO, even though they take up the compound to the same extent and are as sensitive to its toxic effects as are cells that can be induced to differentiate (19). However, these cells retain the ability to differentiate in response to other chemical inducers. DNA from DR-10 cells maintained in the presence of Me₂SO does not differ in its ability to accept methyl groups in vitro from DNA of DR-10 cells grown in the absence of Me₂SO or from DNA of untreated FL cells of other clones. In contrast, DNA from DR-10 cells grown for 4 d in the presence of agents that can induce their differentiation accepts 35–45 pmol of methyl

groups per 100 μg of DNA, a value quite comparable to those obtained for methylation of DNA from differentiating cells of the parental strain and its Me₂SO-sensitive subclone, 5-86.

When DR-10 cells are cultured for an extended period in the absence of Me₂SO, they regain the ability to differentiate in its presence (19). By cultivating DR-10 cells in the absence of Me₂SO for 6 mo, we obtained a Me₂SO inducible culture (55% B+ [benzidine positive] in 5 d). DNA isolated from these cells after 4 d of growth in the presence of Me₂SO was hypomethylated (Table II).

The data presented in Table III provide further evidence that hypomethylation of DNA in FL cells is linked to the differentiation process by demonstrating that FL cell DNA does not become hypomethylated when cells are grown in the presence of an inducing agent, if a compound that blocks differentiation is also present. Bromodeoxyuridine (BrdUrd) added to the medium of FL cells before the addition of inducing agents is an effective inhibitor of heme synthesis, globin mRNA accumulation, and terminal differentiation of FL cells (5, 22, 26). DNA from FL cells grown in the presence of 10^{-6} M BrdUrd accepts methyl groups in vitro to the same extent as DNA from untreated cells.

TABLE II

In Vitro Methylation of DNA from DR-10 Cells

Treatment of cells before DNA isolation	pmol of CH₃/ 100 μg of DNA	% B+
Cells from cultures maintained in Me₂SO*		
None	11.5	1–2
Me₂SO (240 mM)	12.8	1–2
L-Ethionine (6 mM)	35.5	80
HMBA (4 mM)	41.4	55
PMBA (4 mM)	44	60
Cells from cultures maintained 6 mo without Me₂SO		
None	10.8	1
Me₂SO (240 mM)	34.5	55

All details as in Table I.
* Stocks maintained in Me₂SO (240 mM) cultured in Me₂SO-free medium for 1–2 wk before experiment. Methyl acceptance for DNA from stock cultures was 10.5 pmol/100 μg. Methyltransferase used in these experiments was from FL cells of strain 745A, but similar results were obtained with enzyme from DR-10 cells.

TABLE III

In Vitro Methylation of DNA from FL Cells Grown in the Presence of Me₂SO and Inhibitors of Differentiation

Treatment of cells before isolation of DNA	pmol of CH₃/ 100 μg of DNA	% B+
BrdUrd (10^{-6} M), 24 h	10.9	0
BrdUrd (10^{-6} M), 96 h	8.5	0–1
BrdUrd (10^{-6} M) + Me₂SO (240 mM), 72 h*	11.7	1–3
TPA (1.6 × 10^{-7} M), 96 h	10.5	0–1
TPA (1.6 × 10^{-7} M) + Me₂SO (240 mM), 96 h	15.8	7
Me₂SO (240 mM), 72 h	24	25
Me₂SO (240 mM), 96 h	35.2	55
None	11.4	0

* BrdUrd added to culture medium 24 h before addition of Me₂SO. TPA and Me₂SO were added simultaneously. Determination of % B+ cells was performed at the end of the indicated culture period. DNA preparation and details of assay as in Materials and Methods. Values are from a typical experiment and each represents an average of triplicate determinations on a single DNA preparation.

[1] Over a 4- to 5-d culture period, the rate of growth of FL cells in Me₂SO (240 mM) is indistinguishable from that of untreated cells. However, some inducers such as L-ethionine (4 mM) and butyrate (2 mM) slow growth by 50% or more (5).

As shown in Table III, addition of Me₂SO to the medium of FL cells growing in the presence of BrdUrd (10^{-6} M added 24 h before Me₂SO) for 72 h did not induce significant terminal differentiation and did not increase the ability of their DNA to accept methyl groups in vitro. Similar results were obtained with another agent reported to block both spontaneous and induced differentiation of FL cells, the tumor promotor 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) (24, 33). Although TPA itself had no effect on the methyl acceptance of FL cell DNA, it suppressed the increase in methyl acceptance of DNA from FL cells treated with Me₂SO and the ability of Me₂SO to induce terminal differentiation.

It should be noted that both of these inhibitors of differentiation slow the rate of growth of FL cells in the presence of Me₂SO (10, 26). In addition, BrdUrd is incorporated into the DNA in place of thymidine. Both changes in the rate of DNA synthesis and incorporation of base analogs into DNA have the potential to affect the extent to which it is methylated in vivo, and we cannot completely rule out the possibility that these inhibitors block the synthesis of hypomethylated DNA through mechanisms other than interference with differentiation. However, we have previously shown that hypomethylated DNA is synthesized in differentiating FL cells under conditions of both rapid and inhibited growth, indicating that slow growth per se should not attenuate the effect of inducers on DNA methylation (5). We have also analyzed the extent to which DNA containing BrdUrd is methylated in vivo. Even with 30% of dT residues placed by BrdUrd, 3.57% of C residues were methylated in vivo (average of two experiments), indicating that this substitution does not have a major effect on methylation in vivo.

DISCUSSION

The studies presented here provide evidence that a minor percentage of sites normally methylated in FL cell DNA fail to be methylated in DNA synthesized during chemically induced differentiation. Although we have been able to establish a stringent correlation between hypomethylation of DNA and differentiation in these cells, it still remains to be demonstrated whether loss of specific 5MeC residues can affect the expression of the genes whose products characterize the terminally differentiated FL cell.

All evidence in the literature indicates that DNA methylation occurs as an early postreplicative event (4) and that it is restricted to newly synthesized DNA strands (1). This implies that replication of DNA is essential for changing the pattern of DNA methylation and, because no enzyme has been found that "demethylates" DNA, that replication must be accompanied by specific inhibition of DNA methyltransferase to create new unmethylated sites in the DNA. Thus hypomethylation can only play a role in altering gene expression during FL cell differentiation if DNA synthesis is required for the process. Although one possible exception has been reported (14), it appears that FL cells must undergo one to two cell divisions in the presence of inducing agents to become committed to terminal differentiation (15). A link between commitment and DNA hypomethylation is suggested by the findings that DNA hypomethylation can be detected after one or two cell divisions in the presence of an inducing agent and that both the fraction of cells committed to differentiation and the degree of DNA hypomethylation increase with longer exposure to inducing agents.

In vertebrates, 5MeC is found predominantly in the se-

quence 5-MeCp(phosphate)G (9, 11). Using restriction endonucleases that recognize one of the CpG containing sites, it has been possible to obtain evidence indicating that the pattern of methylation of specific C residues may be associated with gene expression. In the most striking example, it was shown that an inverse correlation exists between the level of methylation of specific segments of integrated adenovirus DNA and the extent to which these segments are expressed as mRNA in transformed cells (29). Similarly, > 80% of CCGG sequences are methylated in *Herpesvirus saimiri* DNA isolated from lymphoid cells that do not express the viral genome, whereas the viral sequences are completely unmethylated in virus producing lines (8). These observations, in conjunction with the findings that completely unmethylated, amplified rRNA genes are efficiently expressed in the oocyte of *Xenopus laevis* (7) and that one CCGG site in the β-globin gene of the chicken exists only in an unmethylated state in the DNA of tissues that express or have expressed the gene (17), may indicate that methylation of specific gene sequences acts as an "off" signal. However, in two earlier reports there was no compelling evidence linking gene activity with specific unmethylated CCGG sequences in the β-globin gene of the rabbit (32) or in the chromosomal rRNA genes of *Xenopus laevis* (2).

The experiments reported here provide a measure of the magnitude of the change in methylation of the total genome of FL cells undergoing terminal differentiation, rather than an examination of a few accessible sites in specific genes whose expression is altered during the process. We are currently investigating whether specific sites in or near the globin genes are among those affected. However, it should be noted that the dinucleotide CpG occurs with less than predicted frequency in the sequences in and immediately adjacent to the coding regions of the mouse α, β-major, and β-minor globins. Only 276 CpG per diploid genome occur in these sequenced regions (13, 18). It can be calculated from our data that ~300,000 unmethylated sites arise per haploid FL cell genome during differentiation. Thus, the hypomethylation of DNA that occurs must affect many sites not immediately associated with globin genes and may indicate that terminal differentiation of FL cells involves a change in the pattern of methylation of a number of genome regions.

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