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Replacement of 5-methylcytosine by cytosine: A possible mechanism for transient DNA demethylation during differentiation

(Friend erythroleukemia cells/active demethylation/exchange of cytosine–5-methylcytosine)

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ABSTRACT **In an earlier study it was discovered that when Friend erythroleukemia cells (FELC) were exposed to a variety of chemical agents capable of inducing differentiation, their DNA underwent genome-wide transient demethylation. In an attempt to elucidate the biochemical mechanism responsible for this phenomenon we have induced FELC with 5 mM hexamethylenebisacetamide and labeled the DNA *in vivo* with a density label, 5-bromodeoxyuridine, and a radioactive label, deoxy[5-^{3}H]cytidine. Newly replicated DNA (heavy–light) was separated from parental DNA (light–light) by isopycnic centrifugation. Incorporation of deoxy[5-^{3}H]cytidine into light–light duplex DNA has been observed only in induced cells concomitantly with the demethylation of the DNA, whereas, in parallel experiments, deoxy[G-^{3}H]adenosine was not incorporated into light–light DNA. It was also found that the labeling of light–light DNA with deoxy[5-^{3}H]cytidine is transient since the ^{3}H label was removed from the DNA during the period of *de novo* DNA methylation that follows the demethylation. These results, taken together, strongly suggest that the demethylation of the DNA during differentiation is achieved by an enzymatic mechanism whereby 5-methylcytosine is replaced by cytosine.**

In a previous study, while analyzing changes in DNA methylation during terminal differentiation of Friend erythroleukemia cells (FELC), some of us have observed that genome-wide DNA hypomethylation is induced by a variety of agents that are known to cause differentiation in these cells (1). Genome-wide hypomethylation has also been observed in developing mouse (2) and rabbit (3) early embryos and in teratocarcinoma mouse cells in response to inducers of differentiation (2, 4, 5). It is noteworthy that the hypomethylation observed in FELC was found to be transient: demethylation takes place relatively rapidly (within several hours) and is followed by *de novo* methylation (1). The kinetics of this demethylation process is not consistent with a passive mechanism of hypomethylation—namely, loss of methyl groups by replication of the DNA due to the absence or inhibition of maintenance methylation. In addition to this observation, two other instances have been reported recently that suggest an active mechanism for DNA demethylation. One *Hpa* II site upstream to the chicken vitelogenin II gene undergoes demethylation in response to estrogen treatment in the absence of replication (6), and a region in the Epstein–Barr virus DNA undergoes demethylation in response to induction by phorbol 12-myristate 13-acetate and butyrate before viral DNA amplification takes place (7). In an attempt to elucidate the mechanism by which active demethylation takes place, we have considered three possible mechanisms: (*i*) removal of the methyl group from 5-methylcytosine by direct demethylation; (*ii*) removal of 5-methylcytosine and its replacement with cytosine through an enzymatic mechanism not previously described; (*iii*) removal of a stretch of DNA that would include the 5-methylcytosine moiety by the conventional excision-repair mechanism. The first mechanism may be excluded *a priori* since it would require a reductive cleavage of a C—C bond by a biochemically unprecedented and improbable reaction (8). The other two mechanisms are possible and a number of analogous reactions have been reported in other systems (9–14). In the present study we present experiments that allow us to distinguish between these two mechanisms. The results indicate that the active DNA demethylation observed during FELC differentiation is achieved by a unique mechanism whereby 5-methylcytosine is specifically replaced by cytosine.

MATERIALS AND METHODS

Growth of Cells. Mouse erythroleukemia cells (line 745 originally isolated by C. Friend) were obtained from E. Fibach of the Department of Hematology, Hadassah, Jerusalem. Cells were maintained in Dulbecco's modified Eagle's medium (Biolab, Jerusalem) supplemented with 15% fetal calf serum and 100 units of streptomycin/penicillin per ml. Inductions were performed at a cell density of 1–1.5 × 10^5 cells per ml by the addition of 5 mM hexamethylenebisacetamide (HMBA) (Sigma).

Centrifugal Elutriation and Fluorocytometry. Cells were separated to the various cell-cycle phases by elutriation. Cells (1–5 × 10^8) were suspended in Hanks' balanced salt solution and introduced into the spinning rotor of the Beckman elutriation centrifuge (model JE-6B). Conditions for elutriation were as described by Gambari *et al.* (15). Samples from each fraction were stained with propidium iodide for fluorocytometry and analyzed in a Becton Dickinson FACS 440 cell sorter as described (16).

DNA Labeling *in Vivo*. Two hours before harvesting the cells, newly synthesized DNA was density-labeled by addition of 10 μM BrdUrd and 1 μM 5-fluoro-2′-deoxyuridine (FdUrd) to the growing cultures to inhibit *de novo* synthesis of TMP. One hour later, 2.5 μCi (1 Ci = 37 GBq) of deoxy[G-^{3}H]adenosine (22 Ci/mmol) per ml or 2.5 μCi of deoxy[5-^{3}H]cytidine (22 Ci/mmol) per ml was added to the cell cultures (radioactive material was from Amersham).

DNA Preparation and Analysis. DNA was prepared as described (17); part of the DNA was analyzed for the extent of methylation at CpG sequences and the rest was subjected to isopycnic centrifugation. The degree of methylation of CpG-containing sequences was determined by a modification

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Abbreviations: FELC, Friend erythroleukemia cell(s); HMBA, hexamethylenebisacetamide; FdUrd, 5-fluoro-2′-deoxyuridine.

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of a previously described method (18). According to this method, DNA samples (1–2 μg) were nicked by 15 min of incubation at 37°C in a 20-μl reaction mixture containing 50 mM Tris·HCl (pH 7.4), 5 mM $CaCl_2$, and 1.4 mM mercaptoethanol with DNase I (from Sigma, 0.7 μg/ml). The 3′ ends of the nicks were labeled by an additional 30-min incubation at 15°C with 20 μCi of [α-^{32}P]dGTP (3000 Ci/mmol) and *Escherichia coli* DNA polymerase I (9 units). Unreacted labeled nucleotide was removed by chromatography through a Sephadex G-50 column. The labeled DNA was digested to deoxyribonucleoside 3′-monophosphates using spleen phosphodiesterase and micrococcal nuclease. The digest was chromatographed in two dimensions on cellulose-coated thin-layer chromatography sheets (Eastman Kodak). The chromatograms were autoradiographed and the autoradiograms were scanned by using a Helena Laboratories (Beaumont, TX) Quick Scan. The extent of CpG methylation has been calculated from the radioactivity found in the cytosine and 5-methylcytosine spots (18). For isopycnic centrifugation the density of the DNA solutions was adjusted to 1.71 g/ml. The DNA was sheared by forcing the DNA solutions three times through a 25-gauge needle. The sheared DNA was subjected to 70 hr of ultracentrifugation at 40,000 rpm in a Beckman Ti 50 rotor at 20°C. The CsCl gradients were collected. A_{260} was measured in an LKB Ultraspec II spectrophotometer and aliquots were diluted in H_2O and assayed for radioactivity in Hydrofluor (National Diagnostics, Somerville, NJ) scintillation fluid.

RESULTS

Incorporation of Deoxycytidine into Nonreplicating DNA of FELC During HMBA-Induced Differentiation. To investigate the mechanism by which active demethylation occurs during differentiation we used FELC induced by 5 mM HMBA. In previous experiments, we have observed that induction of FELC is accompanied by a transient genome-wide hypomethylation that peaks at around 12 hr after induction. The experiment described below was designed to examine the possibility that DNA demethylation is based on a mechanism involving the exchange of 5-methylcytosine residues in the DNA with unmethylated cytosine moieties. Cells were induced for 12 and 24 hr as described in *Materials and Methods*. At 10 and 22 hr after the onset of the experiment, 10 μM BrdUrd and 1 μM FdUrd were added to the treated and control cell cultures. At 11 and 23 hr after induction, 2.5 μCi of deoxy[5-^{3}H]cytidine (22 Ci/mmol) per ml was added and cells were harvested 1 hr later. DNA was prepared from all cultures and aliquots of DNA were used to analyze the overall methylation level of CpG sequences (see *Materials and Methods*). The rest of the DNA was sheared and the density of the DNA solution was adjusted by CsCl and subjected to isopycnic centrifugation to assess the distribution of radioactive label between heavy–light DNA (newly replicating DNA) and light–light DNA (nonreplicating DNA). As shown in Fig. 1, label in light–light DNA was detected *only* in the 12-hr-treated cells. Moreover, it can be seen that the ratio of 5-methylcytosine to cytosine in the DNA from cells induced for 12 hr is strikingly lower than that from untreated control cells or from cells incubated for 24 hr after induction. The incorporation of labeled deoxycytidine into light–light DNA can be explained either by an enzymatic mechanism by which 5-methylcytosine is specifically replaced by cytosine or by the conventional nucleotide excision-repair mechanism by which a longer patch of DNA composed of different deoxynucleotides is excised and replaced. It was important to distinguish between these two possible mechanisms, and this could be done (as described in the next section) by comparing the incorporation of deoxycytidine and deoxyadenosine into light–light DNA.

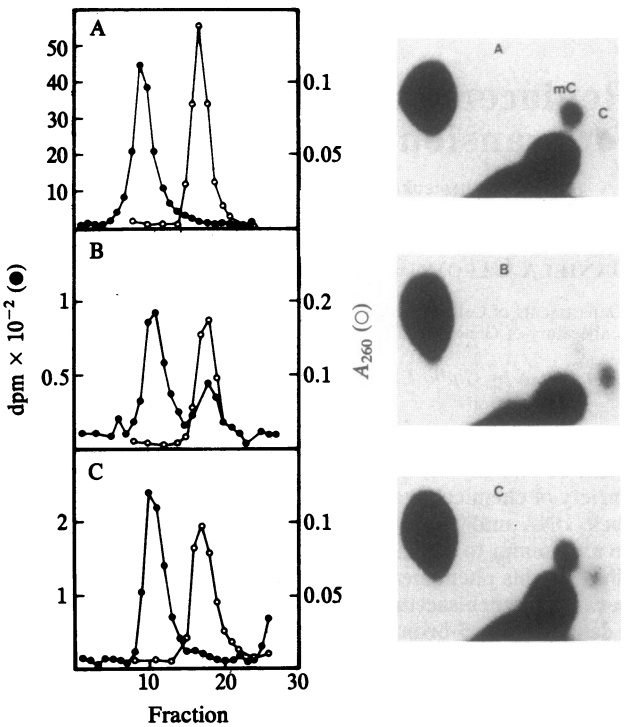

FIG. 1. Extent of methylation and distribution of cytosine incorporated into HMBA-induced FELC. FELC were grown to 1–1.5 × 10^6 cells per ml in Dulbecco's modified Eagle's medium supplemented with 15% fetal calf serum and diluted 1:10 in fresh medium with and without 5 mM HMBA. At 10 and 22 hr, correspondingly, 10 μM BrdUrd and 1 μM FdUrd were added. At 11 and 23 hr, 2.5 μCi of deoxy[5-^{3}H]cytidine was added. After 12 and 24 hr, the cells were harvested and DNA was prepared. Aliquots containing 1 μg of DNA were used for analysis of the content of 5-methylcytosine in CpG sequences (see *Methods and Methods* and ref. 18). Autoradiographs of two-dimensional chromatograms are shown on the right. The radioactive spots represent the nearest neighbors of guanine residues in the DNA. C represents cytosine in CpG and mC represents 5-methylcytosine in CpG. The extent of methylation of CpG was calculated from the density of the mC and C spots obtained by densitometry performed on a Helena Quick Scan densitometer. The values are listed in Table 1. (*A*) Nontreated cell DNA. (*B*) DNA of 12-hr-treated cells. (*C*) DNA of 24-hr-treated cells. The rest of the DNA was subjected to neutral isopycnic centrifugation. Fractions were collected from the bottom of the tube. Radioactivity and A_{260} were measured in each fraction.

DNA Demethylation During Differentiation of FELC Is Due to Specific Replacement of 5-Methylcytosine with Cytosine. In nonsynchronized FELC cultures the time course of demethylation after the addition of HMBA to induce differentiation proved to be somewhat variable. By contrast, we established that in synchronized cells the course of induction of cells at the G_1 phase of the cell cycle was very reproducible and resulted in maximal demethylation at 18 hr after induction (unpublished data). Therefore, in the next series of experiments, we used synchronous FELC cultures obtained by centrifugal elutriation (see *Materials and Methods*). In a typical experiment, cells harvested at a density of 10^6 cells per ml were subjected to centrifugal elutriation and analyzed by fluorocytometry. The fraction corresponding to the G_1 phase was inoculated into two identical cultures of Dulbecco's modified Eagle's medium containing 15% fetal calf serum and 5 mM HMBA. At various times after induction, 10 μM BrdUrd and 1 μM FdUrd were added to both flasks, followed, 1 hr later, by addition of either 2.5 μCi of deoxy[5-^{3}H]cytidine per ml or 2.5 μCi of deoxy[G-^{3}H]adenosine per ml. After an additional period of incubation (60 min) the cells were harvested, and the extracted DNA was analyzed for the

extent of methylation and the distribution of label between replicating and nonreplicating DNA separated on CsCl gradients. The results of a representative experiment shown in Fig. 2 demonstrate that deoxycytidine but not deoxyadenosine was incorporated into the nonreplicating light–light DNA. This incorporation was observed only in response to induction of cell differentiation by HMBA, and the timing of label incorporation correlated well with the timing of demethylation of the DNA at the mCpG sequences (Table 1). These findings confirm the results obtained with nonsynchronous cultures and clearly rule out the possibility that the active demethylation of DNA during induction is due to an excision-repair mechanism whereby a patch of DNA is excised and replaced.

Cytosine Replaces Specifically 5-Methylcytosine Moieties at CpG Sequences. The data reported above indicate that during differentiation the time course of DNA demethylation at the mCpG sequences correlates with incorporation of deoxycytidine into light–light DNA. To determine whether the incorporation of deoxycytidine is limited to mCpG sequences and is specific for these, we took advantage of the fact that the hydrogen atom at position 5 of the cytosine ring is removed during *de novo* methylation and replaced by a methyl group. Thus, when deoxycytidine labeled with ^3H at the 5 position is used, it would be expected that the label would be lost during the *de novo* methylation that follows the period of demethylation. On the other hand, the radioactive label from [5-^3H]cytidine incorporated into nonmethylatable sequences would not be lost. The following experiment was designed to follow the fate of the ^3H atom incorporated into light–light DNA. G_1-phase FELC were treated with 5 mM HMBA for 16 hr. Addition of 10 μM BrdUrd and 1 μM FdUrd was followed by addition of 2.5 μCi of deoxy[5-^3H]cytidine per ml, as described in the previous section. At 18 hr (peak of demethylation), one-half of the cells was harvested, and the other half was washed and resuspended in fresh medium including 5 mM HMBA, 10 mM thymidine, and 2.5 μCi of deoxy[5-^3H]cytidine per ml and incubated for another 6 hr. The radioactive deoxycytidine was added to rule out the possible elimination of label in the light–light DNA by dilution of the cytidine incorporated into DNA as a result of continuous exchange. Cells were harvested and DNA was prepared from the two cultures. Aliquots were used to determine the extent of methylation at CpG sequences (Table 1), and the rest of the DNA was analyzed by isopycnic centrifugation as described above. As shown in Fig. 3, the label in the light–light DNA observed after 18 hr of treatment with HMBA was eliminated during the subsequent 6 hr (18–24 hr) of incubation. At this period *de novo* methylation took place (see Table 1). We conclude from these results that cytosine is incorporated specifically into CpG sequences (replacing 5-methylcytosine), thus becoming prone to methylation, as reflected by the removal of the labeled hydrogen at position 5 of the cytosine ring while these sites become methylated.

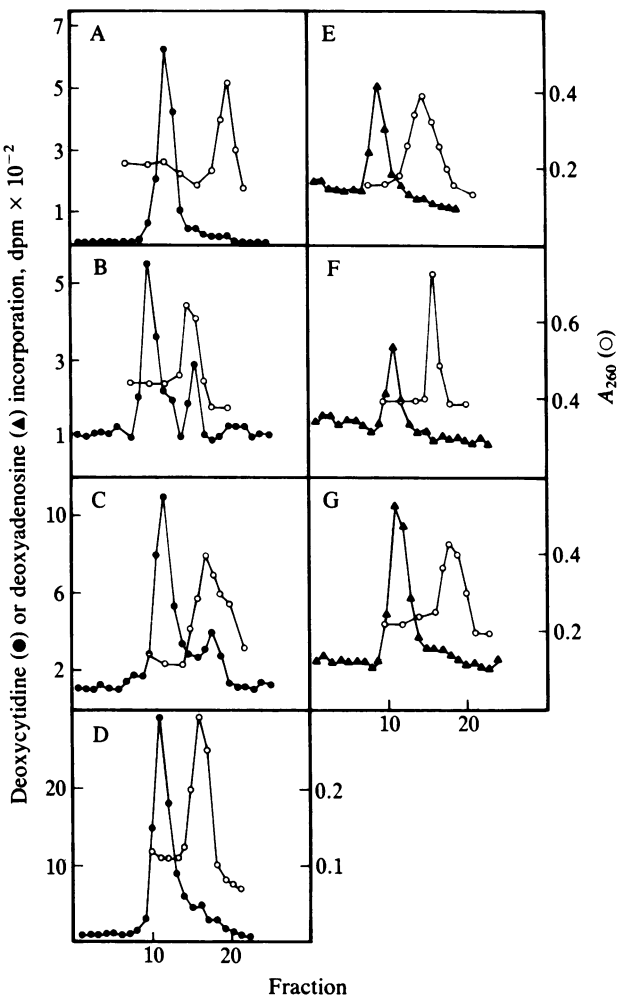

FIG. 2. Incorporation of deoxy[5-^3H]cytidine or deoxy[G-^3H]adenosine into DNA of G_1-phase FELC treated with HMBA. FELC were separated into the various cell cycle phases by elutriation. Cells of the G_1 phase (determined by cytofluorometry) were inoculated (1–1.5 × 10^5 cells per ml) in fresh Dulbecco's modified Eagle's medium, 15% fetal calf serum, and 5 mM HMBA. Two hours before harvest, 10 μM BrdUrd and 1 μM FdUrd were added, followed by the corresponding addition, 1 hr before harvest, of 2.5 μCi of deoxy[5-^3H]cytidine per ml or 2.5 μCi of deoxy[G-^3H]adenosine per ml. Cells were harvested at 18, 21, and 24 hr, and DNA was prepared and analyzed as described in the legend to Fig. 1. (*A–D*) DNA samples labeled with deoxy[^3H]cytidine. (*E–G*) Samples labeled with deoxy[^3H]cytidine. (*A* and *E*) Untreated controls. (*B* and *F*) HMBA-treated (18 hr). (*C* and *G*) HMBA-treated (21 hr). (*D*) HMBA-treated (24 hr). The extent of methylation at CpG sequences was determined as described in the legend to Fig. 1. The values obtained are listed in Table 1.

Table 1. Extent of methylation at CpG sequences of DNA from FELC treated with HMBA

Treatment time, hr	% of total CpG methylated: Unsynchronized cells*	% of total CpG methylated: G_1-phase cells†
Untreated controls‡	83	81
12	40.8	—
18	—	55
21	—	63
24	79.9	71
18 + 6§	—	70

Unsynchronized cells and G_1-phase cells were used for induction.
*See experiment described in text and the legend to Fig. 1.
†See experiments described in text and the legends to Figs. 2 and 3.
‡The extent of methylation of CpG sequences in mammalian somatic tissues is in the range of 75–85%.
§Cells were treated for 18 hr and washed, and treatment was continued for an additional 6 hr (see experiment described in text and the legend to Fig. 3).

DISCUSSION

The principal conclusion that emerges from this study is that the rapid and extensive DNA demethylation, shown earlier to be associated with induction of differentiation, coincides with the specific incorporation of deoxy[5-^3H]cytidine into nonreplicating DNA. A number of experiments were designed to explore the mechanism by which DNA demethylation is accomplished. As noted above, a direct enzymatic removal of

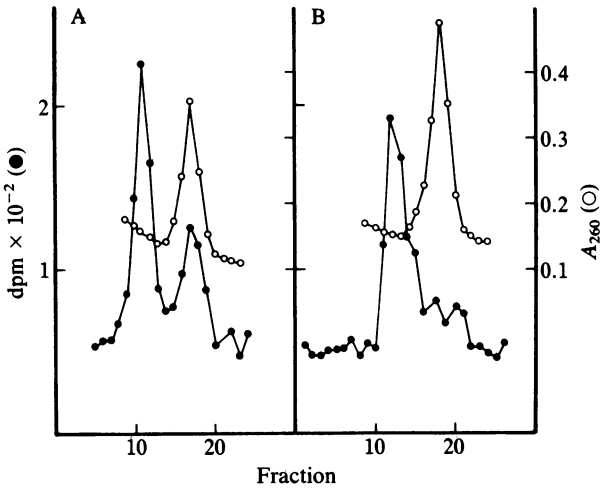

FIG. 3. Elimination of the ^3H label incorporated into light–light DNA during demethylation by an additional incubation at a period of *de novo* methylation. FELC were treated with HMBA and labeled with deoxy[5-^3H]cytidine, and DNA was analyzed. (*A*) Distribution of label between heavy–light and light–light DNA of 18-hr-treated G_1-phase cells. (*B*) Distribution of label following an additional 6 hr of incubation. The extent of methylation at CpG was analyzed. The results are listed in Table 1.

the methyl group from the 5-methylcytosine residues in DNA is mechanistically improbable since the transfer of the methyl group of *S*-adenosylmethionine to carbon, nitrogen, sulfur, or oxygen atoms is irreversible at neutral pH (8). It was possible to visualize two different mechanisms whereby deoxy[5-^3H]cytidine could be incorporated into nonreplicating DNA and these mechanisms were distinguished by showing that deoxycytidine, but not deoxyadenosine, is incorporated into nonreplicating DNA. Finally, it was shown that the incorporation of deoxycytidine is specific to methylated CpG sequences since the ^3H label at the carbon in position 5 of the cytosine ring is eliminated during the period when active *de novo* methylation takes place. All of these results taken together indicate that the mechanism for the demethylation observed during FELC differentiation involves a process whereby 5-methylcytosine moieties at CpG sequences are replaced by cytosine residues. It has not yet been established whether this reaction involves the replacement of 5-methylcytosine with cytosine at the base, deoxyribonucleoside, or deoxyribonucleotide level and the exact mechanism of this unique reaction will have to be determined by experiments in cell-free systems.

The presence of a DNA-demethylating activity in the nucleus of murine erythroleukemia cells had been reported before (19). However, that activity seems to bear no relevance to the demethylation observed by us in FELC in response to induction by HMBA (1). The demethylation observed by us is transient and occurs at the G_1 phase of the cell cycle in cells that have completed a full cell cycle in the presence of the inducer or in a mixed culture of G_1- and S-phase cells (unpublished data). The demethylating activity observed by Gjerset and Martin (19) was found in nuclei from noninduced FELC as well as from induced cells at the time when differentiation was completed. The significance of this reported activity remains unclear and does not contribute to our understanding of the process of demethylation during differentiation.

Based on the results presented here it is tempting to speculate that the observed replacement of 5-methylcytosine by cytosine is a result of a glycosylase and insertase activity induced by a differentiation factor. DNA glycosylases capable of removing 3-methyladenine and 7-methylguanine from DNA have been observed in various mammalian systems (9–13). A protein that binds specifically to partially depurinated DNA and catalyzes the insertion of purines into apurinic sites has been isolated from cultured human fibroblasts (14); similar activity has been reported to exist in *Escherichia coli* (23). Analogous enzymes were discovered that catalyze guanylylation of tRNA (20, 21) and transfer of queunosine into the anticodon wobble position of mature tRNA molecules (22). Efforts must now be focused on exploring the possibility that similar enzymatic activities are triggered by the inducers of differentiation. In this regard, it should be noted that many of the compounds capable of inducing differentiation and, as we have shown earlier, genome-wide demethylation are known inducers of protein kinases. Phosphorylation–dephosphorylation is an attractive possible mechanism for a transient induction of the appropriate enzymatic activities in light of the fact that the demethylation during FELC differentiation has been shown to be a transient phenomenon.

Although the detailed biochemical events leading to the observed changes in DNA methylation during differentiation will require further study, the present report sheds light on the mechanism by which DNA demethylation takes place. The results described here suggest that enzymatic removal of 5-methylcytosine and its replacement by cytosine is responsible for the rapid erasure of a preexisting methylation pattern that is presumably required to set the stage for a new methylation pattern characteristic of the next state of differentiation.

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