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# DNA demethylation in erythroleukaemia cells

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Despite a fall in the proportion of CGs methylated, evidence has not been obtained for significant demethylation of prelabelled DNA when mouse erythroleukaemia cells are induced to differentiate. There is, however, a delay in the methylation of the DNA that is synthesised in the early period of induction, leading to its undermethylation by 30–50% and this may be a contributory cause of the observed fall in CG methylation.

DNA; Methylcytosine; MEL; Friend erythroleukemia; Demethylation

## 1. INTRODUCTION

Agents which cause hypomethylation also induce differentiation of Friend erythroleukaemia (MEL) cells [1–5], yet direct measurement of the level of DNA methylcytosine in cells induced by dimethylsulphoxide (DMSO) or hexamethylene bisacetamide (HMBA) have generally failed to show significant changes. Following such induction the DNA does become a better substrate for DNA methylase suggesting that an increased number of CG dinucleotides are present in an unmethylated state.

Direct evidence for undermethylation was obtained by Razin et al. [6], who showed a dramatic, yet transient, fall in the proportion of CG dinucleotides methylated 12–18 h after induction of MEL cells with HMBA. They presented evidence to support a proposal that the loss of methyl groups is a result of the replacement in the DNA of existing methylcytosine by newly incorporated cytosine. As adenine did not appear to be similarly replaced, they concluded that replacement is not the result of an excision repair reaction but rather was caused by a transglycosylase reaction similar to that which occurs during the insertion of queuosine and inosine into tRNA [7,8] or possibly during purine insertion into apurinic sites in DNA [9].

Another way in which such reactions can be investigated is to look at the fate of prelabelled DNA bases following induction of differentiation and this is the basis of the present communication. 2-[$^{14}$C]Deoxycytidine has been used to label uninduced Friend cells for 3 days. This is incorporated into DNA cytosine (and thereby methylcytosine) but mostly into DNA thymine giving a ratio of counts in cytosine + methylcytosine to counts in thymine (C/T ratio) of about 0.2. This imbalance is probably caused by the action of a high intracellular dCTP/dTTP ratio stimulating dCMP deaminase [10]. The proportion of radioactive cytosine methylated (%mC) at the end of the prelabel is about 3.6%, in close agreement with previously reported figures [4] and to the value obtained by optical analysis (personal observation).

When such prelabelled cells are induced to differentiate, a loss of methylcytosine, however transient, should be evident as a permanent reduction in the proportion of [$^{14}$C]cytosines methylated. Although we have confirmed the results of Razin et al. [6] that there is a reduction in the proportion of CG dinucleotides methylated, no significant fall was observed in the proportion of prelabelled cytosines methylated on induction of MEL cells.

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## 2. MATERIALS AND METHODS

Mouse erythroleukaemia cells growing in RPMI 1640 containing 5% foetal calf serum, were induced to differentiate by addition of DMSO (1.5% v/v) or HMBA (4 mM). Cells were prelabelled by growing for three days in the presence of 2-[$^{14}$C]deoxycytidine (NEN) at 1 $\mu$Ci per 30 ml. The labelled medium was replaced prior to induction. Cells were also labelled for 6 h periods during differentiation using 6-[$^{3}$H]uridine (Amersham) (10 $\mu$Ci/ml).

Cells were harvested and DNA prepared for use as a substrate in a DNA methylase assay and its base composition analysed using an Aminex A6 column (BioRad) as previously described [11]. In addition the DNA was nick translated in the presence of [$\alpha$-$^{32}$P]dGTP (Amersham) and then hydrolysed using micrococcal nuclease and spleen phosphodiesterase (Sigma). The 3′ mononucleotides produced were separated on a Pharmacia MinoRPC column with isocratic elution using 0.1 M tetrabutylammonium phosphate/80 mM NaCl, pH 6.0, and the radioactivity incorporated into dCMP and methyldCMP measured to give an estimate of the proportion of CG dinucleotides methylated [6]. Cells were also analysed for their DNA methylase activity [12] and protein was assayed by the method of Bradford [13].

## 3. RESULTS

MEL cells were induced to differentiate with DMSO and DNA was isolated at various times and the proportion of CG dinucleotides methylated is shown in Fig. 1.

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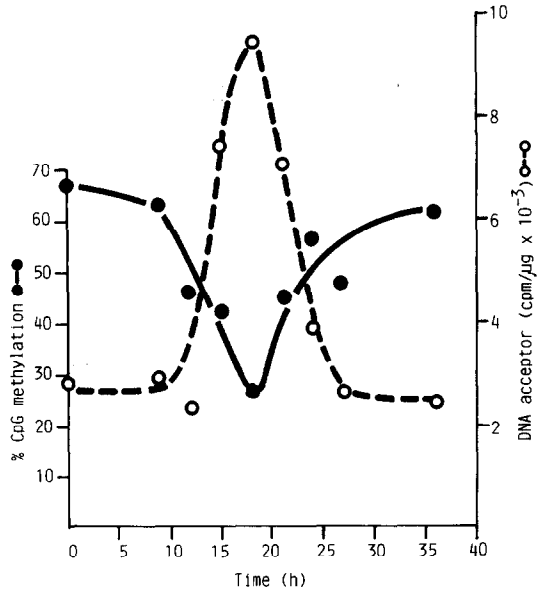


Fig. 1. Transient undermethylation of DNA and CG dinucleotides on induction of MEL cells. DNA was isolated from cells at various times after addition of DMSO and either (a) the proportion of CG dinucleotides methylated measured as described in section 2 (●---●), or (b) used as the substrate in an assay of mouse DNA methylase (○---○).

These results confirm those of Razin et al. [6] and illustrate a halving in the proportion of CGs methylated at 15 and 21 h after induction.

To investigate which DNA was undermethylated, prelabelled MEL cells were induced to differentiate in the absence of further radioactive precursors and samples taken at various times. The results of [$^{14}$C]base analyses are presented in Table I which shows the combined results of four different experiments. Although a decrease was found in the proportion of [$^{14}$C]cytosine methylated at 19 h and 26 h, the extent of the decrease was very variable and cannot be considered significant. The lower value was not maintained; rather a small increase is seen at two and three days to give a %mC of 3.9%. This increase, which was not observed in control cells, was accompanied by synthesis of haemoglobin in the induced cells which were distinctly pink by 50–70 h.

Table I

Base composition of control and induced prelabelled MEL cells

| | %mC | C/T |
|---|---|---|
| 0 h | 3.67 ± 0.08 | 100 |
| 11 h | 3.64 ± 0.06 | 98 |
| 19 h | 3.48 ± 0.36 | 102 |
| 26 h | 3.41 ± 0.58 | 84 |
| 40–48 h | 3.83 ± 0.12 | 86 |
| 68 h | 3.94 ± 0.07 | 78 |

Cells, prelabelled with [$^{14}$C]deoxycytidine as described in section 2, were induced with either DMSO or HMBA. Control and induced cells were harvested when indicated and the base composition of the prelabelled DNA analysed. The %mC results are presented as the means ± SD for four separate experiments combined and the C/T ratios are expressed as the averaged percent of the uninduced value

The reduced methylation observed at 15–18 h may be largely restricted to the DNA made following induction and may not apply to prelabelled DNA. By labelling cells for 6 h periods with [6-$^{3}$H]uridine we have shown that the DNA made in the first day following induction is undermethylated (%mC = 2.5) whereas that made in the second day (when the rate of DNA synthesis is maximum) has 3.9% of its cytosines methylated. Fig. 2 shows that DNA methylase rises 6-fold in activity over the 48 h after induction but is low during the first day and this may account for this reduced methylation of DNA made at this time.

Delayed methylation would result in an increase in hemimethylated sites arising as a result of a failure of the endogenous DNA methylase to keep pace with replication [14]. This undermethylation is confirmed by analysis of the ability of the DNA to act as a substrate for DNA methylase (Fig. 1). These results extend those of Bestor et al. [5] and indicate by an independent method that a dramatic yet transient undermethylation is observed at 18 h after induction.

## 4. DISCUSSION

Although we have confirmed the transient undermethylation of DNA following induction of MEL

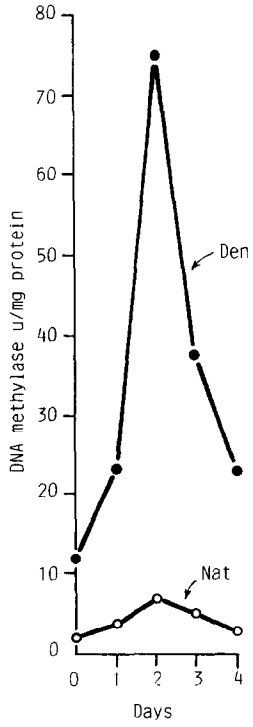


Fig. 2. DNA methylase activity on induction of MEL cells. MEL cells were induced by addition of DMSO and samples taken at various times for assay of DNA methylase in nuclear extracts, using a native or a denatured DNA substrate. The extracted nuclear pellets were also assayed and showed similar changes in activity but at a lower specific activity.

cells, we have shown that this is not the result of selective removal of methylcytosine from the preexisting DNA.

It is possible that both [$^{14}$C]cytosine and methylcytosine are removed from DNA and replaced with newly-synthesised, non-radioactive cytosine. This would result in a fall in the ratios of both [$^{14}$C]methylcytosine and [$^{14}$C]cytosine to [$^{14}$C]thymine. This was observed to some extent (Table I) but cannot wholly account for the size of the effect which would require the $^{14}$C C/T ratio to fall to less than 50% of the initial value, while the observed fall averages only 22%. An alternative explanation is that the removal of $^{14}$C prelabelled methylcytosine is masked by the methylation of previously unmethylated, $^{14}$C-labelled cytosine. Because of the magnitude of the effect this seems unlikely.

Delayed methylation is found at each S-phase [14] and will occur partially when cells are subcultured as this leads to partial synchrony in G1-phase [15]. Razin et al. [6] note that undermethylation is observed more consistently when G1-phase cells are selected by elutriation and we have shown that such cells have very low levels of DNA methylase activity [16].

To what extent undermethylation arising as a result of delayed methylation is a prerequisite for differentiation is not clear. A hemimethylated window, created at the beginning of each S-phase, may provide an opportunity, when changes occur in the environment, for changes also to occur in transcription factor binding or chromatin arrangement.

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