

## Decrease in DNA methylase activity during preimplantation development in the mouse

M. MONK<sup>1</sup>, R. L. P. ADAMS<sup>2</sup> and A. RINALDI<sup>2</sup>

<sup>1</sup>MRC Mammalian Development Unit, 4 Stephenson Way, London NW1 2HE, UK

<sup>2</sup>Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ, UK

### Summary

During early mouse development, there are large-scale changes in DNA methylation. These changes may be due to the availability or stability of the enzyme, DNA methyltransferase (methylase), which is responsible for maintenance of DNA methylation. A microassay for methylase activity in preimplantation embryos shows that the level of maternally inherited enzyme is

extremely high in the egg and that this activity is stable for the first three cleavage divisions. However, from the 8-cell to the blastocyst stage, there is a marked and absolute decrease in enzyme activity.

Key words. DNA methylase, preimplantation embryos, mouse development, DNA methylation.

### Introduction

Early mouse development is associated with large changes in DNA methylation. The sperm and egg genomes are differently methylated, overall (Monk *et al.* 1987), for repetitive DNA sequences, and low copy gene sequences (Sanford *et al.* 1987). The egg is markedly undermethylated. Although overall methylation is lower in sperm than in somatic tissue (Razin *et al.* 1986; Monk *et al.* 1987), genes that will have a specialised function in somatic tissues, and repetitive sequences, are highly methylated in sperm. However, sperm satellite DNA is undermethylated (Sturm and Taylor, 1981; Adams *et al.* 1983a). The CpG islands associated with household genes are also unmethylated in sperm as they are in somatic tissues (Bird, 1986).

During preimplantation development, there appears to be a loss in overall methylation between the 8-cell and the blastocyst stage (Monk *et al.* 1987). The undermethylation of blastocyst DNA could be due to demethylation, or lack of maintenance methylation, occurring solely, or primarily, in trophoblast cell DNA. Certainly, trophoblast cell DNA is less methylated than ICM DNA in the rabbit blastocyst (Manes and Menzel, 1981) and in the late blastocyst in the mouse (Monk, 1988). However, it is also possible that the low methylation of the early blastocyst is due to absent or inefficient *de novo* methylation.

It is not yet clear when *de novo* methylation begins in development. A crude method to look at overall methylation shows detectable *de novo* methylation in the ICM cells of the late blastocyst in the mouse (Monk,

1988) and increasing levels of *de novo* methylation in the fetal precursor cells during the period of implantation and gastrulation. The primordial germ cell DNA, both male and female, is markedly undermethylated as early as 11½ days gestation and it is proposed that the germ line may escape extensive *de novo* methylation (Monk *et al.* 1987). These early differences in methylation in gametes, and large scale changes in early development, could be associated with processes of programming, deprogramming and reprogramming of the genome.

What could be the basis of large scale changes in methylation in early mouse development? One possibility that must be considered is availability and activity of the enzyme, DNA (cytosine-5) methyltransferase (methylase, EC 2.1.1.37), responsible for maintenance of methylation patterns in the DNA (Adams *et al.* 1979; Gruenbaum *et al.* 1982). During preimplantation development, the activity of methylase will be determined by the level of enzyme inherited in the egg, the stability of maternally inherited enzyme, and the timing of activation of transcription of the embryonic gene for the enzyme.

This paper describes the profile of DNA methylase activity during preimplantation development. We see an absolute decrease in maternally inherited activity which could be the basis of the very low methylation observed at the blastocyst stage. However, the startling observation is that methylase activity in the egg is remarkably high, so that, despite the overall loss of activity, the levels at the blastocyst stage are similar to those in other cells.

## Materials and methods

### Isolation of eggs and embryos

Female F<sub>1</sub> (CBA×C57BL/6) mice were superovulated by intraperitoneal injection of 5 i.u. of pregnant mare's serum and, 48 h later, with 5 i.u. of human chorionic gonadotropin. They were then mated with F<sub>1</sub> males (some females were not mated to provide unfertilised eggs). The following day, fertilised or unfertilised eggs were removed by puncturing the oviducts with a needle, and the eggs were then incubated for 5 to 10 min at room temperature with hyaluronidase (500 i.u. per ml) to remove cumulus cells. Eggs were washed in PBI.PVP (PBI, Whittingham and Wales, 1969, with 4 mg ml<sup>-1</sup> polyvinylpyrrolidone instead of albumin) and any remaining adhering cumulus cells were removed by pipetting the eggs with a finely drawn Pasteur pipette of diameter slightly smaller than that of the egg. Some samples of cumulus cells were also collected.

Embryos at the 2-cell and 8-cell stages (second and third day of pregnancy, respectively) were isolated by flushing medium (PBI.PVP) through the oviducts. Blastocysts on the fourth day of pregnancy were isolated by flushing the uterine horns.

All eggs and embryos were washed in PBI.PVP and isolated in groups of 20 in 3  $\mu$ l of medium in 10  $\mu$ l glass capillaries, the ends of the capillaries sealed in a glass flame, and the samples stored at -70°C, until assay.

Mouse embryonal stem cells (CCE) were kindly provided by Robin Lovell-Badge. They were grown, on a gelatin substrate, in a mixture of equal volumes of BRL cell-conditioned medium and Dulbecco's modified Eagle's medium supplemented with 10 per cent fetal calf and 10 per cent newborn calf serum and 10  $\mu$ M mercaptoethanol. Mouse L929 cells were grown in Eagle's medium (Glasgow modification) containing 10 per cent calf serum. Cells were harvested by trypsinisation, counted and the appropriate number placed in a microfuge tube and washed with PBS (phosphate-buffered saline).

### DNA methyltransferase assay

Following replication of DNA, the normal *in vivo* function of the enzyme is to restore the parental pattern of methylation (maintenance methylation). However, it is as a *de novo* methylase that the enzyme is assayed *in vitro*. There is no evidence that these two facets of activity reside in different enzyme molecules. It has been shown that limited proteolysis can increase *de novo* methylase activity (Adams *et al.* 1983b), protease inhibitors are therefore present in the assay.

For assay of DNA methyltransferase, the samples were freeze-thawed four times, either in microfuge tubes (cells), or in glass capillaries (embryos) which were centrifuged twice in either direction before the samples were removed. Because of the small amounts of material involved, all of each sample was used in the methylase assay. For this reason, results are expressed as enzyme activity per embryo or per cell rather than per mg protein. The reaction mixture (23  $\mu$ l) contained Tris-HCl 50 mM, pH 7.8; EDTA, 1 mM; dithiothreitol, 1 mM; glycerol, 10%; Tween 80, 1%; RNAase A 100  $\mu$ g ml<sup>-1</sup>; poly d(I-C)d(I-C), 22  $\mu$ g ml<sup>-1</sup>; <sup>3</sup>H AdoMet (Amersham, 130  $\mu$ Ci ml<sup>-1</sup>, 87 Ci mmol<sup>-1</sup>) and phenylmethylsulphonyl fluoride, 60  $\mu$ g ml<sup>-1</sup>. Incubation was for 2 h at 37°C and was followed by a further 30 min incubation in 1% SDS containing protease K, 1 mg ml<sup>-1</sup>. DNA was isolated in the presence of carrier by phenol extraction and ethanol precipitation, residual RNA removed by treatment with NaOH, and the radioactive product counted in a scintillation spectro-

meter. Details of this highly sensitive assay have been presented elsewhere (Adams *et al.*, 1991).

## Results

Preliminary work indicated that the microassay developed was capable of detecting methylase in 50 to 100 mouse L929 cells and hence was applicable to the tiny amounts of material in embryo samples. Fig. 1 shows the response of increasing numbers of embryonic stem cells in the assay. The blank value obtained in the assay was 55  $\pm$  9 cts min<sup>-1</sup> for 9 estimations. The cumulus cells gave only background levels of incorporation. The embryonic stem cells had an activity of about 0.3 cts min<sup>-1</sup>/cell, whereas L929 cells showed about 0.9 cts min<sup>-1</sup>/cell.

Fig. 2 shows typical results obtained for methylase activity in the early mouse embryos. The points plotted are individual values for different samples. Activity is extremely high in the unfertilised eggs (around

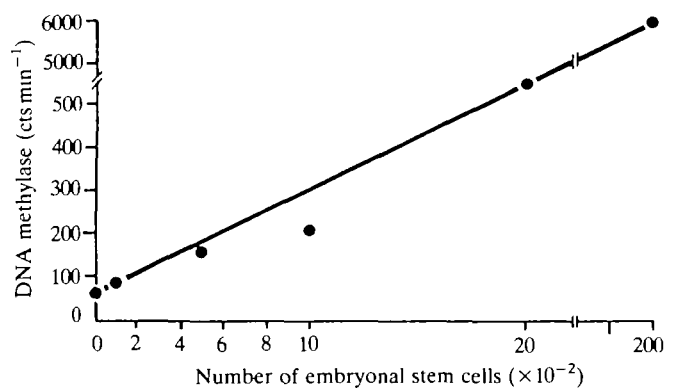


Fig. 1. DNA methylase activity in embryonal stem cells. Lysates from the indicated number of cells were incubated in a standard microassay (see Materials and methods).

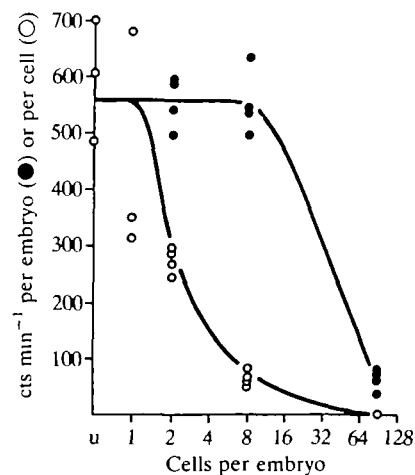


Fig. 2. DNA methylase activity in mouse eggs and early embryos. Lysates from batches of 20 unfertilised (U), 1-cell, 2-cell, 8-cell embryos and blastocysts (approx. 100 cells), were incubated in the standard microassay. Results are given as cts min<sup>-1</sup>/embryo (solid circles) or cts min<sup>-1</sup>/cell (open circles) for individual estimations.

12 000 cts min<sup>-1</sup> for the 20 eggs in each sample, i.e. 600 cts min<sup>-1</sup>/egg) and similar values were found in the fertilised eggs, and 2-cell and 8-cell embryos. Thus the level of activity remains constant on a per embryo basis throughout the first three cleavage divisions. The cause of the variation in activity found, on this occasion, with the fertilised eggs is not known but is unlikely to be a reflection of a variation in time after ovulation which was maintained as constant as possible.

After the 8-cell stage, on a per embryo basis, there is clearly a marked (10-fold) loss of overall DNA methylase activity. On a per-cell basis, the fall in methylase activity between the one-cell and the blastocyst stage (0.6 cts min<sup>-1</sup>/cell) is equivalent to a 1000-fold decrease. However, since the activity in the egg is so high, despite this absolute decrease in activity, and dilution of enzyme due to cell division, the final level of enzyme activity per cell at the blastocyst stage (0.6 cts min<sup>-1</sup>/cell) reaches a similar level to that seen in cultured cells (0.3 cts min<sup>-1</sup>/embryonal stem cell and 0.9 cts min<sup>-1</sup>/mouse ascites Krebs II tumour cell).

## Discussion

For some time, the study of methylation has been directed towards the correlation of degree of methylation with gene activity (Doerfler, 1983; reviewed in Adams and Burdon, 1985), with active and inactive chromatin (Naveh-Manly and Cedar, 1981), with the activity status of the X chromosome (reviewed in Monk, 1986), with the timing of DNA replication in the cell cycle (Shafer and Priest, 1984; Jablonka *et al.* 1985; Selig *et al.* 1988; Adams, 1990), and with the stable heritable nature of the patterns of DNA methylation in differentiated somatic tissues (Wigler *et al.* 1981; Stein *et al.* 1982). Less is known about the role played by changes in methylation during development and tissue diversification (Monk, 1990a). Finely tuned changes in methylation correlated with the onset of tissue-specific gene expression have been the subject of elegant studies by Jost *et al.* (1990) who show demethylation of specific CpG sites in the promoter region of the avian vitellogenin gene associated with the appearance of DNAase I hypersensitivity, changes in specific protein-DNA interactions and onset of transcription. How these specific changes in methylation occur is not known at present. However, during differentiation of Friend erythroleukaemia cells, there is a marked, transient demethylation (Razin *et al.* 1986; Adams *et al.* 1990) which may be associated with replacement of methylated cytosines with non-methylated cytosines; i.e. demethylation could be an enzymic process in this case.

Although the onset of appropriate enzyme activities in specialised cell differentiation may be associated with fine scale adjustment of methylation, the events of programming, deprogramming and reprogramming the genome during gametogenesis, fertilisation, cleavage, separation of the embryonic and extraembryonic lineages and gastrulation, may be characterised by more sweeping changes occurring during a period of

rapidly changing patterns of gene activity (Monk, 1990b). Such changes may be related to the availability of methylase, the level of enzyme inherited in the egg and the time of activation of the embryonic gene for the enzyme. In early development, a supply of household enzymes, and/or maternal messenger RNA (mRNA) for synthesis of these enzymes, is inherited in the egg cytoplasm, to oversee growth, function and development in the initial stages of cleavage. In some cases, the maternal enzyme inherited from the egg cytoplasm is actively degraded between the 8-cell and the blastocyst stage (reviewed in Harper and Monk, 1983). DNA methylase may be another example of this class of enzyme.

A decrease in methylase activity during de-differentiation of *Chlamydomonas* gametes appears to be the reason for a loss of chloroplast methylation (Sano *et al.* 1984). The decrease in methylase in this case was consistent with dilution of the enzyme during cell division. Our experiments show a large decrease in methylase activity in early mouse development. If we assume that the level of activity measured *in vitro* is correlated with the activity of the enzyme *in vivo*, the marked decrease in enzyme activity might account for the overall decrease in methylation at the blastocyst stage. However, the results are not so easily interpreted in this way owing to the fact that the level of methylase in the egg is so remarkably high to begin with.

As explained in the methods section, methylase activity is expressed on a per cell or per embryo basis. This may be more relevant than expressing it per mg protein as it more closely reflects the availability of the enzyme per cell nucleus where the *in vivo* substrate is located. However, as cytoplasmic material is also present in the assay, it is possible that in these early developmental stages the enzyme is not intranuclear, as it is in somatic cells, and that changes in compartmentalisation regulate the functional activity of the enzyme *in vivo*.

As to why methylase activity is so high in the egg is open to speculation at this stage. One possibility is that *de novo* methylation is occurring from the onset of development of the fertilised egg and that the enzyme activity is in excess to requirement so as to compensate for the dilution due to cell division. The efficiency of this *de novo* methylation may be low (see Adams and Burdon, 1985; Adams, 1990) so that an overall increase in methylation only becomes detectable by the ICM stage (Monk, 1988). Alternatively, the high methylase in the egg may ensure that sufficient enzyme survives dilution due to cell division so as to be available for the onset of *de novo* methylation at a later stage.

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