Volume 250, number 2, 134–138 FEB 07249 July 1989

Modulation of DNA methyltransferase during the life cycle of a mealybug *Planococcus lilacinus*

C. Devajyothi and Vani Brahmachari

Department of Microbiology and Cell Biology, ICMR Centre for Genetics and Cell Biology, Indian Institute of Science, Bangalore 560 012, India

Received 30 March 1989

The levels of de novo DNA methyltransferase were studied during the development of a mealybug, *Planococcus lilacinus*. No enzyme activity could be detected in extracts from second instar females. But the enzyme occurs at high levels in third instar females and is maintained at that level during fourth instar when gametogenesis, fertilization and chromosome imprinting occur. These results suggest a developmental stage-specific modulation of levels of DNA methyltransferase. Assays with synthetic polymers showed that the enzyme can methylate not only polymers containing GpG but also those containing CpA and CpI.

Chromosome imprinting; Fertilization; DNA methyltransferase; Sequence specificity; Synthetic polymer

1. INTRODUCTION

Genetic analysis of certain insect species has defined a chromosomal phenomenon termed 'imprinting'. This refers to the remarkable property of particular chromosomes or entire sets of chromosomes to 'remember' their parental origin [1]. The term was first used by Crouse [1] to indicate the differential behaviour of homologous chromosomes in *Sciara*. Brown and collaborators studied this phenomenon in mealybugs and other coccids (Coccoidea; Homoptera; Insecta) [2]. The use of this term was extended to the problem of mammalian X inactivation by Brown and Chandra [3] who defined it as 'a process by which one of two genetically homologous chromosomes is predetermined to function differently from the other at a subsequent stage in development'. In *Planococcus lilacinus*, as in other species of mealybugs, there is a close correlation between inactivation of a haploid set of chromosomes and maleness. In sexually-reproducing mealybugs and other Lecanoid coccids the paternal set of chromosomes is imprinted and inactivated in embryos which develop to be males [4]. There are several lines of evidence suggesting that the mother controls the sex-ratio in her offspring by regulating the proportion of embryos in which such inactivation occurs [2,5,6]. Several authors have suggested that cytosine methylation could be the molecular basis of imprinting and subsequent inactivation of chromosomes [7–9]. Genes located in active and inactive chromosomes show significantly different levels of methylation [10,11]. Recent studies on transgenic mice [12] have provided compelling evidence that DNA methylation is a basis for genomic imprinting. Thus differential methylation of DNA sequences may provide a molecular basis for the observed non-equivalence of parental contributions in embryonic development [13–15].

If methylation is related to imprinting and inactivation of chromosomes in mealybugs, a significant difference in the level of genomic methylation between the two sexes may be expected. In a study of *Planococcus lilacinus*, Deobagkar et al. [16] did

Correspondence address: V. Brahmachari, Department of Microbiology and Cell Biology, ICMR Centre for Genetics and Cell Biology, Indian Institute of Science, Bangalore 560 012, India

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

not observe significant differences between male and female DNAs in the levels of methylation. However, sex-specific differences in methylation levels have been observed in a related species, *Planococcus calceolariae* [17]. Even when the levels of DNA methylation are similar between the two sexes, there may be differences in the sequences methylated. This, in turn, may be due to sex-specific differences in the levels and sequence-specificity of DNA methyltransferase. We report here results suggesting a modulation in sequence specificity of DNA methyltransferase at different developmental stages in a mealybug, *Planococcus lilacinus*.

2. MATERIALS AND METHODS

Stock cultures of mealybugs were obtained from the Horticultural Research Station, Coorg, Karnataka, India. Cultures of a mealybug provisionally identified as *Planococcus lilacinus* (cockerell) were isolated from mass cultures and maintained on pumpkins at room temperature. DNA was extracted from gravid females according to previously published procedures [16].

E. coli cultures (*dcm*$^-$,*dam*$^-$) were obtained from Dr V. Nagaraja and DNA was isolated from them as well as from *E. coli* B following the procedure of Marmur [18]. Calf thymus DNA, ribonuclease and protease were purchased from Sigma Chemical Company, St. Louis, USA. The other reagents used were of analytical grade. The synthetic polymers poly(dI-dC)·poly(dI-dC), poly(dA-dC)·poly(dT-dG) and poly(dG-dC)·poly(dG-dC) were from Pharmacia Chemical Company. *S*-Adenosyl methyl L-[^{14}C]methionine was a gift from Dr C. Vijayasarathy. Specific activity of the sample was 62 μCi/μmol.

2.1. *Preparation of DNA methyltransferase from mealbugs*

Enzyme extraction was carried out following the procedure of Simon et al. [19]. The enzyme was partially purified from male and female mealybugs at different developmental stages through an extraction with 0.8 M KCl followed by DEAE-cellulose chromatography. After DEAE-cellulose chromatography, the enzyme was dialysed against 10 mM Tris-HCl, pH 7.8, 2 mM EDTA, 1 mM DTT, 20% glycerol and 1 mM PMSF for 3 h, aliquoted and stored at −70°C.

2.2. *DNA methyltransferase assay*

The enzyme assay was also performed according to the procedures of Simon et al. [19]. 48 μM *S*-adenosyl [^{14}C]methionine was used in each assay. The reaction was monitored by the method of Parnaik et al. [20]. Non-specific and endogenous methylation was monitored by a control reaction without substrate DNA.

3. RESULTS AND DISCUSSION

Unlike DNA of many other insect species [21,22], *P. lilacinus* DNA contains significant amounts of 5mC [16]. To determine the levels of DNA methyltransferase activity maintained at different stages of development, the enzyme was partially purified from male and female insects starting from second instar upto adulthood. Undermethylated DNAs from various sources were used as substrates. As indicated in table 1, there was no enzyme activity detectable from second instar females with any of the substrates used, whereas significant enzyme activity was observed in third instar females, adults, and at all stages inbetween. Since *E. coli* B lacks 5mC in its DNA, this DNA was used as one of the substrates. Male insects showed detectable levels of methylase from second instar onwards. These results suggested possible stage-specific and sex-specific differences in the levels of DNA methylase.

To understand these differences, reciprocal assays were carried out. Enzymes from different instars of the two sexes were presented with DNA from the same developmental stage 'homologous' as well as from other stages 'heterologous'. The same amount (12 μg) of DNA was used in all the assays. As shown in table 2, no activity was detected in assays where both the enzyme and the substrate DNA were from male insects irrespective of stage of development. But the enzyme extracted from males methylated DNA differed from females to a significant extent (0.4–0.6 pmol/μg protein per h). The low level of methylation obtained in homologous assays with enzyme from both males and females may be due to non-specific methylation in vitro. The enzyme from females exhibited poor activity with both heterologous as well as homologous substrates. These results indicated that there may be sites in the DNA of the female which are undermethylated in vivo but are recognised and methylated by the enzyme from male insects, indicating a possible difference in the site of methylation in the two sexes.

If DNA sequences methylated in the two sexes are different, it may be possible to detect such differences using synthetic polynucleotides. Three polynucleotides containing alternating purine-pyrimidines, representing the dinucleotides CpG, CpA and CpI were chosen. Poly(dI-dC)·poly(dI-dC) does not represent a naturally occurring dinucleotide, but it has been observed that some conformational feature renders this polymer a bet-

Table 1

Methylation of natural substrates

Source of enzyme	Specific activity		
	dcm⁻ dam⁻ E. coli	*E. coli* B	Calf thymus
II instar males	0.25	–	0.19
II & IV instar males	0.55	0.48	0.28
V instar males	0.76	0.75	0.54
II instar females	No detectable activity		
III instar females	0.80	0.75	0.53
IV instar females	0.81	0.79	0.28
Gravid females	0.79	0.73	0.19

Undermethylated DNAs (12 μg) from different sources were used as substrates for the partially purified enzyme isolated from insects in various stages of development. 1 μg of protein was used in all assays. Specific activity is expressed as pmol/μg protein per h. The values are normalised (to) per μg DNA

Table 2

Reciprocal assays

Source of enzyme	Specific activity with DNA from different instars			
	II & IV instar males	V instar males	IV instar females	Gravid females
III & IV instar males	0	0	0.48	0.62
V instar males	0	0.16	0.42	0.65
IV instar females	0.19	0	0.10	0

Assays were carried out using the enzyme from female and male mealybugs with homologous and heterologous substrates. Details are given in the text

ter substrate than poly(dG-dC)·poly(dG-dC) in in vitro reactions [23]. Methylation of the three polymers mentioned above was carried out using enzyme samples isolated from different instars of both sexes. It is to be noted that only de novo methylation is measured using these polymers. Fig.1 depicts a comparison of methylation of CpG and CpA sequences by enzymes from males and females at various stages of development. It can be seen that the preference for the two polymers differs from stage to stage in both males and females. The more preferred substrate for enzyme from young males is poly(dA-dC)·poly(dT-dG) whereas poly(dG-dC)·poly(dG-dC) is the preferred substrate for the enzyme from females. The enzyme from both males and females shows significant methylation of the polymer poly(dA-dC)·poly(dT-dG). It is widely accepted that CpG dinucleotide is the only site for methylation in animal cell DNA [24]. For example enzyme from rat liver did not show any activity with poly(dA-dC)·poly(dT-dG) [19]. However, 5mC has been found in dinucleotides other than CpG in DNA from plant sources [25]. Detection of methylating activity with poly(dA-dC)·poly(dT-dG) in enzyme from mealybugs correlates with the reported presence of 5mC in sequences other than CpG in mealybug genomic DNA [16]. Electrophoretically pure DNA methylase prepared from adult females can methylate poly(dA-dC)·poly(dT-dG) as well as poly(dG-dC)·poly(dG-dC). This suggests that the same enzyme may mediate both CpG and CpA methylation. However in most of our experiments a partially purified preparation of DNA methylase was used and therefore the presence of more than one DNA methylase cannot be ruled out.

Time kinetics was carried out also with poly(dI-dC)·poly(dI-dC). Fig.2 summarises the sequence preference of enzymes from various developmental stages for different polymers normalized to the methylation of CpG sequences. It may be noted that CpA methylation observed with the enzyme from young males (III and IV instar) is comparable to that seen with the enzyme from III instar females. However, CpA methylation with enzyme from early IV instar females reaches significantly high levels when compared to CpG methylation by the same enzyme.

A correlation of the above results with the development of mealybug is shown in fig.3. There are five instars identified in males and four in females. The two sexes can be distinguished morphologically from the second instar onwards. Gametogenesis starts at third instar in both sexes and fertilization occurs during the IV instar stage in females. This means that the control of sex ratio through imprinting and inactivation of paternal chromosomes should manifest itself during fertilization; that is, in late third and early fourth instar females. If DNA methylation is involved in chromosome imprinting, significant levels of de novo methylation would be expected to occur at these stages. The absence of detectable methylase activity in vitro in second instar females could be due either to the enzyme per se or to the presence

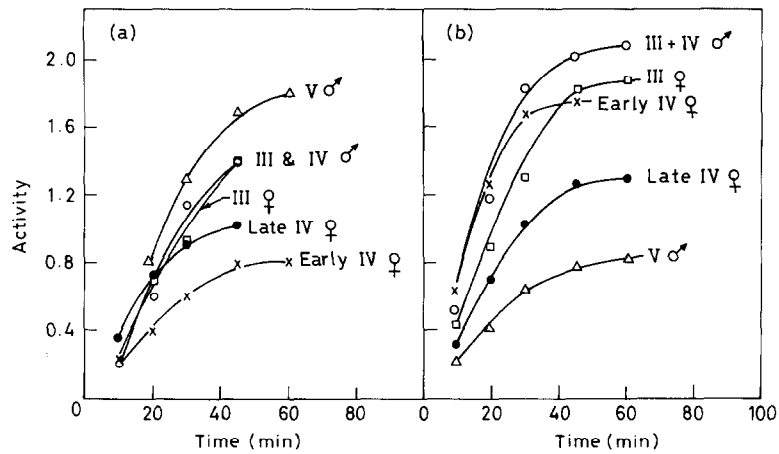

Fig.1. Stage-specific sequence preference. Methylation of synthetic polymers by enzymes from various developmental stages. a, poly(dA-dC)·poly(dT-dG); b, poly(dG-dC)·poly(dG-dC). Activity is expressed as pmol [^{14}C]methyl group incorporated per μg of polymer. Same amount of protein (1 μg) was used in both the assays.

of inhibitors. In either case, it suggests a very low level of DNA methylation at this stage. However, high levels of activity appear in the third instar and are maintained during fourth instar, when fertilization takes place. It may therefore be significant that the rate of methylation also increases at this stage. However, if the relationship between DNA methylation and chromosome imprinting is valid for mealybugs, male insects would be expected to have high levels of maintenance methylase throughout development because most of their nuclei contain a genetically inactive chromosome set. The preference for CpA methylation at certain stages suggests the importance of methylation at sites other than CpG dinucleotides.

In general, de novo methylation is characteristic of early embryonic stages and a high level of enzyme is detected in embryonic cells [26]. Adams et al. [27] have shown that in *Xenopus laevis* there is a rapid rise in nuclear methylase levels soon after fertilization. Methylase levels at various stages in

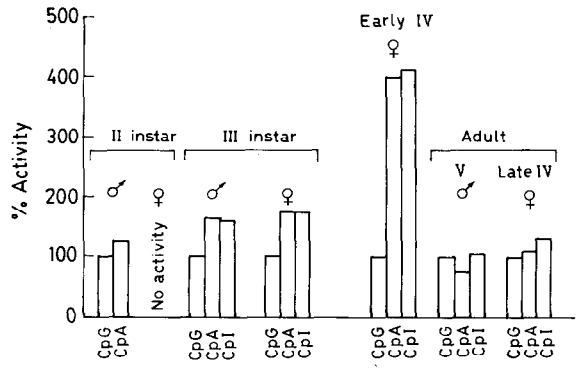

Fig.2. Methylation by enzyme isolated from different instars. Percent activity obtained with polymers containing CpA and CpI which was normalised to values obtained with polymer containing CpG is represented. The activity in the linear range of the reaction is taken. X axis, different polymers; Y axis, percent activity.

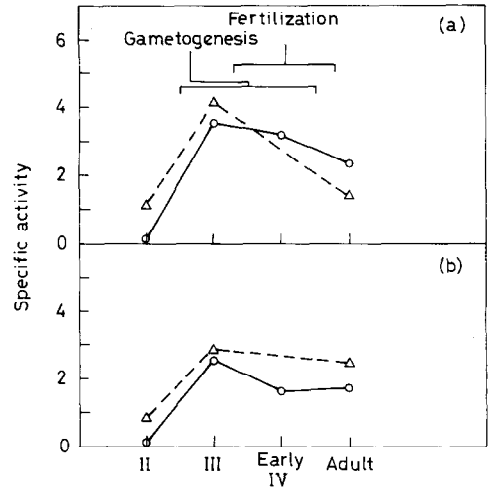

Fig.3. Correlation between developmental stage and level of DNA methyltransferase. X axis, developmental stages; Y axis, specific activity in pmol/μg protein per h. (a) Poly(dA-dC)·poly(dT-dG); (b) poly(dG-dC)·poly(dG-dC). (○—○) Female, (△—△) male.

the life cycle have been studied in *Chlamydomonas* [28]. An increase in methylase levels in gametes and zygotes has been detected and this correlates with the increased methylation that occurs specifically in female chloroplast DNA contained in gametes and zygotes. When these results are viewed in the light of the appearance of high levels of mealybug DNA methyltransferase activity during the third and fourth instars, the observed correlation between increased enzyme activity and the time of occurrence of imprinting and inactivation appears significant. It is of course premature to say whether the two are causally related.

Acknowledgements: We gratefully acknowledge Professor H. Sharat Chandra for his constant encouragement and for a critical review of manuscript. We are thankful to Professor T. Ramakrishnan for going through the manuscript. We thank Dr S.K. Brahmachari for a gift of synthetic polymers. Financial assistance from the Indian Council of Medical Research, India, through a grant to Professor H. Sharat Chandra is acknowledged.

REFERENCES

[1] Crouse, H.V. (1960) Genetics 45, 1429–1443.
[2] Brown, S.W. and Chandra, H.S. (1977) in: Cell Biology; A Comprehensive Treatise (Goldstein, L. and Prescott, D.M. eds) vol.1, pp.109–189, Academic Press, New York.
[3] Chandra, H.S. and Brown, S.W. (1975) Nature 253, 165–168.
[4] Brown, S.W. (1969) Genetics 61 suppl., 191–198.
[5] James, H.C. (1938) Proc. R. Entomol. Soc. London, Ser. A.13, 73–79.
[6] Nelson-Rees, W.A. (1960) J. Exp. Zool. 144, 111–137.
[7] Riggs, A.D. (1975) Cytogenet. Cell Genet. 14, 9–11.
[8] Holliday, R. and Pugh, J.E. (1975) Science 187, 226–232.
[9] Sager, R. and Kitchin, R. (1975) Science 189, 426–433.
[10] Mohandas, T., Sparkes, R. and Shapiro, L.J. (1981) Science 211, 393–396.
[11] Mullins, L.J., Veres, G., Caskey, T. and Chapman, V. (1987) Mol. Cell Biol. 7, 3916–3922.
[12] Swain, J.L., Stewart, T.A. and Leder, D. (1987) Cell 50, 719–727.
[13] McGrath, J. and Solter, D. (1984) Cell 37, 179–183.
[14] Surani, M.A.H., Barton, S.C. and Norris, M.L. (1984) Nature 308, 548–560.
[15] Sapienza, C., Tran, T.-H., Paquette, J., McGawan, R. and Peterson, A. (1989) Prog. Nucleic Acids Res. Mol. Biol. 36, 145–157.
[16] Deobagkar, D.N., Muralidharan, K., Devare, S.G., Kalghatgi, K.K. and Chandra, H.S. (1982) J. Biosci. 4, 513–526.
[17] Scarbrough, K., Hattman, S. and Nur, U. (1984) Mol. Cell Biol. 4, 599–603.
[18] Marmur, J. (1961) J. Mol. Biol. 3, 208–218.
[19] Simon, D., Grunert, F., Acken, U.V., Doring, H.P. and Kroger, H. (1978) Nucleic Acids Res. 5, 2153–2167.
[20] Parnaik, V.K. and Das, M.R. (1981) Biochim. Biophys. Acta 655, 181–188.
[21] Rae, P.M.M. and Steele, R.E. (1979) Nucleic Acids Res. 6, 2987–2995.
[22] Adams, R.L.P., McKay, E.L., Craig, L.M. and Burdon, R.H. (1979) Biochim. Biophys. Acta 563, 72–81.
[23] Pfeifer, G.P. and Drahovsky, D. (1986) FEBS Lett. 207, 75–78.
[24] Adams, R.L.P. and Burdon, R.H. (1983) in: DNA Methylases (Hnilica, L. ed.) vol.1, pp.119–144, CRC Press, Cleveland.
[25] Gruenbaum, Y., Naveh-Many, T., Cedar, W. and Razin, A. (1981) Nature 292, 860–862.