Relationship of DNA Methylation Level to the Presence of Heterochromatin in Mealybugs

KATHRYN SCARBROUGH, STANLEY HATTMAN,* AND UZI NUR

Department of Biology, University of Rochester, Rochester, New York 14627

Purified nuclear DNA from two mealybug species was analyzed for its 5-methylcytosine (m^5C) content by reversed-phase high-pressure liquid chromatography. We observed that the percent m^5C (percentage of cytosines which are methylated) varied between the two species, between males and females of the same species, and between lines with and without supernumerary B chromosomes. This is the first case of a sex-specific difference in overall DNA methylation level. In contrast to a recent report (Deobagkar et al., J. Biosci. [India] 4:513–526, 1982), we found no other modified bases in the DNA. Overall, the percent m^5C in *Pseudococcus obscurus* was two to three times higher than in *Pseudococcus calceolariae*. In both species, the percent m^5C in males was higher than in females, although only in *P. calceolariae* was the difference statistically significant (0.68 ± 0.02 versus 0.44 ± 0.04). The high m^5C content in males was correlated with the presence of a paternally derived, genetically inactive set of chromosomes which is facultatively heterochromatic. The presence of constitutive heterochromatin, however, was associated with a lower m^5C content. Thus, for example, the percent m^5C in females of a *P. obscurus* line with heterochromatic B chromosomes (1.09 ± 0.04) was significantly lower than that of a related line lacking such chromosomes (1.26 ± 0.06). Our findings are discussed with respect to the possible relationship between DNA methylation and heterochromatization.

Heterochromatin is characterized by well-known cytological alterations in the appearance and behavior of chromosomes; e.g., heterochromatin appears to be more highly condensed during most of the cell cycle and to replicate later than euchromatin. Also, heterochromatin is generally genetically nonfunctional. Certain chromosome regions (or even whole chromosomes) are constitutively heterochromatic, e.g., centromeric heterochromatin. In contrast, certain chromosomes (regions) may be either euchromatic or heterochromatic, depending upon their origin, number of homologs present, growth stage, and cell type. This facultative heterochromatization is developmentally regulated and may be reversible (17).

In mammals, facultative heterochromatization is exemplified by the well-known case of X chromosome inactivation (14). Among the insects, one of the best-known examples is the mealybug, or lecanoid, chromosome system (3). In this system, both males and females start development as diploids with euchromatic chromosome complements. In males, however, the paternally derived set of chromosomes becomes heterochromatic during early embryogeny; it remains heterochromatic in most tissues and appears to be transcriptionally inactive. During spermatogenesis, the heterochromatic set is eliminated and only the maternally derived (euchromatic) chromosome set is included in the sperm. In contrast, both chromosome sets remain euchromatic and genetically functional in females, and both are transmissible during sexual reproduction. Gender is apparently determined by whether the early embryo possesses one or two active sets of chromosomes.

It is evident that, in the developing male embryo, there is a mechanism for distinguishing between chromosome sets of maternal and paternal origins. The notion of imprinting was proposed as a basis for this distinction (3, 4). Sager and

Kitchin (20) proposed a model to explain imprinting and heterochromatization based on DNA modification (such as DNA methylation) and restriction: here the notion of restriction was meant to be "not only immediate [DNA] degradation, but also more complex events . . . , including chromosome elimination from the nucleus, intranuclear destruction of particular chromosomes, and heterochromatization" (20). According to this model, the euchromatic set of chromosomes transmitted to the offspring by the adult male mealybug is unmodified and is then either modified or restricted in the fertilized egg. If the paternally derived chromosomes are modified after fertilization, then the zygote develops into a female; if they are restricted in the egg cytoplasm, then the chromosomes become heterochromatic and the zygote develops into a male.

The most likely way in which the DNA may be modified is through methylation. Thus, there might be a difference in the DNA methylation content or pattern of males and females. A previous study on mealybugs indicated that there are no methylated bases present in the DNA (13); however, in that study, in which paper chromatography was used, the analysis was not sensitive enough to detect 5-methylcytosine (m^5C) at a level of 1% of the cytosine residues. Because this result was not consistent with the current DNA imprinting models, we decided to reinvestigate this question. This communication demonstrates that mealybug DNA is, in fact, methylated and that the level is species and sex specific.

MATERIALS AND METHODS

Origin and collection of animals. In the present study, DNA from two species of mealybug (*Pseudococcidae:Coccoidea:Homoptera*), *Pseudococcus obscurus* Essig and *Pseudococcus calceolariae* (Maskell), was analyzed. The first species was used because it was known to possess supernumerary B chromosomes which are heterochromatic in both sexes (17), and the second was used because in this species one can obtain embryos with different sex ratios

---

* Corresponding author.

(proportion of males) by aging the females before they are mated (U. Nur and B. L. Brett, unpublished data). Two *P. obscurus* lines were used: line 72, which contains an average of 5 B chromosomes per cell, and line 76, which lacks B chromosomes. The lines were chosen from a series of lines established in 1973 from individual females collected on oleander at Davis, Calif. The single line of *P. calceolariae* used was established in 1975 from a single female collected on myrtle, also at Davis. The lines were maintained on sprouting potatoes in Mason jars at 19°C.

Females (1 to 2 g) from each line were collected as third instar larvae or as adults (fourth instar) before they started oviposition. The males were picked out of cocoons (which only the males form at the end of the second instar). Thus, the males collected were in the late second, third, fourth, and early fifth (adult) instar stages. It may be noted that an adult male weighs less than 50 μg, and DNA of several hundred males was needed for each assay of base composition. Embryos were obtained by collecting ovisacs, since at the time that eggs are laid they already contain embryos. The embryos were freed of adults and ovisac material by sieving through a fine-mesh nylon screen. All the material collected was stored at −20°C until ready for DNA isolation.

In *P. obscurus*, the embryos were obtained from unaged females; their sex ratio was about 50% males. In *P. calceolariae*, embryos were obtained from unaged females and from females which were aged for 60 days before they were mated. Among the embryos of the former, the sex ratio was about 35% males, and among those of the latter, about 75% males. The sex ratio of embryos was estimated cytologically by fixing the ovisacs of about 10 females in a mixture of chloroform, 99% ethanol, and glacial acetic acid (4:3:1, vol/vol/vol), staining the embryos with acetolacmoid, and examining 200 embryos of each collection for the presence of a heterochromatic set.

Preparation of nuclei. Chloroform was used to dissolve the wax filaments adhering to batches of males and females. The material was filtered through a fine-mesh nylon screen, rinsed with 95% ethanol, and transferred to a cold (−20°C) Waring microblender cup. Individuals were disrupted and nuclei were isolated by a procedure modified from Eckhardt and Gall (7). A mealybug suspension was prepared in 10 ml of ice-cold buffer 1 (50 mM Tris-hydrochloride [pH 7.6]–25 mM KCl–5 mM magnesium acetate–0.35 M sucrose). After filtration through a fine-mesh nylon screen into a centrifuge tube, the nuclei and some cell debris were harvested by centrifugation at 5,000 × *g* for 10 min. The pellet was resuspended in cold buffer 1, and centrifugations were repeated to remove cytoskeletal material. The final pellet was homogeneously resuspended in 2.2 M sucrose and centrifuged at 25,000 rpm for 30 min at 5°C in either a Beckman SW41 or an IEC SB283 rotor.

The supernatant, including the material floating on top of the 2.2 M sucrose, was poured into a cold Dounce tissue grinder, rehomogenized, and centrifuged as described above. The pellets were pooled and resuspended in cold saturated sucrose. A final centrifugation at 25,000 rpm at 5°C for 30 min left nuclei floating at the meniscus, whereas fungi (possibly from the potatoes) and other debris were pelleted.

Extraction of DNA. Nuclei were washed in 10 mM Tris-hydrochloride (pH 7.5)–10 mM NaCl–3 mM $MgCl_2$ and then lysed with four volumes of 0.5 M EDTA–1% sodium dodecyl sulfate–10 mM Tris-hydrochloride (pH 9.5) which had been previously equilibrated at 65°C. The nuclear lysate was then heated at 65°C for 20 min with occasional mixing. Protein was digested at 37°C for 1.5 to 3 h with freshly dissolved proteinase K (Boehringer-Mannheim Corp.) at a final concentration of 300 μg/ml. Deproteinization was completed by extraction with a 1:1 mixture of water-saturated phenol (redistilled) and isoamyl alcohol-stabilized chloroform (chloroform-isoamyl alcohol, 24:1, vol/vol), followed by several chloroform-isoamyl alcohol extractions. Repeated chloroform extractions were required to remove the cellular fat deposits from adult females. RNA was hydrolyzed for 14 to 18 h in 0.3 M KOH at 37°C. The hydrolysate was then dialyzed extensively against 10 mM Tris-hydrochloride (pH 8.0)–1 M NaCl to remove ribonucleotides and pigmented components which absorb strongly at 230 nm.

High-pressure liquid chromatography analysis of DNA methylation content. A total of 2.5 to 25 μg of DNA was ethanol precipitated, resuspended in 0.2 M NH_4HCO_3 (pH 8.5)–0.01 M $MgCl_2$, and digested to deoxyribonucleosides at 50°C for a minimum of 4.5 h with a mixture of 50 U of DNase I (Worthington Diagnostics or Boehringer-Mannheim), 0.5 U of snake venom phosphodiesterase (Boehringer-Mannheim), and 0.02 U of bacterial alkaline phosphatase (Worthington). Twenty microliters of the digest was applied to a Supelcosil reversed-phase column (25 cm by 4.6 mm, type LC-18-DB, fitted with a guard pre-column [5 cm by 4.6 mm; Supelco Chromatography Supplies]), and the deoxyribonucleosides were separated by the method of Gama-Sosa et al. (9) in 50 mM KH_2PO_4–5% methanol (pH 4.1) at 35°C.

RESULTS AND DISCUSSION

Species- and sex-specific levels of DNA methylation. DNA was isolated from purified nuclei obtained from two different species of mealybug. The DNA was enzymatically digested and analyzed for base composition by high-pressure liquid chromatography (see Materials and Methods). The results are summarized in the left half of Table 1. The salient findings are summarized as follows. (i) Mealybugs contain m^5C in their nuclear DNA; the DNA does not contain N^6-methyladenine (<1 N^6-methyladenine per 2,000 adenine residues) or N^7-methylguanine (data not shown). (ii) The m^5C content varies according to species. For example, adult females of *P. obscurus* lines 72 and 76 contained about 2.5 to 3.0 times more m^5C than adult females of *P. calceolariae* (Table 1). (iii) The m^5C content appears to be sex specific; this is the first case of its kind to be reported. A comparison of males and females of *P. calceolariae* (as well as embryos of different sex ratios) indicate a higher level of m^5C content in males. This sex-specific difference in DNA methylation is not as readily apparent in the *P. obscurus* lines, possibly because of the higher level of m^5C already present in females. The higher m^5C content in males is correlated with the presence of a heterochromatic set of chromosomes of paternal origin. This correlation is analyzed in a later section and is presented in the right half of Table 1. (iv) Presence of supernumerary B chromosomes, which are constitutively heterochromatic, is not associated with an increase in the overall level of DNA methylation. Therefore, heterochromatin per se is not necessarily associated with hypermethylation.

Our results are in contrast to those of a much earlier study (13) which failed to detect m^5C in the DNA of the mealybug *Planococcus citri* (formerly *Pseudococcus citri*). While our manuscript was in preparation, another group (5) reported an extraordinarily high m^5C content (13.5% of the cytosines) in a different *Planococcus* species, *Planococcus liliacenus* (Cockerell). These authors also reported high levels of N^6-methyladenine (12.5% of total adenine) and N^7-methylguanine (12.8% of total guanine). These results differ greatly

TABLE 1. High-pressure liquid chromatography analysis of m^5C in mealybug DNA[a]

Organism[b]	Developmental stage	Sex	% m^5C[c]	% H[d]	Slope[e]	y intercept[f]	Sum[g]
P. calceolariae line 19	Adult	Females	0.44 ± 0.04 (4)	0			
	Embryo	Males (~35%)	0.36 ± 0.03 (5)	17.5	0.58 ± 0.04	0.36 ± 0.04	0.94
	Embryo	Males (~75%)	0.59 ± 0.05 (4)	37.5			
	Second instar to adult	Males	0.68 ± 0.02 (3)	50			
P. obscurus line 76 (no B chromosomes)	Adult	Females	1.26 ± 0.06 (7)	0	0.71 ± 0.36	1.26 ± 0.05	1.97[h]
	Third instar and young adult	Females	1.26 ± 0.11 (5)	0			
	Embryo	Males (~50%)	1.44 ± 0.06 (6)	25	0.28 ± 0.23	1.28 ± 0.05	1.56[i]
	Second instar to adult	Males	1.34 ± 0.13 (3)	50			
P. obscurus line 72 (with B chromosomes)	Adult	Females	1.09 ± 0.04 (7)	0	0.69 ± 0.39	1.09 ± 0.05	1.78
	Embryo	Males (~50%)	1.21 ± 0.05 (8)	16.7			

[a] See text.

[b] Lines 72 and 76 were originally isolated from the same hedge. Line 72 contained an average of about five heterochromatic, supernumerary B chromosomes.

[c] A minor peak corresponding to deoxyuridine (dU) often appeared in our chromatograms. Because the dU could arise only from deamination of deoxycytidine (dC), it was included in the calculations: percent m^5C = {[m^5dC/(m^5dC + dU + dC)] × 100} ± standard error. The numbers in parentheses represent the number of high-pressure liquid chromatography determinations used to calculate the compositions. We did not include data from all chromatographic runs, however. We discarded data from runs when, on the basis of areas digitized by hand, the percent deoxyadenosine differed from the percent thymidine or the percent deoxyguanosine differed from the percent dC plus dU plus m^5dC by more than 2.5%. Also, because of the high sensitivity used in recording the UV absorption profiles, unknown peaks occasionally appeared in the vicinity of the m^5dC. Depending on the amount of proximity of these peaks, it was sometimes not possible to determine with confidence the area of the peak corresponding to m^5dC, and these data were also excluded.

[d] Numbers represent the percentage of facultative heterochromatin (H) expected in the sample and were calculated on the assumption that in males without B chromosomes, 50% of the DNA is from the facultatively heterochromatic set. The value of 16.7 (for *P. obscurus* line 72 embryos) is based on a sex ratio of 50% males and the assumption that, in the males of this line, one-third of the DNA is from the B chromosomes, one-third is from the euchromatic set, and only one-third is from the heterochromatic set.

[e,f,g] Based on the linear regression of percent m^5C (y) on percent facultative heterochromatin (x) in which $y = a + bx$, where a = y intercept and b = slope. On the assumption that the correlation between percent m^5C and the percent facultative heterochromatin is due to the higher level of m^5C in the DNA of the heterochromatic set, the y intercept should represent the percent m^5C in the euchromatic set; the slope should represent the increase in percent m^5C in the heterochromatic set, and the sum of these values should represent the percent m^5C in the heterochromatic set.

[h] Values based on data from the first three groups.

[i] Values based on data from all four groups.

from ours in that we did not detect any N^6-methyladenine or N^7-methylguanine, and the levels of m^5C we observed were 10- to 30-fold lower. Moreover, the genetically programmed presence of N^7-methylguanine has not been verified in other types of DNA.

DNA methylation in constitutive heterochromatin. Lines 72 and 76 were observed to have the same overall base composition (33% guanine plus cytosine); thus, the B chromosomes and the regular chromosomes do not differ markedly in overall base composition. This result is in agreement with the observation of Klein and Eckhardt (11). The analysis of DNA from lines 72 and 76 provides information about the relationship between DNA methylation and the presence of constitutively heterochromatic B chromosomes. B chromosomes are not homologous with the regular chromosomes, they are dispensable, and they generally do not affect the phenotype. In *P. obscurus*, the B chromosomes are heterochromatic in both sexes (16). In males they resemble the paternal heterochromatic set cytologically, and both types of heterochromatin replicate later than the euchromatin (10). The DNA of the B chromosomes contains little or no satellite DNA and is not particularly enriched in highly repetitive sequences (12). Females and embryos of line 72 (containing B chromosomes) have significantly lower DNA methylation levels than their counterparts in line 76 (lacking B chromosomes) (ANOVA, F = 4.77; P < 0.05) (Table 1). This result was unexpected because it differed from those of other studies in which constitutive heterochromatin was found to be more heavily methylated than euchromatin (1, 8).

There are several possible causes for this lower extent of methylation in the line with B chromosomes. Their presence may reduce the DNA methylase activity or the concentration of the substrate (S-adenosylmethionine); alternatively, the increased DNA content may prevent DNA methylation from keeping pace with DNA replication. These possibilities are deemed unlikely, however, because line 72 embryos have a higher methylation level than do line 72 females. The presence of the facultatively heterochromatic set in male embryos elicited an increase in overall DNA methylation level; therefore, it is likely that, in line 72 females, the rate of DNA methylation is not limiting. It is also possible that there is a genetic difference between the two lines which is not related to the presence or absence of B chromosomes. We think this is unlikely because the two females used to start the lines were collected from the same population, the lines readily intercross, and the high frequency of B chromosomes in line 72 was attained only after the line was in the laboratory for several generations.

We favor the notion that the DNA of the B chromosomes is undermethylated. If one assumes that, in line 72, the presence of five B chromosomes (in addition to the 10 regular chromosomes) increases the amount of DNA per cell by 50% and that the lower m^5C content in this line is due specifically to the undermethylation of the B chromosomes, then the level of m^5C in the B chromosomes is calculated to be ~0.75% (compared with 1.26% in the euchromatin). The same level of B chromosome undermethylation is obtained by comparing line 76 and line 72 females and by comparing embryos (Table 1). The B chromosomes are shorter than the

regular chromosomes, and thus their presence is likely to increase the amount of DNA per cell by less than 50%; therefore, their level of methylation might be even less than 0.75% m⁵C.

DNA methylation associated with facultative heterochromatin. As indicated earlier, the analysis of the DNA from *P. calceolariae* strongly suggests that in this species, the m⁵C content in males is higher than in females (Table 1). We propose that this difference is due to a higher level of m⁵C in the facultatively heterochromatic set, because such an interpretation is consistent with the inverse correlation generally observed between gene activity and (site-specific) DNA methylation (1, 6, 8, 10). To obtain an estimate of the possible increase in percent m⁵C (percentage of cytosines which are methylated) in the heterochromatic set relative to the level in the euchromatic set, we calculated the linear regression of the percent m⁵C on the percentage of facultative heterochromatin calculated to have been present in the samples of each type. This percentage was calculated on the basis of the sex ratio observed in the sample and on the assumption that in males without B chromosomes, 50% of the chromatin consists of heterochromatin. For *P. calceolariae*, the percent m⁵C and the percentage of chromatin which is heterochromatic are strongly correlated, because the slope of the regression line (0.58 ± 0.04) is significantly different from zero ($P < 0.01$) (Table 1). On the basis of the assumptions made in calculating the regression of percent m⁵C on the percentage of facultative heterochromatin in the samples, the y intercept should represent the percent m⁵C in the euchromatic set; the slope (b) should represent the difference in percent m⁵C between facultative heterochromatin and euchromatin, whereas the sum of these values should represent the percent m⁵C in heterochromatin. In *P. calceolariae*, these values were calculated to be 0.36, 0.58, and 0.94, respectively. Thus, under the assumption that in males the increase in percent m⁵C is confined to the heterochromatic set, the heterochromatic set is calculated to have about 0.6% more m⁵C than the euchromatic set; this corresponds to a methylation content about 2.5-fold higher in the heterochromatic set.

The results of a similar analysis of the regression of percent m⁵C on percent facultative heterochromatin in *P. obscurus* are also presented in Table 1. In this species, the slopes are also positive and of about the same magnitude as the slope in *P. calceolariae*, although they are not significantly different from a slope of $b = 0$. Because in both lines, however, the slopes based on comparing females and embryos are very similar, it is likely that the percent m⁵C and percent facultative heterochromatin are also correlated in this species. One possible reason why the slopes are not significantly different from zero might be the higher level of m⁵C in the DNA of this species, which made it more difficult to detect a small difference in methylation level. One cannot rule out the possibility that in *P. obscurus* the differences in percent m⁵C between females and embryos is due to the developmental stage and not to the fact that about half the embryos are males. This is unlikely, however, on the basis of the observation that in *P. calceolariae* the differences in percent m⁵C were correlated with the proportion of males in the sample, and not with stage (Table 1).

The increase in percent m⁵C in the *P. obscurus* lines, as estimated from the slopes (0.28, 0.69, and 0.71), is fairly similar to the value calculated for *P. calceolariae* (0.58). This similarity contrasts with the pronounced difference in percent m⁵C in females of the two species, and thus, presumably in the percent m⁵C of their euchromatin (about 0.36 in

P. calceolariae and about 1.26 in *P. obscurus*). These observations suggest that the process of facultative heterochromatization in the two species may be associated with a similar increase in the percent m⁵C in the heterochromatic set and that this increase is independent of the basal level of DNA methylation in the female.

For a guanine-plus-cytosine content of 33%, the observed increase in m⁵C in *P. calceolariae* males represents one additional m⁵C per 1,100 to 1,200 bases in the DNA of the heterochromatic set. It is possible that the additional methylation involves specific sequences necessary for maintaining the paternal set in the heterochromatic state.

Relevance to the concept of DNA imprinting. One of the major reasons we embarked on this study was to explore the possibility that DNA modification might be the basis of DNA imprinting, as proposed by Sager and Kitchin (20). According to their model, the DNA of the female is modified, whereas that of the male is not (at least not in the germ line). In the fertilized egg, the paternal chromosome set is either restricted or modified, and restriction leads to heterochromatization. On the assumption that DNA modification is due to the methylation of some of the base residues, one might expect that the DNA from females would be methylated to a greater extent than the DNA from males. Our results, however, indicate just the opposite. This does not necessarily invalidate the model, because modification may involve only a limited number of sites. Nevertheless, if the model is to be valid, the factors determining overall level of methylation in males and females must be different than those which are responsible for imprinting.

The validity of the model of Sager and Kitchin may also be questioned on the basis of its inability to account for heterochromatization in males of certain soft scale insects, such as *Lecanium cerasifex* (18). In this species, unfertilized eggs develop into males and fertilized eggs develop into females. In unfertilized eggs, the haploid female pronucleus divides once and the two products fuse. A few divisions later, one of the two sets of chromosomes becomes heterochromatic, and such eggs develop into males. In fertilized eggs, which later develop into females, the nuclei of the sperm and egg fuse and neither set becomes heterochromatic. The model of Sager and Kitchin cannot explain how one of two identical sets of maternal origin becomes heterochromatic or why in the same egg neither set becomes heterochromatic once the egg is fertilized. It is therefore likely that, as presented, the Sager and Kitchin model is not applicable even to one of the groups whose heterochromatization the model was designed to explain.

Implications for the mammalian X chromosome. Recent studies have attempted to determine whether facultative heterochromatization of the mammalian X chromosome is associated with an increased level of m⁵C; however, the results are conflicting. Treatment of mouse-human hybrid cells with 5-azacytidine led to reactivation of several genes on the heterochromatic X chromosome (21). This reactivation was attributed to an inhibition of DNA methylation. In contrast, staining in association with antibodies specific for m⁵C failed to detect any difference between the euchromatic and heterochromatic X chromosomes in cells of human and owl monkey females (15). Similarly, use of the restriction enzymes *Hpa*II and *Msp*I to analyze specific regions of the X chromosome (for which cloned probes were available) failed to find consistent differences between the fragment sizes of DNA from males and females (24).

Facultative heterochromatization of the X chromosome in the mammalian female and the paternal chromosome set in

mealybugs share several common features. These features include increased condensation, genetic inactivation, delayed replication, and even the preferential inactivation of chromosomes of paternal origin, as for example in marsupials and in certain extraembryonic tissues (3, 22). These similarities led to the suggestion that both types of facultative heterochromatization evolved from the facultative heterochromatization of the X chromosome during spermatogenesis in most animals (2, 12) and that both may employ a similar molecular mechanism (20). Thus, our finding that in mealybugs the presence of a facultatively heterochromatic paternal set is correlated with an increased level of m^5C makes it more reasonable to expect that such an increase will be found in the inactive X chromosome in mammals. Moreover, if DNA methylation is directly involved in establishing or maintaining the heterochromatic state, then it is important to look carefully at the methylation status of the X chromosome during spermatogenesis, especially in *Drosophila* spp., in which it appears that there is no DNA methylation (23; D. Swinton and S. Hattman, unpublished observations).

As discussed earlier, the increase in m^5C associated with the heterochromatic set of the male mealybug was estimated to be about 0.6%. If the increment is similar for inactive X chromosomes, it could not have been detected in the cytological analysis of stained preparations with antibodies specific for m^5C (15). By this method, one can apparently detect only a twofold increase (15); in humans, this would correspond to an increase of 4 to 5% m^5C (based on the m^5C contents observed in human DNA [9]). The studies in which *Hpa*II and *Msp*I were used to detect methylation at specific (but undefined) regions of the X chromosome (24) could probably have detected such an increase, but only if most of the methylated sites were CCGG and these were distributed fairly evenly along the X chromosome. Thus, this question must await investigation with probes specific for known X chromosome genes.

ACKNOWLEDGMENTS

LITERATURE CITED

1. Adams, R. L. P., and R. H. Burdon. 1982. DNA methylation in eukaryotes. Crit. Rev. Biochem. 13:349–384.
2. Brown, S. W. 1977. Adaptive status and genetic regulation in major evolutionary changes of coccid chromosome systems. Nucleus 20:145–157.
3. Brown, S. W., and H. S. Chandra. 1977. Chromosome imprinting and the differential regulation of homologous chromosomes,

p. 109–189. *In* L. Goldstein and D. M. Prescott (ed.), Cell biology, vol. 1. Genetic mechanisms of cells. Academic Press, Inc., New York.
4. Crouse, H. V. 1960. The controlling element in sex chromosome behavior in *Sciara*. Genetics 45:1429–1443.
5. Deobagkar, D. N., K. Muralidharan, S. G. Devare, K. K. Kalghatgi, and H. S. Chandra. 1982. The mealybug chromosome system I: unusual methylated bases and dinucleotides in DNA of a *Planococcus* species. J. Biosci. (India) 4:513–526.
6. Doerfler, W. 1983. DNA methylation and gene activity. Annu. Rev. Biochem. 52:93–124.
7. Eckhardt, R. A., and J. G. Gall. 1971. Satellite DNA associated with heterochromatin in *Rhynchosciara*. Chromosoma 32:407–427.
8. Ehrlich, M., and R. Y.-H. Wang. 1981. 5-Methylcytosine in eukaryotic DNA. Science 212:1350–1357.
9. Gama-Sosa, M. A., R. M. Midgett, V. A. Slagel, S. Githens, K. C. Kuo, C. W. Gehrke, and M. Ehrlich. 1983. Tissue-specific differences in DNA methylation in various mammals. Biochim. Biophys. Acta 740:212–219.
10. Huang, C. C. 1971. Replication of euchromatin and heterochromatin in a mealybug. Chromosoma 34:230–242.
11. Klein, A. W., and R. A. Eckhardt. 1976. The DNAs of the A and B chromosomes of the mealybug, *Pseudococcus obscurus*. Chromosoma 57:333–340.
12. Lifschytz, E., and D. L. Lindsley. 1972. The role of the X-chromosome inactivation during spermatogenesis. Proc. Natl. Acad. Sci. U.S.A. 69:182–186.
13. Loewus, M. W., S. W. Brown, and A. D. McLaren. 1964. Base ratios in DNA in male and female *Pseudococcus citri*. Nature (London) 203:104.
14. Lyon, M. F. 1961. Gene action in the X-chromosome of the mouse (*Mus musculus* L.). Nature (London) 190:372–373.
15. Miller, D. A., E. Okamoto, B. F. Erlanger, and O. J. Miller. 1982. Is DNA methylation responsible for mammalian X chromosome inactivation? Cytogenet. Cell Genet. 33:345–349.
16. Nur, U. 1962. A supernumerary chromosome with an accumulation mechanism in the lecanoid genetic system. Chromosoma 13:249–271.
17. Nur, U. 1967. Reversal of heterochromatization and the activity of the paternal chromosome set in the male mealybug. Genetics 56:375–389.
18. Nur, U. 1972. Diploid arrhenotoky and automictic thelytoky in soft scale insects (Lecaniidae:Coccoidea:Homoptera). Chromosoma 39:381–401.
19. Razin, A., and A. D. Riggs. 1980. DNA methylation and gene function. Science 210:604–610.
20. Sager, R., and R. Kitchin. 1975. Selective silencing of eukaryotic DNA. Science 189:426–433.
21. Shapiro, L. J., and T. Mohandas. 1982. DNA methylation and the control of gene expression on the human X-chromosome. Cold Spring Harbor Symp. Quant. Biol. 47:631–637.
22. Takagi, N., N. Wake, and M. Sasaki. 1978. Cytological evidence for preferential inactivation of the paternally derived X chromosome in XX mouse blastocysts. Cytogenet. Cell Genet. 20:240–248.
23. Urieli-Shoval, S., Y. Gruenbaum, J. Sedat, and A. Razin. 1982. The absence of detectable methylated bases in *Drosophila melanogaster* DNA. FEBS Lett. 146:148–152.
24. Wolf, S. W., and B. R. Migeon. 1982. Implications for X-chromosome regulation from studies of human X-chromosomal DNA. Cold Spring Harbor Symp. Quant. Biol. 47:621–630.