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The mealybug chromosome system I: Unusual methylated bases and dinucleotides in DNA of a *Planococcus* species

DILEEP N. DEOBAGKAR, K. MURALIDHARAN, SUSHILKUMAR G. DEVARE*, KRISHNA K. KALGHATGI** and H. SHARAT CHANDRA†

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

* Laboratory of Cellular and Molecular Biology, National Cancer Institute, NIH, MD 20205, USA

** Department of Chemical Engineering, Yale University, New Haven, CT 06520, USA

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Abstract. The methylation status of the nuclear DNA from a mealybug, a *Planococcus* species, has been studied. Analysis of this DNA by High Performance Liquid Chromatography and Thin Layer Chromatography revealed the presence of significant amounts of 5-methylcytosine. Since analysis of DNA methylation using the Msp I/Hpa II system showed only minor differences in susceptibility of the DNA to the two enzymes, it seemed possible that 5-methylcytosine (5mC) occurred adjacent to other nucleotides in addition to its usual position, next to guanosine. This was verified by dinucleotide analysis of DNA labelled *in vitro* by nick translation. These data show that the total amount of 5-methylcytosine in this DNA is slightly over 2.3 mol %, of which 0.61% occurs as the dinucleotide 5mCpG, 0.68% as 5mCpA, 0.59% as 5mCpT and 0.45% as 5mCpC. 5mCpG represents approximately 3.3% of all CpG dinucleotides. The experimental procedure would not have permitted the detection of 5mCp5mC, if it occurs in this system. Unusually high amounts of 6-methyladenine (approximately 4 mol %) and 7-methylguanine (approximately 2 mol %) were also detected, 6-methyladenine and 7-methylguanine occurred adjacent to all four nucleotides. The total G+C content was 33.7% as calculated from dinucleotide data and 32.9% as determined from melting profiles.

Keywords. Mealybug *(Planococcus);* chromosome imprinting; chromosome inactivation; diffuse centromere; DNA methylation; 7-methylguanine.

Introduction

Among the several unusual genetic systems found in the coccids (Coccoidea; Homoptera; Insecta), the "lecanoid" system was the first to be described (Hughes-Schrader, 1948; Brown and Kur, 1964; Brown and Chandra, 1977). This chromosome system, named after the taxonomic group in which it was discovered,

Abbreviations used: 5mC, 5-Methylcytosine; 6mA, 6-methyladenine; 7mG, 7-methylguanine; HPLC, high performance liquid chromatography; TLC, thin layer chromatography; UV, ultra-violet; dNTP, deoxynucleotide triphosphates; DEAE, diethylaminoethyl; Tris, Tris-(hydroxymethyl) amino methane.

† To whom reprint requests should be addressed.

has been studied extensively in the citrus mealybug, *Planococcus citri* (Risso) (Brown and Nelson-Rees, 1961; Chandra, 1963a; Brown and Weigmann, 1969; Brown and Nur, 1964; Brown and Chandra, 1977). In *P. citri,* there are no sex chromosomes, and both males and females start development with apparently identical chromosome complements. During the early cleavage divisions of embryos which develop as males, the paternal set of chromosomes becomes genetically inactive and heterochromatic; the maternal set remains transcripitionally active or potentially active and appears euchromatic. In embryos which develop as females no such distinction between paternal and maternal chromosome complements is seen. In adults, in the male, there is no recombination during meiosis and the paternal, heterochromatic chromosomes are eliminated during meiosis II and sperm are formed only from the maternal chromosomes. Thus the maternal chromosomes of one generation become the paternal chromosomes of the next generation. In females, there is recombination, and meiosis leads to the production, as usual, of haploid eggs.

The behaviour of the paternal chromosomes in the male suggests the existence of a mechanism by which a set of chromosomes "remember" their parental origin in violation of the rules of Mendelism. The mechanism by which this impriting or "memory" is achieved is not understood. There is one other aspect of the mealybug chromosome system which is of interest from the view-point of molecular biology. This is the observation that zygotes, which are apparently genetically identical, can follow either of two developmental pathways, male or female (Nur, 1963; Chandra, 1963b; Nur, 1971). In sexually reproducing species, a strict correlation appears to exist between maleness and the inactivation or elimination of the paternal set of chromosomes. In the female, as mentioned earlier, both haploid sets function normally; there is neither inactivation nor elimination.

Inactivation or elimination of a chromosome or a whole haploid set of chromosomes when genetically similar or identical homologs in the same nucleus remains unaffected has also been described in mammals (the X chromosome) and *Sciara,* among others (Chandra and Brown, 1975; Brown and Chandra, 1977). Riggs (1975), Holliday and Pugh (1975) and Sager and Kitchin (1975) have recognized the possibility that if initiation and maintenance methylases exist in these animal systems, they would provide an attractive model for understanding such differential regulation of homologous chromosomes. Recent experiments (Liskay and Evans, 1980; Mohandas *et al.,* 1981) suggest a relationship between methylation of cytosine and the inactivation of the mammalian X-chromosome. These observations prompted us to search for modified bases in the DNA of a sexually reproducing mealybug belonging to the genus *Planococcus.*

The chromosomes of mealybugs and other coccids have an unusual centromeric organization. As first shown by Hughes-Schrader and Ris (1941), the centromeric property is not localized as in most higher organisms but distributed over the entire chromosome. As a result, chromosome fragments, whether naturally-occurring or induced, are capable of perpetuation as independent entities. By means of Cobalt-60 irradiation of males, it has been possible to show, in *Planococcus citri,* that even

very small fragments are capable of anaphase movement during mitosis, although at a slower rate than undamaged chromosomes (Chandra, 1963a). This diffuse centromere provides an additional reason for investigation of the sequence organisation and modified bases in the DNA of this insect species.

Materials and methods

Stock cultures of mealybugs were obtained from the Horticultural Research Station, Chettahalli, Coorg, Karnataka. These mass cultures were mixtures of *P. citri* and at least one other mealybug species. Cultures of a mealybug provisionally identified as *P. lilacinus* (Cockerell) were isolated from these mass cultures and maintained on pumpkins at room temperature. The taxonomic identification was kindly provided by Dr. B. K. Rajagopal, Department of Entomology, University of Agricultural Sciences, Bangalore. Cytology of chromosome behaviour in males showed that it resembled chromosome behaviour in *P. citri* and that it was typically lecanoid (Hughes-Schrader, 1948; Brown, 1959).

DNA isolation

Nuclei were isolated from adult females, many of which were gravid. Gravid females would be expected to contain a proportion of male embryos in which the paternal set of chromosomes would be inactive and heterchromatic. Isolation of nuclei was done in the presence of citrate (Busch and Daskal, 1977) in order to prevent the formation of phenolic compounds. During initial attempts at isolating DNA from this organism, we found that due to the polyphenolic oxidases present in the haemolymph (Banks, 1976) of this insect, phenolic compounds were formed during the isolation procedure. Since these phenolic compounds bind readily and firmly to DNA, it was necessary to adopt the citrate method for isolating nuclei. The acidic conditions of the citrate method prevent the formation of phenolic compounds. Following their isolation, nuclei were suspended in saline-citrate containing 0.1% sodium lauryl sulphate, and then incubated at 37°C in the presence of proteinase K (10 µg/ml) for 1 h. DNA was extracted using buffer-saturated phenol, followed by extraction with isoamyl-alchohol: chloroform (1 : 24, v/v) and precipitation with ethanol. RNA was removed by treatment with DNase-free RNase (50 µg/ml). The DNA was re-extracted as above and precipitated with ethanol.

Digestion with Restriction enzymes

Restriction enzymes were obtained from New England Biolabs, USA. Digestion was carried out according to the conditions specified by the manufacturer. Electrophoresis of DNA was carried out in 1% agarose gels in Tris-(hydroxyl-methyl)-aminomethane (Tris) (40 mM)-acetic acid (20 mM)-EDTA (2 mM) at pH 8.1. Gels were stained with ethidium bromide (5 µg/ml) for 30 min. DNA bands were visualised using ultra-violet illumination and photographed using a red filter. A 400 ASA, 35 mm negative film was used. Lambda DNA digested with Eco RI and Bam H1 was used as marker.

In vitro radio-labelling of DNA

DNA was labelled with α–^{32}P by nick translation as described by Rigby *et al.* (1977). DNA (5 µg) was nicked with pancreatic DNase I (0.5 µg/ml) for 6 min at

37°C. Nicked DNA was incubated for 10 min at 15°C in a 50 µl reaction mixture containing 50 mM potassium phosphate (pH 7.2), 5 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 0.1 mM cold deoxynucleotide triphosphates and 1.2 µM (250 µCi) of one of the dNTPs labelled as α–^{32}P. Specific activities were in the range of 400 Ci/mmol to 3000 Ci/mmol in the various dNTPs, all obtained from Radiochemical Centre, Amersham, England. Incorporation of radioactivity into DNA was monitored in aliquots. After the completion of the reaction, the reaction mixture was loaded on to a diethylaminoethyl (DEAE)-cellulose column equilibrated with 0.01 M Tris-HCl (pH 7.5) and 0.1 M NaCl. After extensively washing the column with the same buffer, DNA was eluted in high salt buffer (1.5 M NaCl). The DNA was ethanol precipitated overnight at –20°C.

Dinucleotide analysis

The labelled DNA was dissolved in 16 µl of water and digested to 3′-monophosphates using micrococcal nuclease (140 µg/ml) and spleen phosphodiesterase (7 µg/ml) (Boehringer-Mannheim GMBH, Mannheim, W. Germany) in 100 mM Tris-HCl (pH 8.5), 10 mM $CaCl_2$ buffer. The digestion was carried out for 3 h at 37°C. An aliquot of the digest was applied to cellulose thin layer chromatography (TLC) sheets (Eastman Kodak) and chromatographed in two dimensions using isobutyric acid: water: NH_4OH (66:20:1, v/v/v) in the first dimension and saturated $(NH_4)_2SO_4$: *iso*-propanol: 1 M sodium acetate (80:2:18, v/v/v) in the second dimension (Cedar *et al.,* 1979). Chromatograms were exposed to X-OMAT (Eastman Kodak) films and autoradiograms were developed. Using these autoradiograms as templates, the respective spots from the TLC plates were scraped of and the ^{32}P radioactivity in them was measured. The spots on the autoradiograms were identified by comparison with the mobilities of Standard deoxymonophosphates in the same solvent system. These standards were obtained from P.L. Biochemicals, Wisconsin, USA. Three separate isolates of DNA were studied. Each of these three samples was nick-translated and analysed in duplicate experiments.

High performance liquid chromatography

The ^{32}P-labelled 3′-monophosphates were analysed by high performance liquid chromatography (HPLC) for the presence of modified nucleotides. A 5µ Spherisorb ODS column, 15 cms long, was used. Decyltrimethyl ammonium bromide, 10 mM, in 50 mM sodium phosphate buffer, pH 6.0 was used as the eluent. The flow rate was 0.8 ml/min at a temperature of 23±1°C. The eluent was monitored at 260 nm using a Schoefel-770 variable wavelength detector. The standard monophosphates (P.L. Biochemicals) were the same as those used for TLC. After the elution times of the standards had been determined, 20 µl of the sample was injected along with 50 µl of the non-radioactive, standard deoxynucleotides. The fractions corresponding to the known elution times of the standards were collected and their radioactivity measured.

HPLC was also performed on acid hydrolysed unlabelled DNA. A C_{18}µ-Bondapak reverse phase column was used on a Waters instrument (Model ALC–

GPC-244). The solvent used was water: methanol: acetic acid (96:4:0.25, v/v/v). The eluent was monitored at 254 nm.

Calculation of tetranucleotide frequencies

From the observed frequencies of the various dinucleotides, the probable frequencies of all possible tetra-nucleotides were calculated on a computer. The programme generated tetranucleotides as a product of the frequencies of overlapping dinucleotides within each tetranucleotide. For example, the frequency of CCGG would be the product of the experimentally observed frequencies of CC, CG and GG. Similarly, the frequency of CCCC was calculated by multiplying three times the observed frequency of CC.

Results

Consistently good preparations of DNA were obtained by use of the method mentioned earlier. Each sample of DNA was characterized spectrophotometrically as well as electrophoretically. The DNA preparations exhibited a sharp and smooth melting profile, with a Tm of 83.4°C (figure 1). The G+C content was calculated to be 33.7% on this basis (Mandel and Marmur, 1968).

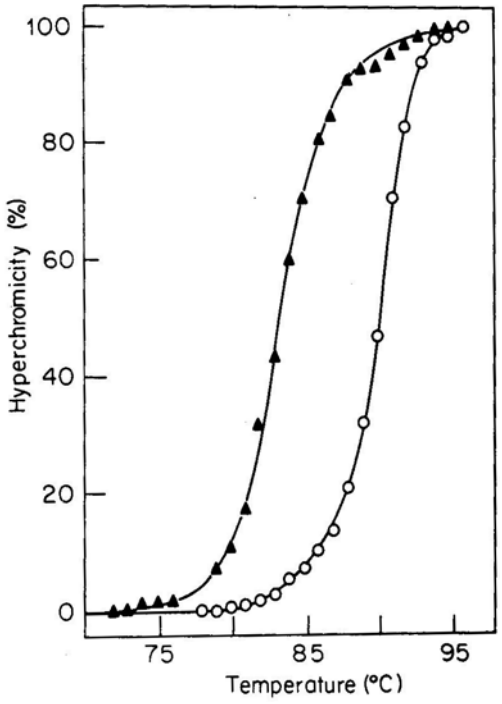

Figure 1. Thermal denaturation of DNA. Native DNA in 0.12M sodium phosphate buffer, pH 6.8, was melted at 1°C increments in a Gilford Spectrophotometer 250 equipped with a thermoprogrammer (model 2527). *Escherichia coli* DNA was used as standard. The DNA melting temperature (Tm) and G+C content were calculated according to Mandel and Marmur (1968). The open circles represent *E. coli* DNA and the closed triangles, DNA from mealybug (gravid females).

The restriction enzymes Msp I and Hpa II both recognize the sequence CCGG and are useful for studying the methylation status of the CpG dinucleotide. While Msp I cuts this sequence whether or not the internal C is methylated, Hpa II will cut it only if the internal C is unmethylated. There was a small but recognizable difference in the susceptibility of mealybug DNA to these two enzymes (figure 2, lanes 2 and 3). When the DNA was digested by a mixture of the two enzymes (figure 2, lane 4), the extent of digestion was similar to that observed with Msp I alone. These results suggested that a small proportion of the CCGG sequences in

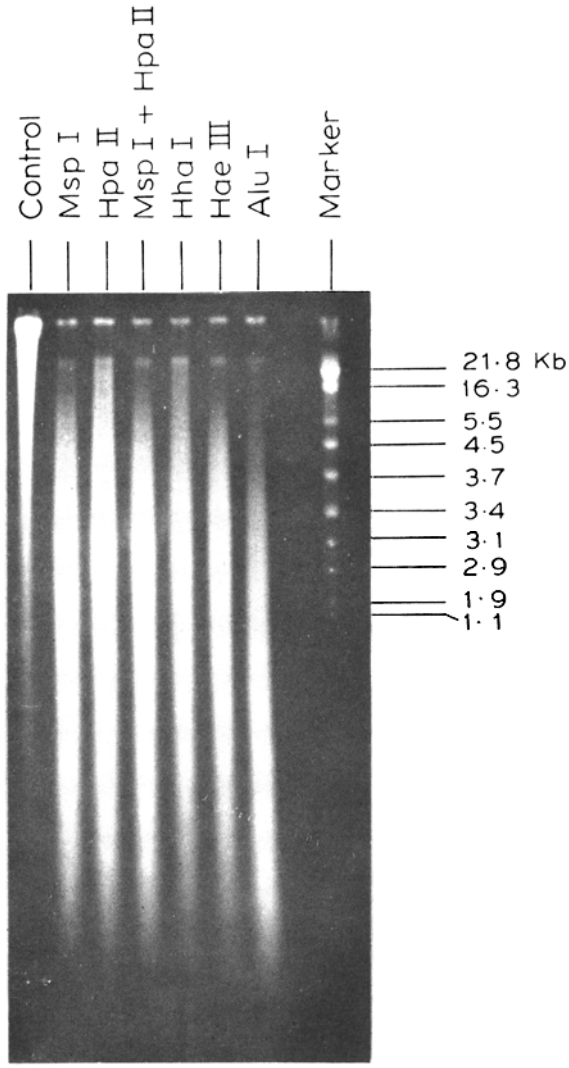

Figure 2. Restriction enzyme digestion of DNA. Two µg of mealybug DNA was digested for 6 h at 37°C with 4 units of the enzyme. DNA fragments were separated by electrophoresis at 50 volts for 8 h on 1% agarose gel. λ DNA digested with EcoRl and Bam H1 was used as molecular weight marker.

this DNA was resistant to digestion by Hpa II because the internal C was methylated. On the other hand, when bases prepared by perchloric acid digestion of DNA were analysed by HPLC (MCH 10 C-18 Micropack RP column, Varian LC 5000), large amounts of 5-methylcytosine and 6-methyladenosine (6mA) were detected (data not shown). In view of the apparently high content of 5mC in this DNA, one would expect significant differences in the susceptibility of this DNA to the enzymes Msp I and Hpa II. However, as seen from figure 2, there is only a small difference in the susceptibility of this DNA to the two enzymes, suggesting that only a fraction of the total 5mC is present in CCGG sequences. It therefore seemed likely that even if 5mC occurred in the dinucleotide CpG it was predominantly in sequences other than CCGG or that it occurred in other dinucleotides as well.

Dinucleotide analysis of labelled DNA was done to identify the dinucleotides with 5mC. The results are summarised in table 1. The two-dimensional TLC resolved, in addition to the four usual 3′-deoxy mononucleotides, d6mAMP and d7mGMP (figure 3 and figures 4a and b). On extended autoradiography, additional unidentified spots of minor components could also be visualised (figure 4c).

Following HPLC, the four normal deoxynucleotide monophosphates as well as 5mCMP and 7mGMP, are well resolved under the solvent conditions used (figure 5a). 5mCMP and 7mGMP together with the four normal deoxynucleotide monophosphates were mixed with the hydrolysate of the labelled mealybug DNA and subjected to HPLC analysis. The fractions corresponding to the known elution times of the standards were collected and radioactivity in them was counted (figure 5b). The presence of significant amounts of radioactivity in the HPLC fractions corresponding to the elution times of the standard 5mCMP and 7mGMP was taken as qualitative evidence for the presence of these modified nucleotides in mealybug DNA. Since there was more than one unidentified peak in the HPLC profiles, these data were not considered sufficient for accurately determining the extent of methylation.

HPLC of perchloric acid hydrolysate of unlabelled mealybug DNA provided additional evidence for the presence of 7mG (figures 6a, b). The identity of the 7 m G peak was confirmed by peak enhancement.

As shown in table 1, 5mC in mealybug DNA occurs as extensively in CpA and CpT dinucleotides as in CpG. 5mCpC occurs to a lesser extent. 6mA and 7mG were found as 5′ neighbours of A, T, C and G. Although dinucleotide analysis was done twice each on three separate samples of DNA, the quantitative data reported in table 1 may be subject to some error, for the following reason. Since the modified bases frequently moved close to their respective unmodified bases, there would be chances of cross contamination when such spots are scraped off for radioactivity measurements. As a result, accurate data about the relative proportions of the modified and unmodified bases and their relationship, if any, to the sex and stage of development of the insect should await careful HPLC analysis of the DNA of this species. Further studies along these lines are in progress.

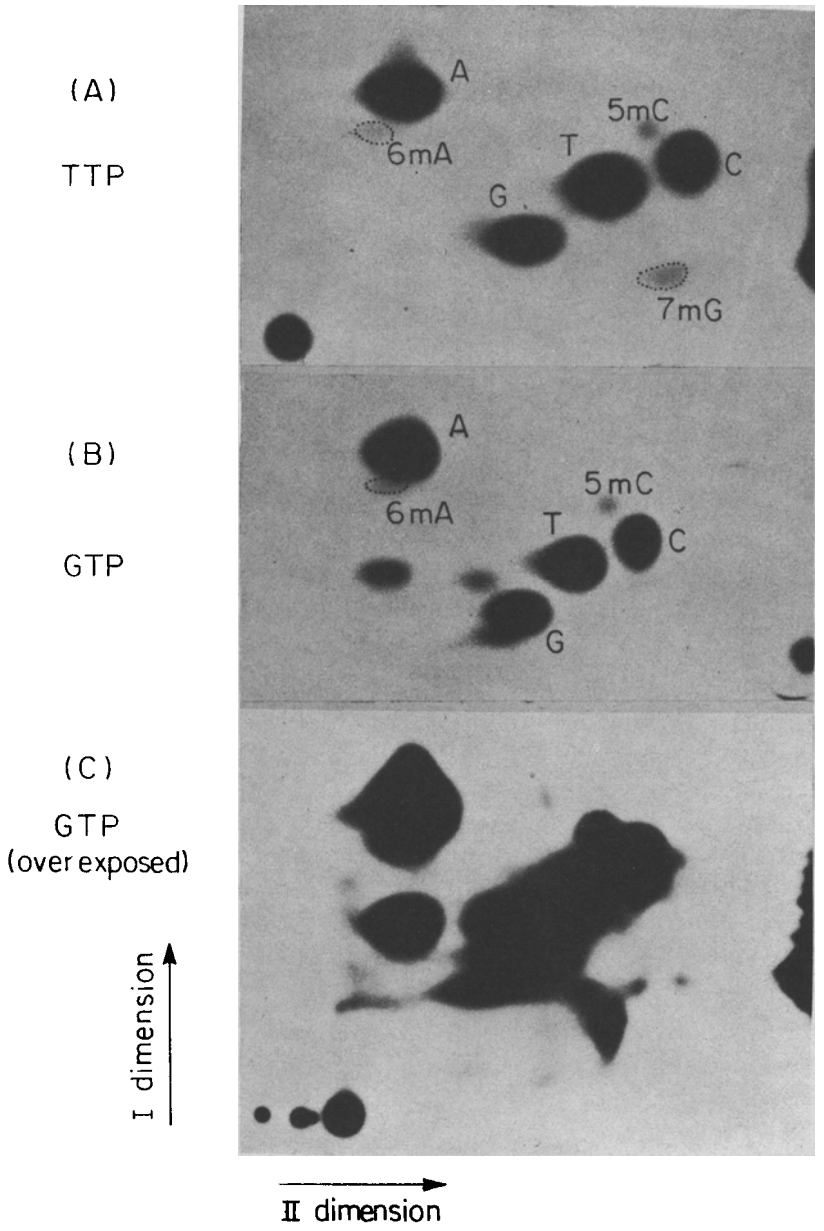

Figure 3. Dinucleotide analysis. 3' α-^{32}P labelled nucleotide monophosphates were chromatographed under the same conditions as those employed for separation of the Standard dNMPs (figure 4).

A. Autoradiogram of 3' dNMPs following nick translation in the presence of α-^{32}P dTTP.

B. Autoradiogram of 3'-dNMPs following nick translation in the presence of α-^{32}P dGTP.

C. Overexposed autoradiogram of 3 '-dNMPs following nick translation in the presence of α-^{32}P dGTP.

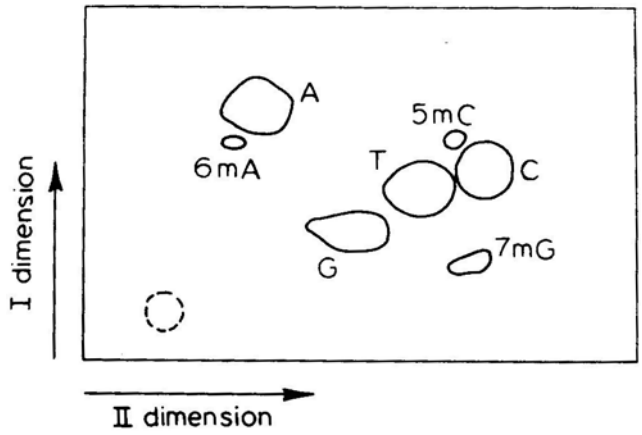

Figure 4. Separation of deoxynucleotide monophosphates on two dimensional TLC. Diagrammatic representation of observed separation of standard dNMPs following chromatography on cellulose TLC plates using Isobutyric acid: water: NH_4OH (66:20: 1, *v/v*/v) in the first dimension and saturated $(NH_4)_2SO_4$: in the second dimension.

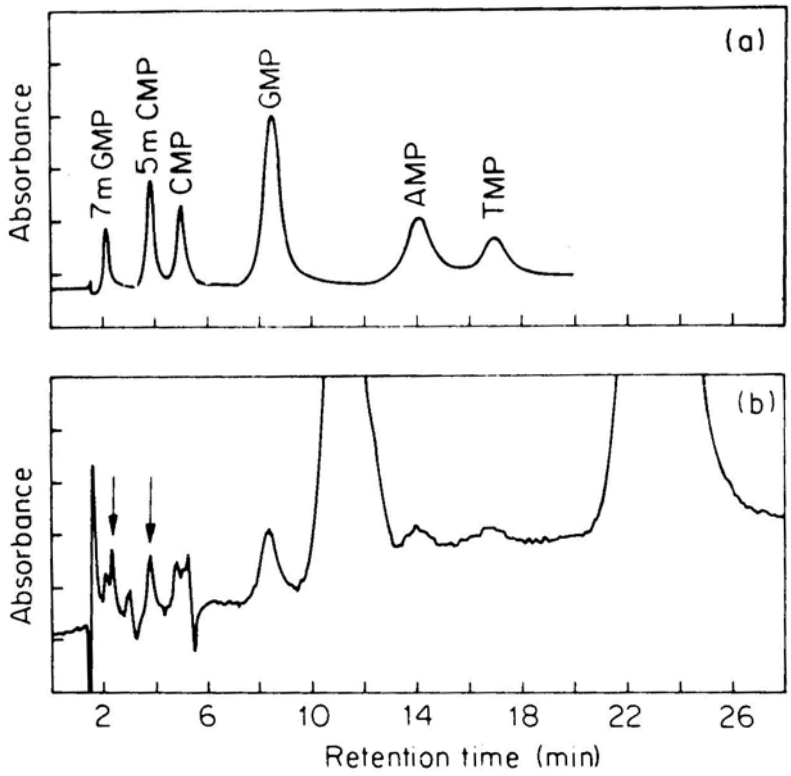

Figure 5. HPLC of deoxynucleotide monophsophates. The solvent conditions and other experimental details were as described under Materials and methods.
A. Separation of standards.
B. HPLC profile of ^{32}P-deoxynucleotide monophsphates obtained after nick translation of mealybug DNA. The two arrows indicate the peaks corresponding to 7mG and 5mC.

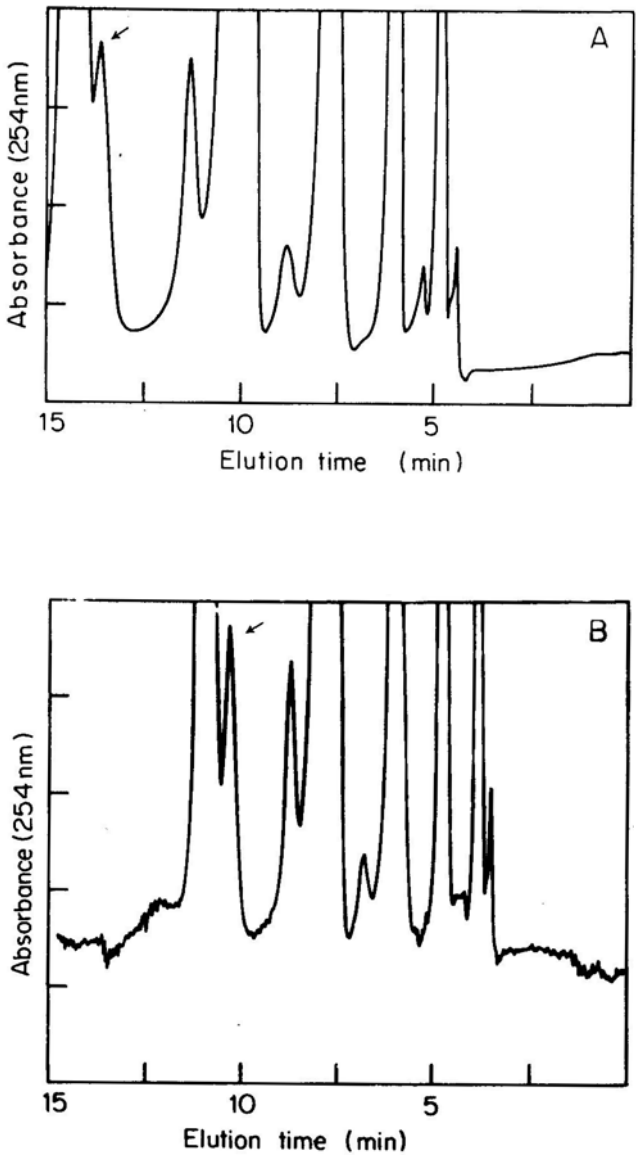

Figure 6. HPLC profiles of acid hydrolysed unlabelled mealybug DNA.

A. Separation of bases from mealybug DNA on a C_{18}μ-Bondapak column at a flow rate of 0.61 ml per minute.

B. Peak enhancement observed following injection of authentic sample of 7 mG along with hydrolysate of mealybug DNA. The flow rate was 0.81 ml per minute. Arrows in the two panels indicate the 7 mG peaks.

Table 1. Nearest neighbour anlysis of methylated bases in mealybug DNA.

	5′ end				
	5mC	6mA	7mG		
	0.68	0.67	0.34	A	3′ end
	0.59	0.61	0.92	T	
	0.61	0.92	0.37	G	
	0.45	1.81	0.55	C	
Total	2.33	4.01	2.18		

The G+C content calculated from the dinucleotide analyses was 32.9%, which is in agreement with the estimate from DNA melting profiles (33.7%). Of the total cytosine, 13.5% occurred as 5mC. Further, 12.5% of total adenine occurred as 6mA and 12,8% of guanine as 7mG. The frequencies of the various dinucleotides were used to compute tetranucleotide frequencies. The tetranucleotides of interest and their estimated frequencies are given in table 2.

Table 2. Calculated frequencies of certain tetranucieotides which are recognized by restriction enzymes.

DNA sequence	Frequency of the tetranucleotide, in per cent*	Enzyme recognizing the sequence
CCGG	0.44	Msp I
C5mCGG	0.07	Msp I
CCGG	0.44	Hpa II
GCGC	0.57	Hha I
GGCC	0.57	Hae III
AGCT	0.61	Alu I
GATC	1.72	Sau 3A
G6mATC	0.10	Sau 3A

* These numbers were derived from dinucleotide data. For details, see Materials and methods.

Whereas the restriction enzymes Msp I and Hpa II are useful for characterizing methylation in CCGG sequences, other enzymes such as Hha I, Hae III and Alu I are useful for studying the methylation of CpG in other tetranucleotide sequences. As seen in table 2, the recognition sequences for Hha I, Hae III and Alu I all

occurred in nearly equal proportions. From these calculated frequencies one would expect that mealybug DNA would be equally susceptible to digestion by these three enzymes. However, as seen in figure 2, (lanes, 5, 6 and 7) digestion with Alu I yielded much smaller fragments than digestion with either Hha I or Hae III. One explanation for this observation could be that the recognition sequences for the enzymes Hha I and Hae III are sometimes methylated in mealybug DNA.

Discussion

Loewus *et al.* (1964) reported that they did not detect any 5mC by paper chromatography of acid-hydrolysed DNA isolated from a mealybug, *Planococcus citri* (Risso), which belongs to the same genus as *P. lilacinus.* Nor did their data on buoyant density and melting profiles of DNA from *P. citri* suggest differences between DNAs of males and females in the content of any modified base. Subsequently, Rao and Chandra reported, in abstract form (1980a, b), significant amounts of 5mC in *P. citri* DNA as well as large differences between the two sexes in the amount of 5mC. They also reported finding, in DNA isolated from males, two buoyant density peaks whereas only one such peak was observed in DNA isolated from virgin females. It now appears that the results of Rao and Chandra (1980a, b) will have to be reassessed because the procedure adopted for isolation of DNA did not take into account the presence of polyphenolic oxidases (Wyatt, 1961) in mealybugs. If care is not taken, phenolic compounds would be formed during the isolation procedure and these would bind firmly to DNA. Since many of these phenolic compounds contain methyl groups (Banks, 1976), they could introduce artefacts during TLC and other methods of estimation of DNA methylation.

There is one other complexity of the mealybug system which is relevant to the results reported in this paper. This pertains to the presence of certain yeast-like symbionts in both sexes. They are transmitted by the mother to the egg. The symbionts invade certain polyploid cells which form a small organ called the mycetome whose function is not known. When DNA is isolated from whole animals, the possibility of "contamination" of mealybug DNA by symbiont DNA cannot be avoided. This may become important, particularly when one is studying differences between DNAs isolated from males and females, because the two sexes are vastly different in size. *P. lilacinus* appears to contain far fewer symbionts than *P. citri.* At least in the stock we are working with, it is often difficult to demonstrate symbionts in adult females, whether virgin or gravid. In addition, care was taken during this investigation to isolate DNA from purified preparations of nuclei and not directly from whole animals. As a result, we believe that contamination of mealybug DNA by symbiont DNA, if it had occurred, was negligible.

DNAs from a wide variety of organisms have been shown to contain 5mC (Ehrlich and Wang, 1981). Rather large amounts of 5mC have been reported in DNAs of certain plants whereas insects generally contain low amounts or, as in the case of Drosophila, apparently none at all. 6-Methyladenine has been reported to occur in several lower eukaryotes such as the protozoa, and in algae such as *Chlamydomonas.* It has also been reported in DNA of mosquito cells in culture. 7mG has been detected in DNA of the Shigella phage DDVI (Nikolskaya *et al.,*

1976). We are aware of only one report on the occurrence of this rare modified nucleotide in eukaryotes (Culp *et al.,* 1970). The presence of very small amounts of 7mG and two other modified forms of G were reported by Culp *et al.* (1970) in DNA isolated from HeLa cells.

In the DNA of *P. lilacinus,* high levels of C methylation (5mC representing 13.5 % of total C), A methylation (6mA representing 12.5% of total A) and of the unusual modification of guanine (7mG representing 12.8% of G) were observed. In other systems, 5-methylcytosine has been reported to occur most often in the dinucleotide CpG. In the data reported here, it occurs in CpA and CpT almost as often as in CpG. The frequency of its occurrence in CpC is slightly lower. The technique of dinucleotide analysis would not have permitted the detection of 5mCp5mC if it occurs in mealybug DNA (table 1). Occurrence of the unusual methylated dinucleotides 5mCpA, 5mCpT and 5mCpC has been reported in wheat-germ DNA (Gruenbaum *et al.,* 1981). However, in wheat-germ, the most commonly seen modified dinucleotide is still 5mCpG which represents 82% of all CpG dinucleotides; 5mCpT and 5mCpA occur in far lower amounts, 19% each, of the respective dinucleotide populations.

In the mealybug data reported here, 5mCpA and 5mCpT occur in high frequencies and in about equal proportions. It is therefore likely that, as in the case of wheat-germ DNA (Grauenbaum *et al.,* 1981), C methylation often occurs in the trinucleotide CXG, where X is either A or T.

As seen in table 2, the estimated frequency of CCGG sequences, which are the recognition and cleavage sites for the enzyme Hpa II, is 0.44% of all tetra-nucleotides. Msp I recognizes and cuts the above sequence both when it is unmethylated as well as when the internal C is methylated. These sites are estimated to occur at a frequency of 0.51 % of all tetranucleotides. It follows from these data that the proportion of Hpa II resistant sites in this DNA would be approximately 0.07%. This would mean that only about 14% of the total CCGG sequences have a methylated internal C. This is consistent with the observation that the difference in susceptibility of this DNA to Msp I and Hpa II is very small (figure2, lanes 2 and 3). Further, while 6mA occurs in all possible dinucleotides, it occurs most often as 6mApC.

The occurrence of high levels of 6mA, 7mG and 5mC, and their presence in a variety of dinucleotide combinations, may prove to be of interest in further analysis of this unusual chromosome system.

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