Copyright © 1999 by the Genetics Society of America

The Relationship Between DNA Methylation and Chromosome Imprinting in the Coccid *Planococcus citri*

Silvia Bongiorni,[1] Orietta Cintio and Giorgio Prantera

Dipartimento di Agrobiologia e Agrochimica, Università della Tuscia, 01100 Viterbo, Italy

Manuscript received August 5, 1998
Accepted for publication December 11, 1998

ABSTRACT

The phenomenon of chromosome, or genomic, imprinting indicates the relevance of parental origin in determining functional differences between alleles, homologous chromosomes, or haploid sets. In mealybug males (Homoptera, Coccoidea), the haploid set of paternal origin undergoes heterochromatization at midcleavage and remains so in most of the tissues. This different behavior of the two haploid sets, which depends on their parental origin, represents one of the most striking examples of chromosome imprinting. In mammals, DNA methylation has been postulated as a possible molecular mechanism to differentially imprint DNA sequences during spermatogenesis or oogenesis. In the present article we addressed the role of DNA methylation in the imprinting of whole haploid sets as it occurs in Coccids. We investigated the DNA methylation patterns at both the molecular and chromosomal level in the mealybug *Planococcus citri.* We found that in both males and females the paternally derived haploid set is hypomethylated with respect to the maternally derived one. Therefore, in males, it is the paternally derived hypomethylated haploid set that is heterochromatized. Our data suggest that the two haploid sets are imprinted by parent-of-origin-specific DNA methylation with no correlation with the known gene-silencing properties of this base modification.

THE phenomenon of chromosome, or genomic, imprinting reveals the relevance of parental origin in determining functional differences between homologous alleles or differences in the behavior of homologous chromosomes (for reviews see Moore and Haig 1991; Peterson and Sapienza 1993). Imprinting is now widely recognized as an important reversible mechanism of epigenetic regulation at the gene and chromosome levels. In mammals, imprinting induces parent-of-origin-specific expression or repression of certain genes. Moreover, the loss of imprinting has been shown to be responsible for genetic syndromes such as Prader-Willi, Angelman, and Becwith-Wiedemann syndromes in humans (for review see Lalande 1997).

Historically, the first evidence for imprinting and its consequences for development was obtained from cytological studies of insects. In some insects, imprinting can affect the behavior of whole chromosomes or of an entire set of chromosomes. For example, in the fungal gnat Sciaridae, the zygote has three X chromosomes, two of paternal and one of maternal origin. During the syncytial embryonic divisions, maternal factors regulate whether one or two X chromosomes are eliminated. The embryos eliminating one X chromosome develop as females, while those eliminating two develop as males. In the germ line of both sexes, only one X chromosome is eliminated. Both in the germ line and in the soma, the elimination always affects the paternally derived X chromosomes; hence, the X chromosomes are parentally imprinted (Crouse 1960; Gerbi 1986). In Pseudococcids or mealybugs (Homoptera, Coccoidea), both females and males develop from fertilized eggs and there are no sex chromosomes. In females, all of the chromosomes remain euchromatic and functional. However, in embryos destined to be males, one haploid set of chromosomes becomes heterochromatic after the sixth cleavage division, and remains so in most of the tissues (for review see Hughes-Schrader 1948; Brown and Nur 1964). Males are functionally haploid due to heterochromatization. It has been shown that the heterochromatic complement is invariably that of paternal origin (Brown and Nelson-Rees 1961). The different behaviors of the two haploid sets, which depend on their parental origin, represent one of the clearest and most striking examples of chromosome imprinting (Nur 1990). As such, the mealybug chromosome system provides an excellent model system to study the mechanism of imprinting and its consequences at the chromosomal level.

In vertebrates, considerable data exist suggesting that methylation of cytosine residues at CpG doublets in DNA plays a role in the establishment, maintenance, and erasure of the parent-of-origin imprinting at the gene level (for review see Razin and Cedar 1994). This DNA modification has also been correlated with gene

Corresponding author: Giorgio Prantera, Dipartimento di Agrobiologia e Agrochimica, Università della Tuscia, Via S. Camillo De Lellis, 01100 Viterbo, Italy. E-mail: prantera@unitus.it

[1] *Present address:* Department of Zoology, University of Washington, Seattle, WA 98195-1800.

regulation in mammals since CpG-rich promoters of housekeeping genes on the female inactive X chromosome are heavily methylated, while those on the active X chromosome are unmethylated. However, this DNA modification seems to be a secondary event since the methylation of X-linked genes is subsequent to their inactivation at blastula. Moreover, in the case of imprinted genes, the simple correlation of methylation and inactivation is controversial (see discussion).

Elucidating the molecular mechanisms underlying the imprinting of entire chromosomes or haploid chromosome sets as occurs in insects should be informative for examining the relationship between imprinting, methylation, and gene activity. In Coccids the presence of both cytosine methylation (Achwal *et al.* 1983) and of a cytosine-specific DNA methyltransferase (Devajyothi and Brahmachari 1992) has already been demonstrated. A possible role of DNA methylation in the genomic imprinting of Pseudococcids was previously addressed in a study using HPLC as an assay to compare methylation levels in male *vs.* female DNAs. The authors found a significantly different level of cytosine methylation between the two sexes in only one of the two mealybug species analyzed, with the male DNA more methylated than female DNA (Scarbrough *et al.* 1984).

In these studies, we examined the relationship between DNA methylation and chromosome imprinting in mealybugs using two approaches. First, our molecular assays confirmed the presence of 5-methylcytosine in the DNA of the Coccid *Planococcus citri.* We did not detect differences in the overall methylation levels when male and female genomic DNAs were compared. We also used a method that permitted the detection of DNA methylation patterns *in situ*, at the chromosomal level. This method is called restriction enzyme-directed *in situ* nick translation (RE/NT) and had previously been proven to be a powerful tool to investigate DNA sequence organization at the chromosomal level (Bullerdiek *et al.* 1985; de la Torre *et al.* 1991; Ferraro *et al.* 1993). Specifically, when *Msp*I and its methyl-sensitive isoschizomer *Hpa*II were used as nicking agents, DNA methylation differences between homologous chromosomes and chromosome regions could be detected (Ferraro and Prantera 1988; Adolph and Hameister 1990; Prantera and Ferraro 1990). We applied the RE/NT technique and revealed differences in DNA methylation between the heterochromatic and euchromatic haploid chromosome sets in *P. citri.* In male embryos, we found that the heterochromatic, paternally derived haploid set was undermethylated with respect to the euchromatic maternally derived set. In female embryos, half of the chromosome complement was undermethylated with respect to the other half. We present suggestive evidence that the undermethylated chromosomes in females represented those of paternal origin. Our results suggest that DNA methylation could be at the basis of the imprinting phenomenon at the chromosomal level in Coccids. Moreover, they confirm recent evidence (Tweedie *et al.* 1997) that, in invertebrates, DNA methylation is not correlated with gene inactivation.

MATERIALS AND METHODS

Mealybug cultures: *P. citri* were raised in our laboratory on sprouting potatoes at 27°, inside glass food containers covered with gauze. The potatoes were kept in the dark to sprout for 1 mo before use.

X-ray treatment: About 25 males at different stages of the life cycle were treated with 4000 rad of X rays delivered at a dose of 100 rad per minute. Following treatment, the males were mated *en masse* to virgin females. After fertilization, gravid females, containing a sufficient number of embryos for cytological analysis, were dissected as described below.

Genomic DNA extraction, digestion, and electrophoresis: Total genomic DNA was separately isolated and purified from virgin females, gravid females, or adult males, following the method of Epstein *et al.* (1992). About 30 insects were homogenized with a plastic pestle in HB buffer (0.1 m Tris-HCl pH 8, 10 mm EDTA pH 8, 0.35 m NaCl, 7 m urea). One volume of phenol-chloroform (1:1) was added and the mixture was gently stirred and then centrifuged (13,000 rpm) for 5 min. DNA was isolated by ethanol precipitation. Restriction enzyme digestions were performed using standard techniques.

Chromosome preparation: Chromosome spreads were obtained by a modification of previously described methods (Odierna *et al.* 1993; Manicardi *et al.* 1996) from embryos that were dissected and maintained in a solution of 0.8% sodium citrate and 3×10^{-3} m colchicine for 1 hr. The embryos were then transferred to minitubes and centrifuged at 3000 rpm for 2 min. Bradley-Carnoy fixative (chloroform:ethanol:acetic acid, 4:3:1) was added to the pellet. The pellet was dispersed by repeated passage through a gauge hypodermic needle attached to a 1-ml syringe for 30 min. The suspension was centrifuged at 4500 rpm for 8 min. After the pellet was resuspended in methanol-acetic acid (3:1), 60 μl of cellular suspension was dropped onto clean slides and air-dried.

***In situ* nick translation:** *In situ Msp*I, *Hpa*II, *Alu*I, and *Eco*RI/nick-translation (NT) experiments were carried out on slides containing fresh chromosome spreads (Ferraro and Prantera 1988). Thirty units of restriction enzyme (Boehringer Mannheim, Monza, Italy) in the appropriate buffer were added to chromosomes for 1.5, 3, 10, and 30 min, at 37°. Each slide was treated for 10 min at room temperature with 50 μl of NT mixture: 4 μm dNTP(-dTTP), 4 μm biotinylated-16-dUTP (Boehringer Mannheim), 10 units/ml DNA polymerase I (Boehringer Mannheim) in 1× NT buffer (NT buffer is 50 mm Tris-HCl pH 7.9, 5 mm $MgCl_2$, 10 mm 2-β-mercaptoethanol, 50 μg/ml BSA). In each RE/NT experiment control slides were processed exactly as above, except for the omission of the relative restriction enzyme in the digestion mixture.

Detection and fluorescence observation: After two washes in 10 mm Tris-HCl pH 7.4 for 5 min, the slides were treated with blocking reagent (4× SSC, 30 mg/ml BSA, 0.1% Tween 20; 1× SSC is 0.15 m NaCl, 0.015 m sodium citrate) at 37° for 30 min. Detection was carried out with avidin fluorescein isothiocyanate conjugate (avidin-FITC; Vector Laboratories, Burlingame, CA) 1:300 in detection solution (2× SSC, 1 mg/ml BSA, 0.1% Tween 20) for 30 min at 37° in a wet chamber. The slides were washed three times in 4× SSC and 0.1% Tween 20, at 42° for 5 min. The signal was amplified with biotinylated antiavidin antibody (Vector Laboratories) 1:100 in PBS for 30 min at 37° in a wet chamber. After four washes in 4× SSC

and 0.1% Tween 20, at 42° for 5 min, the slides were treated again with avidin FITC for 30 min at 37° in a wet chamber. The chromosomes were counterstained with 0.2 μg/ml DAPI (Boehringer Mannheim) in 2× SSC for 5 min. After counterstaining in DAPI, the slides were mounted in antifade medium [DABCO (Sigma, St. Louis) 23.3 mg/ml, 10 mm Tris-HCl pH 7.5–8, 90% sterile glycerol] and observed with a Zeiss Axiophot fluorescence microscope equipped with a 100-W mercury light source. The filter combinations used were 01 for DAPI (365/11 nm excitation range) and 09 for FITC (450–490 nm excitation range). Fluorescent images were captured with a CCD camera (series 200; Photometrics, Tucson, AZ) using IPLab software (Signal Analytics Corp., Vienna, VA) and processed with a Power Macintosh 8100 using Adobe Photoshop software.

RESULTS

Probing genomic DNA of *P. citri* with *Hpa*II and *Msp*I: The two isoschizomers, *Hpa*II and *Msp*I, have the same recognition sequence (C/CGG), but are differentially sensitive to the methylation state of the internal cytosine. *Hpa*II does not cut the sequence when the cytosine is methylated, whereas *Msp*I is insensitive to its methylation state. Figure 1 shows ethydium bromide stain of mixed male and female *P. citri* genomic DNA after digestion with *Hpa*II (lane 1) or *Msp*I (lane 2). As expected, both enzymes produced a wide range of restriction fragments, but with *Hpa*II the smear began just below the 23.1-kb marker band, while with *Msp*I the smear was shifted toward the lower molecular weights, starting below the 9.4-kb band of the marker. In lanes 3 and 4 the results of the digestion with *Hpa*II of male (lane 3) and unmated-female (lane 4) genomic DNA are shown. The two patterns are nearly identical and indistinguishable from the pattern of *Hpa*II-digested genomic DNA from the mixed population of males and females (lane 1). Hence, this method did not detect any sex-specific differences in DNA methylation in *P. citri.*

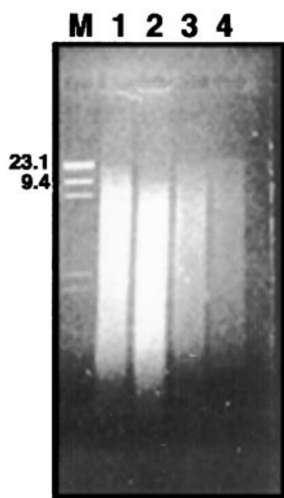

Figure 1.—Methylation pattern of *P. citri* genomic DNA. Two aliquots of the same genomic DNA preparation from *P. citri* gravid females were digested with *Hpa*II (lane 1) or *Msp*I (lane 2). Each gravid female before ovoposition contains ∼400 embryos of both sexes. The smear produced by *Msp*I is shifted toward the low molecular weights as compared to that produced by *Hpa*II. In lanes 3 and 4, respectively, the digestion patterns with *Hpa*II of male and virgin female genomic DNA are shown. The two patterns are identical. Marker (M) is bacteriophage lambda DNA digested with *Hin*dIII.

Methylation patterns of *P. citri* chromosomes *in situ*: In *P. citri* males the haploid set of paternal origin becomes heterochromatized during midcleavage stages, while in females both sets appear euchromatic. To detect *in situ* possible differences in DNA methylation patterns between haploid sets in *P. citri* male and female cells ($2n = 10$) we digested chromosome preparations with the restriction enzymes *Msp*I or *Hpa*II. The endonucleolytic nicks were then expanded by nick translation in the presence of a biotinylated dUTP. The incorporation of biotinylated dUTP after nick translation is evidenced by the binding of an avidin-fluorescein complex. FITC fluorescence is thus indicative of susceptibility to the enzyme of the corresponding chromosome material. Both male and female embryonic tissues were simultaneously present in the same preparation (see materials and methods), permitting comparison within an experiment. Male and female tissue patches can be easily distinguished because in male cells the heterochromatic haploid set formed a conspicuous, darkly staining structure in interphase cells. This morphological distinction between the two sets is still distinguishable in mitosis (compare male and female cells in Figures 2 and 3, a1–e1) until late metaphase when both euchromatic and heterochromatic sets become highly condensed.

At digestion times of 10 min or higher, *Msp*I and *Hpa*II produced a similar fluorescent pattern along the chromosomes of both female and male cells, with no differential labeling of the two haploid sets in the latter. However, at the shortest digestion times (1.5 and 3 min) the two enzymes yielded different patterns along *P. citri* chromosomes, and the results reported below refer to these observations.

Figure 2 shows the results obtained after the *Msp*I digestion in male (a–c) and female (d and e) embryonic cells. The FITC-fluorescence pattern indicates the susceptibility of the *P. citri* chromosomes to digestion with *Msp*I. In Figure 2, a1–e1, the same metaphases were counterstained with the fluorescent DNA dye DAPI. Note that in both male and female cells all the chromosomes of a metaphase were FITC-labeled and that the labeling intensity of the chromosomes of a metaphase parallels their DAPI staining intensity.

The pattern obtained after *Hpa*II-directed *in situ* nick translation of *P. citri* chromosome preparations is shown in Figure 3. In both male (Figure 3, a–c) and female (Figure 3, d and e) embryonic cells only five chromosomes of the diploid complement are FITC labeled, while the remaining five are faint or totally unlabeled. From the comparison with DAPI images of male nuclei

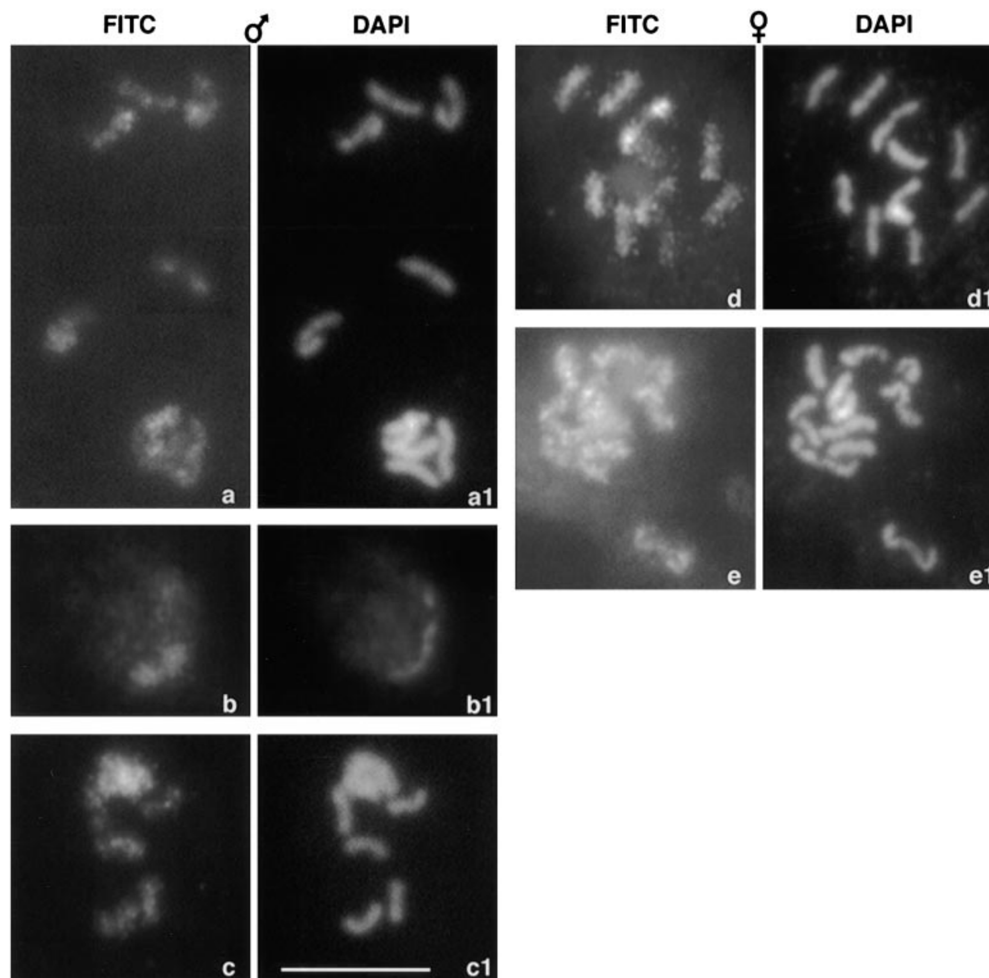

Figure 2.—*Msp*I-directed RE/NT of *P. citri* chromosomes and nuclei. *Msp*I nicking in male (a–c) and female (d, e) cells is evidenced by FITC fluorescence after RE/NT. DAPI counterstaining of the same cells is in a1–e1. Note that in both male and female cells, all the chromosomes ($2n = 10$) show uniform FITC fluorescence with only slight differences that parallel the differences in their DAPI staining. Bar, 10 μm.

and metaphases (Figure 3, a1–c1) it was readily apparent that the five labeled chromosomes corresponded to the heterochromatic haploid set. These results therefore indicated that the paternally derived heterochromatic haploid set was more sensitive to the digestion by the methyl-inhibited *Hpa*II enzyme than the maternally derived euchromatic chromosomes.

The RE/NT of *P. citri* chromosomes after digestion with *Alu*I (AG/CT) or *Eco*RI (G/AATTC) never produced a differential labeling of the heterochromatic and euchromatic sets in male cells, or the appearance in female cells of five FITC-labeled and five unlabeled chromosomes (data not shown).

In parallel control experiments, the chromosome preparations were nick translated after incubation with the digestion buffer without any restriction enzyme. At incubation times of 1.5 and 3 min the mock digestion did not produce any FITC labeling along the chromosomes, while with higher incubation times a faint, uniform labeling was produced along the chromosomes of both male and female embryonic cells.

Parental origin of the undermethylated chromosomes in female cells: We wanted to ascertain if the five *Hpa*II-sensitive and the five *Hpa*II-resistant chromosomes of female embryonic cells also represented two separate haploid sets and, in this case, if they were of nonrandom parental origin. To this aim, since it is difficult to reconstruct the *P. citri* karyotype after DAPI staining, we carried out a different experimental approach. We applied *Hpa*II RE/NT to the progeny of crosses between virgin females and males treated with heavy doses of X-irradiation. The X-ray-induced chromosome fragments and rearrangements were retained during spermatogenesis and transmitted to the F_1 progeny because of the holocentric nature of Coccid chromosomes (Brown and Nelson-Rees 1961). Therefore, we expected that all fragmented and rearranged chromosomes in the F_1 offspring were of paternal origin. The analysis of chromosome preparations from this F_1 offspring (Figure 4) showed that 31 out of 39 chromosome fragments observed in female embryonic cells were FITC labeled, due to their sensitivity to *Hpa*II nicking activity. Moreover, in

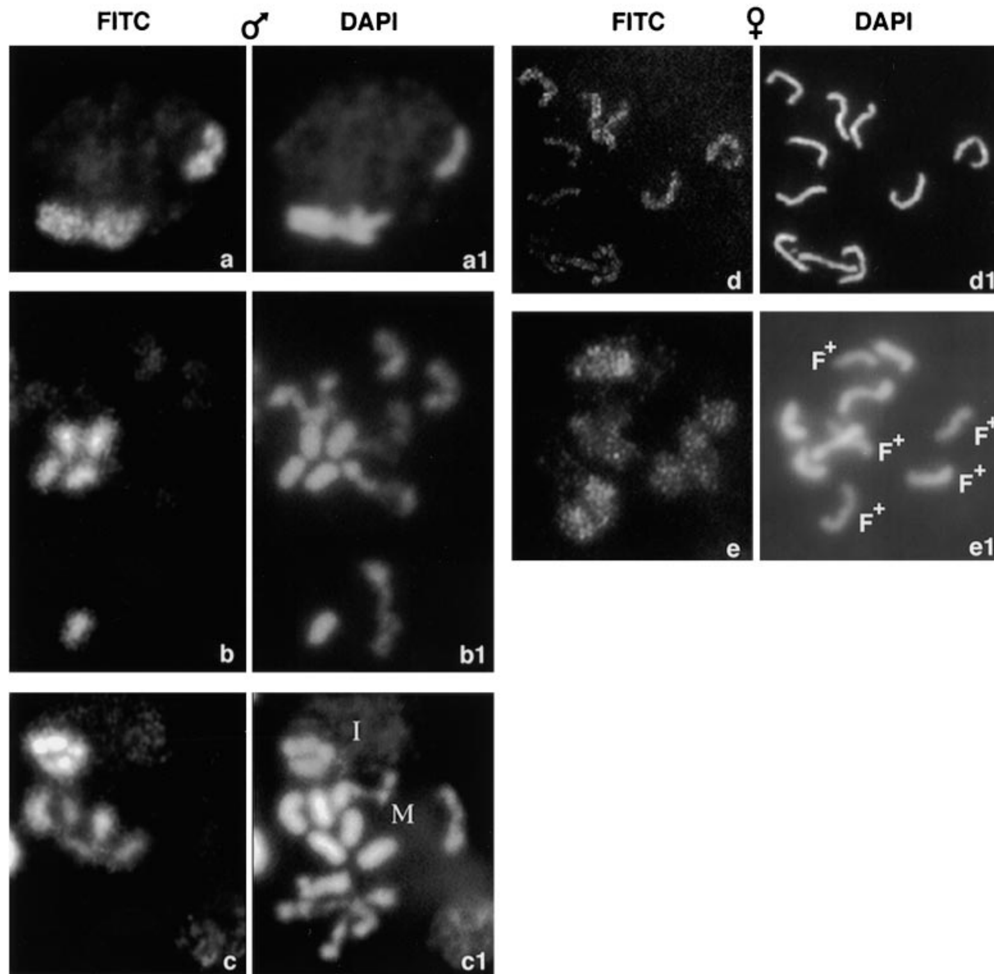

Figure 3.—*Hpa*II-directed RE/NT of *P. citri* chromosomes and nuclei. In both male (a–c) and female (d, e) cells, chromosomes are not uniformly FITC labeled in that five chromosomes are resistant to the nicking activity of the methyl-inhibited enzyme *Hpa*II. From the comparison with the DAPI counterstaining images, it clearly emerges that in male cells (a1–c1) the *Hpa*II-resistant, hence methylated, chromosomes correspond to the euchromatic haploid set. In c1, one interphase nucleus (I) and one metaphase plate (M) are indicated. In e1, the five chromosomes that are FITC labeled in e are indicated (F^+).

cells showing fragments, five unlabeled chromosomes can be always recognized, whereas five normally shaped FITC-labeled chromosomes cannot.

DISCUSSION

From the comparison of genomic DNA digestion patterns with *Hpa*II and *Msp*I, we expected information on the pattern of DNA methylation in the *P. citri* genome. In fact, these two isoschizomers cut the same DNA sequence (CCGG), but *Hpa*II is inhibited by the methylation of the internal cytosine, while *Msp*I is insensitive to it. Our results clearly show that *Msp*I produces lower molecular weight DNA fragments than *Hpa*II in DNA from a mixed population of male and female individuals, thus confirming the presence of a methylated DNA fraction in the *P. citri* genome, in accordance with the data obtained in mealybug species by other groups with immunochemistry (Achwal *et al.* 1983) and HPLC (Scarbrough *et al.* 1984). When male and female genomic DNA were probed separately with *Hpa*II, they yielded nearly identical patterns. Our results do not necessarily mean that there are no methylation differences between *P. citri* male and female genomic DNAs, but only that these were not detectable by analyzing bulk DNA digestion patterns.

We obtained a confirmation and extension of the results described above using the RE/NT technique. The RE/NT technique uses the differential sensitivity of *Hpa*II and *Msp*I to methylated cytosine residues to detect *in situ* DNA methylation patterns at chromosome and cellular levels (Ferraro and Prantera 1988; Adolph and Hameister 1990; Prantera and Ferraro 1990; del Mazo *et al.* 1994). In our experiments, *Msp*I and *Hpa*II produced different digestion patterns along *P. citri* chromosomes only at the shortest digestion times (1.5 and 3 min). The lack of differential patterns with the two enzymes at longer incubation times could be due to the extraction of chromosomal DNA as a result of the extensive digestion. The consequent loss of an

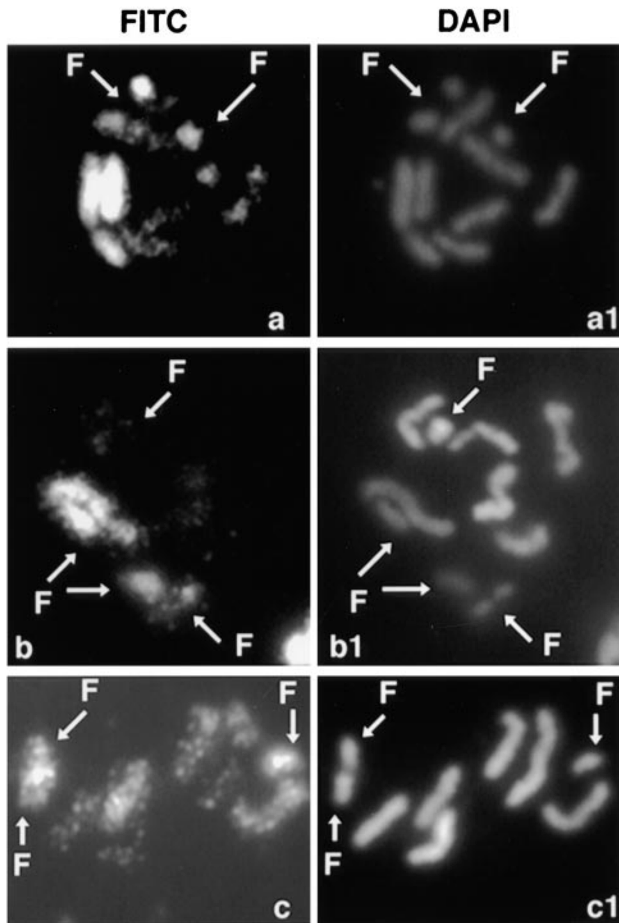

Figure 4.—Parental origin of the hypomethylated chromosomes in *P. citri* females. *Hpa*II RE/NT analysis of chromosome preparations from daughters of irradiated males shows that the great majority of the paternally inherited chromosome fragments (F) are sensitive to *Hpa*II and, hence, hypomethylated. In the illustrated examples, only the uppermost fragment in b is unlabeled. Note that we can always recognize five unlabeled chromosomes, but not five labeled, normally shaped chromosomes.

intact template prevents the repair by DNA polymerase I and the incorporation of sufficient labeling. The result is the masking of the true digestion pattern, as already described in human (de la Torre *et al.* 1992) and insect (Manicardi *et al.* 1998) chromosomes. For these reasons, in the following discussion we considered only the results obtained with short incubation times.

The digestion with the methylation-sensitive *Hpa*II does not uniformly affect all the chromosomes of the *P. citri* complement ($2n = 10$). In fact, in both male and female embryonic cells five chromosomes of the complement are intensely FITC labeled, *i.e.*, sensitive to *Hpa*II, while the other five are faint or unlabeled, *i.e.*, insensitive to *Hpa*II. These findings are in perfect accordance with the above-reported molecular evidences. In fact, these observations confirm both the presence of a methylated DNA fraction in the *P. citri* genome and the similarities of male and female genomic DNA with respect to the methylation pattern. In male cells, the five highly labeled chromosomes consistently correspond to the heterochromatic set. The paternally derived heterochromatic set is therefore more sensitive to *Hpa*II and, hence, less methylated than the maternally derived euchromatic one.

The efficiency of enzyme nicking is certainly dependent upon DNA sequence and it could also be sensitive to the level of chromatin packaging (Burkholder 1989; Prantera and Ferraro 1990; de la Torre *et al.* 1991). However, we believe that our results were not influenced by differences in chromatin accessibility for two reasons.

First, it is the heterochromatic set that, because of its higher degree of chromatin compaction, would be predicted to be less accessible to enzyme digestion than the euchromatic one. But in this context it should be noted that in the mealybug *P. lilacinus* only the 3–5% of DNA in males is in a chromatin conformation resistant to nuclease digestion. The remaining 95–97% of male chromatin exhibits the same level of nuclease resistance as female chromatin (Khosla *et al.* 1996). This means that the male heterochromatic haploid set is not in a nuclease-resistant chromatin conformation as compared to the euchromatic chromosomes.

Second, the two chromosome sets do not show differential sensitivity to the methyl-insensitive isoschizomer *Msp*I. In fact, the heterochromatic set is only slightly more labeled by *Msp*I RE/NT than the euchromatic one, but this difference, if any, parallels an analogous difference after DAPI staining (compare FITC and DAPI images of Figure 2). The two haploid sets have obviously the same DNA base compositions, and hence their differential staining with the AT-specific DNA ligand DAPI is only determined by the more condensed chromatin conformation of heterochromatic chromosomes. The higher DNA amount per chromosome unit-length of the heterochromatic material can also well account for the slightly higher FITC-labeling intensity of the heterochromatic set after *Msp*I RE/NT. The similar sensitivity to *Msp*I of euchromatic and heterochromatic sets is well in accordance with the above-mentioned results (Khosla *et al.* 1996) on sensitivity to nuclease digestion. In fact, a 3–5% difference in nuclease sensitivity of the two haploid sets is clearly undetectable by *in situ* RE/NT. These observations are further confirmed by the RE/NT experiments with the restriction enzymes *Alu*I and *Eco*RI, which do not contain a CpG doublet in their recognition sequence. RE/NT with these two enzymes, in fact, does not produce a differential labeling of the two haploid sets in male cells, thus confirming that they are equally accessible to the enzyme action.

Our results suggest that parent-of-origin-based differences in DNA methylation levels of the two haploid sets could account for parental chromosome imprinting in the *P. citri* males. The paternally derived unmethylated chromosomes undergo inactivation by heterochromati-

zation, while their methylated maternally derived homologs remain active. From what is known about vertebrates, the findings that the functional inactivation affects the less-methylated homolog and that methylation does not hinder genetic activity appear to be surprising. In this context two considerations must be taken into account. First, for those invertebrate species in which the presence of DNA methylation has been demonstrated, this base modification is not correlated with gene inactivation. In fact, recently it has been shown that several constitutively active housekeeping genes reside in the methylated fraction of the invertebrate genome (Tweedie *et al.* 1997). Second, in vertebrates, imprinted genes exhibit parental-specific DNA methylation profiles, but the correlation between methylation and allele silencing is too simplistic. In fact, a well-studied imprinted region of the mouse chromosome 7 contains two genes, *Igf2* and *H19*, which are methylated on the paternally derived chromosome 7 and hypomethylated on the maternally derived homolog. Yet these two genes are imprinted in opposite ways. In fact, for *H19* it is the maternally derived hypomethylated allele that is active, while in the case of *Igf2*, the paternally derived methylated allele is active (Feil *et al.* 1994). Moreover, in the case of the imprinted mouse gene *Igf2r*, it has recently been shown that the imprinting signal resides in an intronic CpG island, which is methylated on the maternally derived expressed allele (Stoger *et al.* 1993; Wutz *et al.* 1997). These findings led to the hypothesis that the imprinted allele inactivation occurs by the binding of a repressor to the promoter of the allele that is parentally unmethylated (Nicholls *et al.* 1998).

We observed also that in female embryonic cells 5 chromosomes of the complement are more sensitive to *Hpa*II than the other 5, while with *Msp*I the 10 chromosomes are equally digested. The *Hpa*II RE/NT analysis of chromosome preparations from the daughters of irradiated fathers showed that the great majority (31 out of 39) of paternally derived chromosome fragments scored in female cells were sensitive to *Hpa*II. Furthermore, in cells showing fragments, 5 unlabeled chromosomes can always be recognized, but 5 normally shaped FITC-labeled chromosomes cannot, indicating that the rearranged chromosome contribution of irradiated fathers is hypomethylated.

Taken as a whole, our data show that in *P. citri* the zygote receives from the male gamete a haploid set that is hypomethylated with respect to that received from the female gamete. In male embryos, after midcleavage stage, the heterochromatization cascade affects the hypomethylated paternally derived chromosomes. In *P. citri* female embryos, in the absence of the heterochromatization cascade, the paternally derived hypomethylated haploid set remains active. In conclusion, we suggest that in Coccids parental genomic imprinting is mediated by DNA methylation and that there is no correlation with the known gene silencing effects of this base modification.

We are deeply indebted to Barbara Wakimoto for helpful discussions and valuable comments on the manuscript. We also thank Julie Brill for critical reading of the manuscript. We are grateful to Angelo Schinoppi who carried out X-ray treatments and to Bruno Fazzini for his help in photographic reproduction. This work was supported by grants from the Italian Ministero dell'Università e della Ricerca Scientifica e Tecnologica and from the Consiglio Nazionale delle Ricerche of Italy (contract no. 95.02924.CT14).

LITERATURE CITED

Achwal, C. W., C. A. Iyer and H. S. Chandra, 1983 Immunochemical evidence for the presence of 5mC, 6mA and 7mG in human, Drosophila and mealybug DNA. FEBS Lett. **158:** 353–358.

Adolph, S., and H. Hameister, 1990 *In situ* nick translation of human metaphase chromosomes with restriction enzymes *Msp*I and *Hpa*II reveals an R-band pattern. Cytogenet. Cell. Genet. **54:** 132–136.

Brown, S. W., and W. A. Nelson-Rees, 1961 Radiation analysis of a lecanoid genetic system. Genetics **46:** 983–1007.

Brown, S. W., and U. Nur, 1964 Heterochromatic chromosomes in the coccids. Science **145:** 130–136.

Bullerdiek, J., J. Dittmer, A. Faehre, S. Bartnitzke, V. Kasche *et al.*, 1985 A new banding pattern of human chromosomes by *in situ* nick translation using EcoRI and biotin-dUTP. Clin. Genet. **28:** 173–176.

Burkholder, G. D., 1989 Morphological and biochemical effects on isolated mammalian chromosomes in vitro. Chromosoma **97:** 347–355.

Crouse, H. V., 1960 The controlling element in sex chromosome behavior in Sciara. Genetics **45:** 1429–1443.

de la Torre, J., A. R. Mitchell and A. T. Sumner, 1991 Restriction endonuclease/nick translation of fixed mouse chromosomes: a study of factors affecting digestion of chromosomal DNA *in situ.* Chromosoma **100:** 203–211.

de la Torre, J., A. T. Sumner, J. Gosalvez and L. Stuppia, 1992 The distribution of genes on human chromosomes as studied by in situ nick translation. Genome **35:** 890–894.

del Mazo, J., G. Prantera, M. Torres and M. Ferraro, 1994 DNA methylation changes during mouse spermatogenesis. Chromosome Res. **2:** 147–152.

Devajyothi, C., and V. Brahmachari, 1992 Detection of a CpA methylase in an insect system: characterization and substrate specificity. Mol. Cell. Biochem. **110:** 103–111.

Epstein, H., T. C. James and P. B. Singh, 1992 Cloning and expression of *Drosophila* HP1 homologs from a mealybug, *Planococcus citri.* J. Cell. Sci. **101:** 463–474.

Feil, R., J. Walter, R. D. Allen and W. Reik, 1994 Developmental control of allelic methylation in the imprinted mouse *Igf2* and *H19* genes. Development **120:** 2933–2943.

Ferraro, M., and G. Prantera, 1988 Human NORs show correlation between transcriptional activity, DNase I sensitivity, and hypomethylation. Cytogenet. Cell Genet. **47:** 58–61.

Ferraro, M., V. Predazzi and G. Prantera, 1993 In human chromosomes telomeric regions are enriched in CpGs relative to R-band. Chromosoma **102:** 712–717.

Gerbi, S. A., 1986 Unusual chromosome movements in Sciarid flies, pp. 71–104 in *Results and Problems in Cell Differentiation*, Vol. 13, edited by W. Hennig. Springer-Verlag, Berlin.

Hughes-Schrader, S., 1948 Cytology of Coccids (Coccoidea-Homoptera). Adv. Genet. **2:** 127–203.

Khosla, S., P. Kantheti, V. Brahmachari and H. Sharat Chandra, 1996 A male-specific nuclease-resistant chromatin fraction in the mealybug *Planococcus lilacinus.* Chromosoma **104:** 386–392.

Lalande, M., 1997 Parental imprinting and human disease. Annu. Rev. Genet. **30:** 173–195.

Manicardi, G. C., D. Bizzaro, E. Galli and U. Bianchi, 1996 Heterochromatin heterogeneity in the holocentric X chromatin of *Megoura viciae* (Homoptera, Aphididae). Genome **39:** 465–470.

Manicardi, G. C., M. Mandrioli, D. Bizzaro and U. Bianchi, 1998

Patterns of DNaseI sensitivity in the holocentric chromosomes of the Aphid *Megoura viciae.* Genome **41:** 169–172.
Moore, T., and D. Haig, 1991 Genomic imprinting in mammalian development: a parental tug-of-war. Trends Genet. **7:** 45–49.
Nicholls, R. D., S. Saitoh and B. Horsthemke, 1998 Imprinting in Prader-Willi and Angelman syndromes. Trends Genet. **14:** 194–200.
Nur, U., 1990 Heterochromatization and euchromatization of whole genomes in scale insects (Coccoidea: Homoptera). Development, Supplement: 29–34.
Odierna, G., F. Baldanza, G. Aprea and E. Olmo, 1993 Occurrence of G-banding in metaphase chromosomes of *Encarsia berlesei* (Himenoptera: Aphelinidae). Genome **36:** 662–667.
Peterson, K., and C. Sapienza, 1993 Imprinting the genome: imprinted genes, imprinting genes, and a hypothesis for their interaction. Annu. Rev. Genet. **27:** 7–31.
Prantera, G., and M. Ferraro, 1990 Analysis of methylation and distribution of CpG sequences on human active and inactive X chromosomes by *in situ* nick translation. Chromosoma **99:** 18–23.
Razin, A., and H. Cedar, 1994 DNA methylation and genomic imprinting. Cell **77:** 473–476.
Scarbrough, K., S. Hatiman and U. Nur, 1984 Relationship of DNA methylation level to the presence of heterochromatin in mealybugs. Mol. Cell. Biol. **4:** 599–603.
Stoger, R., P. Kubicka, C.-G. Liu, T. Kafri, A. Razin *et al.*, 1993 Maternal-specific methylation of the imprinted mouse *Igf2r* locus identifies the expressed locus as carrying the imprinted signal. Cell **73:** 61–71.
Tweedie, S., J. Charlton, V. Clark and A. Bird, 1997 Methylation of genomes and genes at the invertebrate-vertebrate boundary. Mol. Cell. Biol. **17:** 1469–1475.
Wutz, A., O. W. Smrzka, N. Schweifer, K. Schellander, E. F. Wagner *et al.*, 1997 Imprinted expression of the *Igf2r* gene depends on an intronic CpG island. Nature **389:** 745–749.

Communicating editor: V. G. Finnerty