Chromosoma (2001) 110:93–101
DOI 10.1007/s004120000124

ORIGINAL ARTICLE

Marina Ferraro · Giovanni L. Buglia
Francesca Romano

Involvement of histone H4 acetylation in the epigenetic inheritance of different activity states of maternally and paternally derived genomes in the mealybug *Planococcus citri*

Received: 14 June 2000 / In revised form: 13 November 2000 / Accepted: 28 November 2000 / Published online: 20 March 2001
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Abstract Modification of histones by acetylation is a well-known mechanism for the establishment and maintenance of specific chromatin structures with different activity states. In *Planococcus citri* males the paternal genome, early in development, becomes mostly inactive and heterochromatic. As we had not found methylation in the genome of *P. citri*, we analyzed the acetylation state of histone H4. We report here that, in males, differences in the level of histone H4 acetylation are indeed present in the two genomes of different parental origin; these differences were confirmed by treatment with the histone deacetylase inhibitor Trichostatin A. There is also evidence of acetylation of histone H4 on metaphase chromosomes. Our data therefore suggest a role of histone H4 acetylation in the imprinting of the paternal genome in *P. citri* males, thus supporting a role of modification of chromatin-related structural proteins in the epigenetic transmission of imprinting.

Introduction

Epigenetics refers to the transmission from parent cells to progeny cells of a specific pattern of activity, of genes or chromosomes, which is not based on the DNA sequence. Epigenetic modifications are responsible for the phenomenon of imprinting, defined as the differential expression of genes depending on their parental origin. Different models to explain how an epigenetic imprint might be maintained have been proposed. They involve alterations at the level of DNA, through cytosine methylation at the dinucleotide CpG, or of chromatin structure. The latter could occur in organisms in which imprinting has been demonstrated but cytosine methylation is lacking.

Edited by: P.B. Becker

M. Ferraro (✉) · G.L. Buglia · F. Romano
Department of Genetics and Molecular Biology,
University “La Sapienza”, Rome, Italy
e-mail: mferraro@axcasp.caspur.it

In the mealybug *Planococcus citri* (Homoptera, Coccoidea) both males and females start development as diploids from fertilized eggs in which both maternally and paternally derived chromosomes are euchromatic. However, early in the development of embryos that will develop as males, the haploid set of chromosomes of paternal origin becomes heterochromatic (Brown and Nelson-Rees 1961) and remains so in most tissues (Nur 1966). It was later demonstrated by the use of genetic markers that in those cells in which it is in the heterochromatic state the paternal genome is also mostly inactive (Brown and Wiegmann 1969). The heterochromatic set does not participate in gamete formation since, following an inverted type of meiosis, during the second division the heterochromatic and the euchromatic chromosomes segregate to opposite sides of the cell and only the euchromatic derivatives proceed to form sperm (Brown and Nur 1964). In females, on the contrary, meiosis is canonic with one germ cell giving rise to four functional gametes (Hughes-Schrader 1948) (Fig. 1). The same haploid chromosome set can therefore be somatically active and transmitted to the next generation, or inactive and not transmitted, depending on the germ line it passes through. The mealybug *P. citri* therefore represents a clear example of parental imprinting, evidenced at the cytological level by the acquisition of a typical heterochromatic appearance.

The phenomenon of imprinting is considered as representing one type of cell memory since the epigenetic modification(s) determining functional differences between the two alleles of different parental origin and the committed expression pattern will be propagated unchanged from one cell to progeny cells.

The two most common epigenetic mechanisms involved in the control of gene expression and cell memory are methylation of DNA at CpG residues and chromatin structure. In organisms such as *Drosophila* and yeast, cytosine methylation is lacking, nevertheless epigenetic control of gene expression has been demonstrated (for examples see Klar 1987; Golic et al. 1998). As for Coccids, data in the literature are often ambiguous and

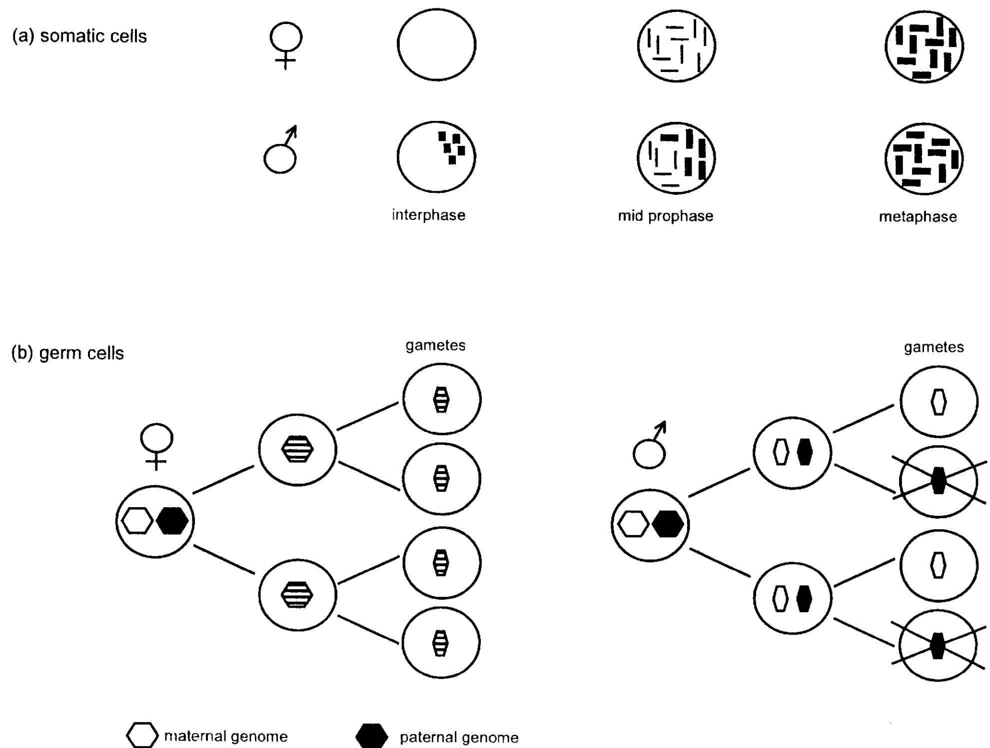

Fig. 1a, b Different behaviors shown by maternally and paternally derived genomes of *Planococcus citri*. **a** Somatic cells: the different chromatin condensation cycles from interphase to metaphase are shown. **b** Germ cells: the different fate of the paternal genome when passing through male or female germline is schematized

controversial even about the same species, and from our previous investigations DNA methylation does not seem to play a decisive role in the silencing of the paternal genome in *P. citri* males (Buglia et al. 1999). The widely accepted alternative way to create an epigenetic state and to store cell memory is the modification of chromatin-related structural proteins. Evidence favoring the retention of specific nucleoprotein complexes on metaphase chromosomes comes from both cytogenetic studies (Kerem et al. 1983) and from studies on isolated chromosomes (Gazit et al. 1982).

Histone acetylation is found throughout the eukaryotes, from unicellular organisms to the most complex of higher plants and animals (for review see Jeppesen 1997) and it is well known that the post-translational modifications that histones undergo might influence higher-order chromatin packaging. Moreover, histone acetylation is a reversible process and is also maintained through cell division. Actually, in some cases, the way through which an epigenetic type of control of gene expression is achieved seems to be a differential level of acetylation/deacetylation at the four acetylatable lysine positions of histone H4 in active and inactive chromatin (Braunstein et al. 1993; O'Neill and Turner 1995; Struhl 1998).

Here we investigate the possible role of histone H4 acetylation in the epigenetic control of different activity states of the genomes of different parental origin in *P. citri* males. Indirect immunofluorescent staining with antibodies to acetylated histone H4 revealed that in *P. citri* male cells the paternal and maternal genomes, recognizable one from the other by their differing levels of condensation, have quite different levels of acetylation. Exposure to the deacetylase inhibitor Trichostatin A (TSA) leads to a statistically significant, clear increase in immunolabeling, particularly evident on the heterochromatic paternally derived genome of male cells, which is otherwise almost completely devoid of fluorescent signals. Finally, we show that on metaphase chromosomes residual spots of acetylated histone H4 are still present which, according to the model proposed by Wolffe (1994), could represent "seeding" elements for the reassembly, after DNA replication, of the former chromatin state.

Materials and methods

Animals and cytological preparations

Mealybugs were grown on sprouting potatoes at 25°C. Dissections were carried out in 0.9% NaCl. For most preparations to be exposed to antibodies to histone H4, ovaries excised from gravid females, bodies of females, or adult males were fixed essentially according to the technique of Turner et al. (1990). Briefly, dissected materials were sequentially exposed to (1) *Chironomus* Ringer's solution (87 mM NaCl, 3.2 mM KCl, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$), pH 6.3 for 20 min in ice; (2) *Chironomus* Ringer's solution, pH 6.3, plus 2% Nonidet P-40, 1% Triton X-100 for 20 min at room temperature; (3) *Chironomus* Ringer's solution, pH 7.3, plus 2% Nonidet P-40, 1% Triton X-100, 3.2% formaldehyde for 10 min at room temperature; (4) 45% acetic acid, 10 mM $MgCl_2$ for 1 h at room temperature. Fixed preparations were squashed between glass slides and coverslips, frozen in liquid nitrogen and the coverslips snapped off. Slides were then immersed in 80% ethanol overnight. Preparations to be exposed to MPM-2 antibody or to be stained by the Methyl green/DAPI (4′,6-diamidino-2-phenylindole) technique were fixed in 3:1 methanol:acetic acid.

Cell type analyzed

Cells to be analyzed were chosen according to two criteria. First, male and female cells, in order to be compared, must be in a similar cell cycle. For this reason we choose as the parameter the compaction level of euchromatin, i.e., chromatin of female cells (in which both genomes are euchromatic) should show a degree of compaction similar to that of the single euchromatic genome of male cells. Second, the cell cycle stage considered suitable for our analysis was the G2 stage due to the scheduled experiment with TSA, which is known to block cells at the G1/G2 and G2/M transitions. A preliminary check of G2 cell morphology was done by immunolabeling with a mouse monoclonal antibody to mitotic proteins (not shown).

Trichostatin A treatment

Some ovaries, bodies of females, and adult males, were exposed to the histone deacetylase inhibitor TSA (Sigma) at 0.1 μg/ml (Belayev et al. 1996) for 3 h. Control preparations were simultaneously mock-treated. After TSA treatment, squashing and fixation were as described above.

Antibodies

The antibodies used were: a rabbit antiserum to non-acetylated human histone H4 (Serotec); a rabbit antiserum to the acetylated isoform of human histone H4 (Serotec); a mouse monoclonal antibody to mitotic proteins (DAKO MPM-2).

Immunolabeling

Slide preparations were immersed sequentially in 40% ethanol for 10 min, phosphate-buffered saline (PBS) for 10 min, then in blocking solution (PBS, plus 1% bovine serum albumin, 0.5% Triton X-100) for 1 h, and finally washed three times (5 min each) in PBS. For both rabbit antisera, slide preparations were incubated for 4 h at 4°C with a 1:200 dilution in blocking solution. After three 5 min washes in blocking solution and one in PBS, the slides were incubated for 2 h at 37°C with the secondary antibody (Rhodamine-conjugated goat anti-rabbit IgG, whole molecule, Cappel) diluted 1:20 in blocking solution. For mouse anti-MPM-2, the dilution was 1:50 with an incubation period of 1 h at 37°C. After-incubation washes were as above. The secondary antibody used was a fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse (Boehringer) diluted 1:20 in blocking solution, incubated for 90 min at 37°C. In all cases, after incubation with the secondary antibodies the slides were washed three times (5 min each) in PBS, then counterstained in 0.2 μg/ml DAPI (Boehringer) for 5 min. An FITC-conjugated serum of non-immunized rabbit (ChromoPure, Jackson ImmunoResearch) was used as the control. Some slides obtained by a modified air-drying technique were also exposed to immunolabeling, as described above, with the only difference being the counterstaining by Methyl green/DAPI.

Methyl green/DAPI staining

Chromosome preparations stained by this technique were obtained by the air-drying technique originally described by Odierna et al. (1993), modified in the handling of specimens during cell dissociation and the fixative used (3:1 methanol:acetic acid). Preparations obtained were stained in a solution of Methyl green (0.352 mg/ml in McIlvaine's buffer, pH 7.0) for 20 min at room temperature, rinsed twice in the same buffer, then stained in DAPI (Donlon and Magenis 1983).

Microscopy and image analysis

All preparations were examined with a Zeiss Axioplan microscope (equipped with an HBO 50 W mercury lamp for epifluorescence) and with a cooled charge-coupled device (Sensys). Grayscale digital images were collected separately with IP Lab Spectrum software (Signal Analitics), converted to Photoshop 3.0 format (Adobe), pseudocolored and merged. Final images were printed using a dye sublimation process.

Results

In male cells the two genomes of different parental origin are labeled at strikingly different levels by antibodies specific for the acetylated histone H4

The mealybug *P. citri* can be considered a paradigmatic case of genomic imprinting. In fact, in male embryos, early in development, one of the two genomes, invariably that of paternal origin, becomes heterochromatic and mainly inactive in most tissues (Nur 1966; Brown and Wiegmann 1969). Nonetheless, not all male cells have a heterochromatic genome as, after heterochromatization has taken place in all tissues, in some of them this same genome reverts to the euchromatic state. We investigated the possible involvement of histone H4 acetylation in the epigenetic control of the different chromatin conformations and activity states shown by the two genomes of different parental origin. We used indirect immunofluorescence with antibodies to histone H4 acetylated at all four acetylatable lysines. The accessibility of the heterochromatic paternal structures to antibodies was verified by indirect immunofluorescence with an antibody to non-acetylated histone H4 since in this case a similar reaction would be expected from the paternal and maternal genomes. In Fig. 2 it is clearly shown that anti-H4 antibodies can "penetrate" the heterochromatic genome of paternal origin as well as the euchromatic maternal one. The distribution of acetylated histone H4 on male cells, where the two genomes are distinguishable one from the other by their different levels of compaction, is shown in Fig. 3 (a, from embryo; b and c, from adults). In both cases the two genomes show quite different levels of histone H4 acetylation. The euchromatic maternally derived genome shows widespread fluorescence with a few spots of higher intensity, which are consistently more frequent in cells from embryos. The heterochromatic paternal genome, on the contrary, is in most cases devoid of signals, only rarely showing a few faintly fluorescent spots of immunolabeling. Histone H4 seems therefore to be acetylated in both genomes even though to quite different extents, the heterochromatic genome being much less acetylated.

We also analyzed and compared the distribution of acetylated histone H4 of male cells in which both genomes are euchromatic (Fig. 3d), and compared it with that shown by cells that had never undergone heterochromatization, i.e. female cells (Fig. 3e). Since the reversal of heterochromatization starts in male embryos when they

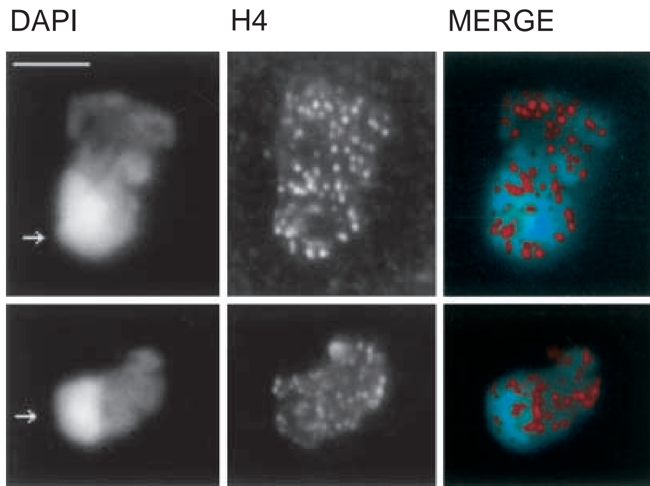

Fig. 2 Immunolabeling of interphase male cells by antibodies to histone H4. Immunofluorescent signals decorate to similar extents both the maternally derived euchromatic and the paternally derived heterochromatic genomes, indicating that the heterochromatic paternal structures are also capable of being reached by anti-H4 antibodies. *Arrows* indicate the heterochromatic paternal genome. *DAPI* 4′,6-diamidino-2-phenylindole staining. *Bar* represents 10 μm

are still in the ovary, female cells utilized for this analysis were from adult females after removal of the ovary. As can be seen, no fluorescence differences worthy of note between the cells of different origin were readily observed.

Exposure to the deacetylase inhibitor TSA leads to an increase in immunolabeling by antibodies specific for the acetylated histone H4 and affects chromatin compaction

The known relief of transcriptional repression in different organisms by the deacetylase inhibitor TSA (for review see Yoshida et al. 1995) prompted us to use TSA as a tool for verifying the actual involvement of histone H4 underacetylation in the inactivation of the paternal genome in *P. citri* males. We therefore analyzed the distribution of acetylated histone H4 in the two genomes of different parental origin of male cells after exposure to TSA. The results are shown in Fig. 3. In male cells in which the two genomes are euchromatic (Fig. 3i) and in female cells (Fig. 3l), an increase in both the number and intensity of fluorescent signals is apparent as compared with untreated cells (Fig. 3d, e, respectively). What is more, Fig. 3 also shows that in male cells, both from embryos (Fig. 3f) and adults Fig. 3g, h), in which the paternal genome is heterochromatic, the exposure to TSA has the effect of both enhancing the fluorescent signals on the euchromatic maternally derived chromosome set, and of producing, in most of these types of cells, fluorescent signals on the paternally derived genome also. Table 1 shows that the labeling increase after TSA treatment, while only reaching the limit of significance for the euchromatic genome, is highly significant ($P<0.001$) for the heterochromatic one. These results indicate that, although both genomes are partially underacetylated, the heterochromatic one is a much better substrate for deacetylating agents.

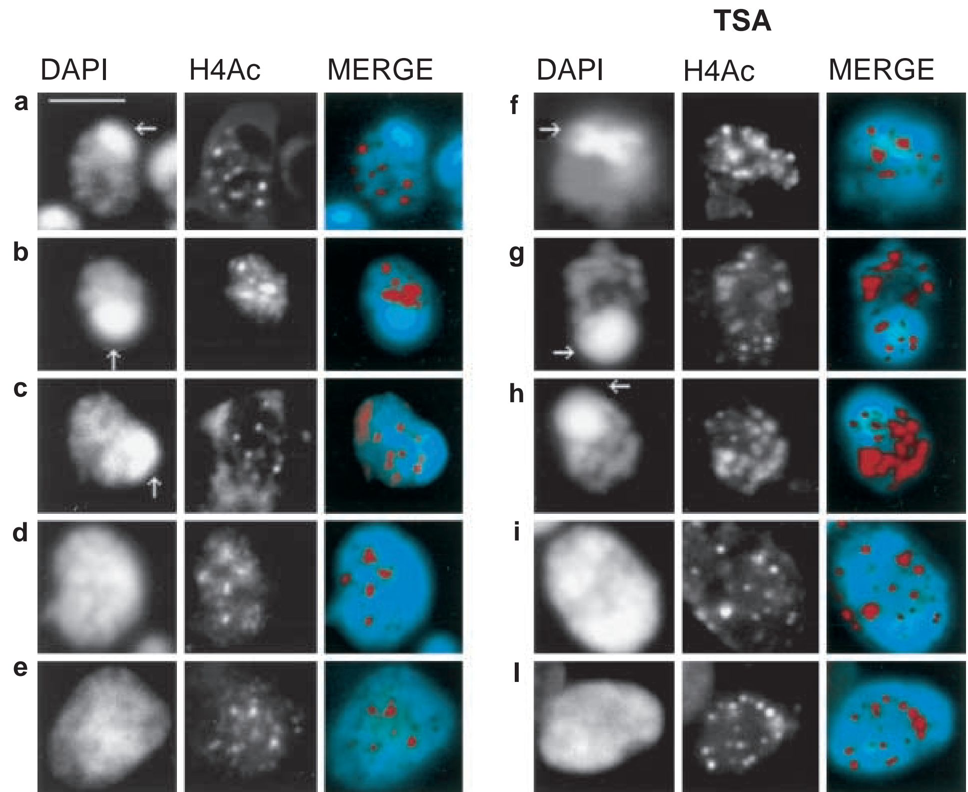

Fig. 3a–l Immunolabeling of interphase *P. citri* cells by antibodies to acetylated histone H4. **a–e** Cells without any treatment; **f–i, l** cells exposed to Trichostatin A (*TSA*). In untreated male cells in which the genome of paternal origin is heterochromatic (**a** from embryos, **b, c** from adults) the immunolabeling signals are only seldom observed on the heterochromatic genome (*arrowed*). After exposure to TSA this genome also is generally immunolabeled (**f** from embryos, **g, h** from adults). Male cells in which the heterochromatic paternal genome has reverted to the euchromatic state (**d**) show a distribution of labeling signals quite similar to that observed in “true” female cells, i.e. cells from females deprived of the ovary (**e**). Both types of cells show an increased number of immunolabeling signals after TSA treatment (**i** and **l**, respectively). *Bar* represents 10 μm

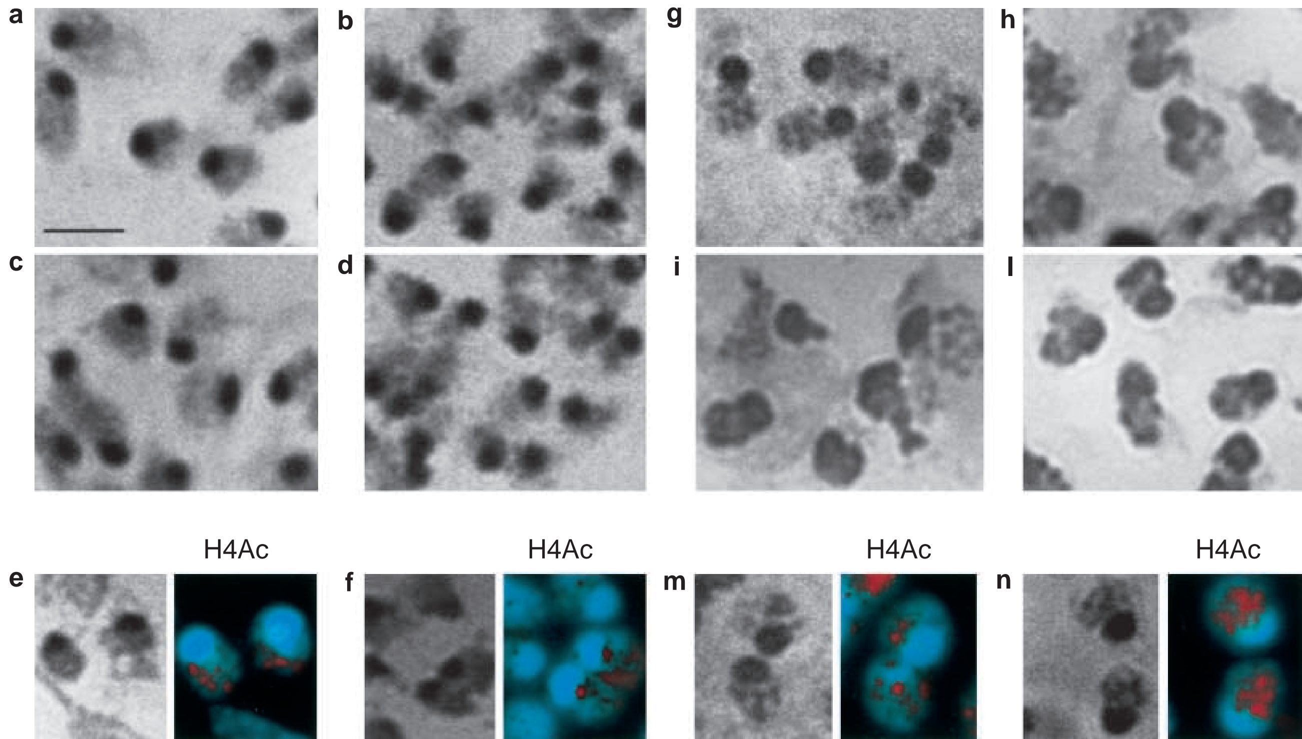

Fig. 4a–n Effect of TSA treatment on chromatin compaction. Giemsa staining of interphase male cells from control samples (**a–d**) and from samples exposed to TSA (**g–i, l**). In the lower part of the figure are shown two examples from controls (**e, f**) and from cells exposed to TSA (**m, n**) of the same cells stained by Giemsa after being immunolabeled and destained from DAPI. The decondensing effect of TSA is evident both for euchromatin and heterochromatin. *Bar* represents 10 μm

In order to check the possible link between the histone H4 acetylation level and the chromatin state of the paternal genome of male cells, we analyzed the effect of TSA treatment on chromatin compaction. Preparations from both TSA-exposed and control cells were analyzed after staining by Giemsa. Giemsa staining was chosen since it would allow us to detect compaction variations that otherwise would most probably be masked by the "spreading" effect of a fluorescent dye like DAPI. Figure 4(g–n) shows that, in male interphase cells, after TSA treatment, the staining intensities of both genomes, but particularly that of the paternal one, are fainter as compared with control cells (Fig. 4a–f). Finally, in Fig. 4(e, f, m, n) are reported two examples of cells photographed after immunolabeling, destained in 3:1 methanol:acetic acid, stained with Giemsa, and photographed again after relocation.

Taken together the results obtained with TSA treatment strengthen the likelihood of a role for histone H4 underacetylation in the epigenetic control of the imprinting of the inactive state of the genome of paternal origin in *P. citri* males, evidenced at the cytological level by its heterochromatic conformation.

Acetylation of histone H4 is maintained by metaphase chromatin

Cell memory mediated by chromatin structure implies that any given chromatin-based information must be retained throughout the cell cycle. Epigenetic modifications involved in this process (involving either DNA or proteins) should therefore be self-templating and maintained by metaphase chromatin. We extended the analysis of acetylated histone H4 distribution to metaphase chromosomes, looking for the possible presence of his-

Table 1 Effect of Trichostatin A (TSA) on euchromatic and heterochromatic genomes of male cells, measured by comparing the number of immunofluorescent spots in treated (+TSA, n=28)) and untreated cells (Controls, n=30)

	Total	Euchromatic	Significance	Heterochromatic	Significance
Controls	322	297		25	
+TSA	507	375	$0.02<P<0.05$*	132	$P<0.001$*

*Chi-square analysis

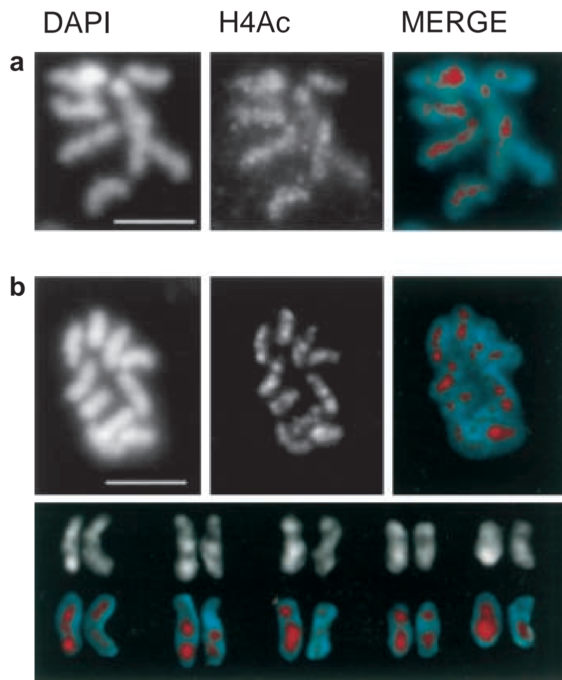

Fig. 5a, b Metaphase spreads, obtained from ovaries of gravid females, immunolabeled by antibodies to acetylated histone H4. After DAPI staining all the chromosomes show quite a similar level of fluorescence and immunofluorescent signals are present on all of them. These signals, however, are more often evenly distributed along the chromosome length (**a**) or sometimes show a more band-like distribution (**b**). From this last type of metaphase it was sometimes possible to arrange immunolabeled chromosomes in pairs in which it is apparent that one of the two homologs is more strongly labeled. This is shown for the metaphase in **b** (*lower row*). *Bar* represents 10 µm

tone H4 acetylation at this level. The best technique for obtaining a consistently high number of good-quality metaphase spreads with *P. citri* is the air-drying technique. Unfortunately, the manipulation rather than the fixation procedures of this technique do not allow it to be used in making preparations for immunolabeling. As for males, in particular, their very short life span as adults, only a couple of days, is also a possible limiting factor as high cell proliferative activity in their tissues may not be necessary. For all these reasons, even by squashing we have been unable to obtain metaphase spreads from adult males in sufficient amounts to be analyzed separately. We therefore analyzed metaphase spreads on preparations obtained by squashing from ovaries of gravid females, well aware that we were dealing with both male and female metaphase cells. Immunolabeling fluorescent signals were consistently observed on metaphase spreads (more than 60 metaphases analyzed) more or less evenly distributed along the chromosome length (Fig. 5a), or showing a more discrete, band-like, distribution (Fig. 5b). Interestingly, even if after DAPI staining all chromosomes showed quite a similar intensity of fluorescence, it was sometimes possible from this last kind of metaphase, actually only seldom observed, to make a “karyotype” where in each pair of homologs one of the two chromosomes was clearly more labeled than the other (Fig. 5b, lower row).

Staining by Methyl green/DAPI reveals two subsets of metaphases whose chromosomes are differentially labeled by antibodies to acetylated histone H4

Methyl green is a non-fluorescent DNA ligand with AT base-pair specificity that has been demonstrated as identifying specific chromosomal regions when used in combination with DAPI (Donlon and Magenis 1983). Based on several considerations Donlon and Magenis speculated that the technique differentiates chromosomes on the basis of DNA conformation in addition to base-pair specificity. We checked the possible presence of metaphase spreads in which all the chromosomes, not differing on any other morphological grounds, showed different staining levels with the Methyl green/DAPI technique. The preparations used for this analysis were obtained, by slightly modifying the usual air-drying technique, from ovaries of gravid females, allowing us to analyze numerous, good-quality metaphase spreads. Actually, two subsets of metaphases where all the chromosomes had the same degree of compaction were found, either with all chromosomes equally stained (Fig. 6a) or with five chromosomes less intensely stained (Fig. 6g), thus witnessing the likely presence, in some metaphases, of two chromosome sets in different chromatin conformations. Nevertheless we cannot say whether those cells are from male or female embryos. We would like to note that the present finding is consistent with our previous observations (Ferraro et al. 1998) of different staining intensities shown by homologous chromosomes of *P. citri* after staining with a different compound having affinity for AT-rich DNA (D287/170) that is also known to recognize different classes of heterochromatin (Sumner 1990 and references cited therein).

Figure 6 also reports some examples of the two different types of metaphases, counterstained by Methyl green/DAPI after immunolabeling with anti-acetylated histone H4 antibodies. Due possibly just to the technique used, immunolabeling is of a lower quality as compared with that obtained on squashed preparations. Nonetheless, it can be seen that in metaphases where all the chromosomes are equally fluorescently stained by Methyl green/DAPI, all the chromosomes have immunolabeling spots (Fig. 6d, f). On the contrary, in metaphases where five chromosomes are less intensely stained, in general these chromosomes are the ones to be immunolabeled (Fig. 6l, n). Based on the fact that Methyl green/DAPI staining reveals different chromatin conformations, and that a general characteristic of heterochromatin is that of being more intensely stained as compared with euchromatin because of its higher compaction, in our opinion these cells could indeed represent male cells. The more fluorescently stained (i.e. more condensed) chromosomes of these metaphases could represent the hetero-

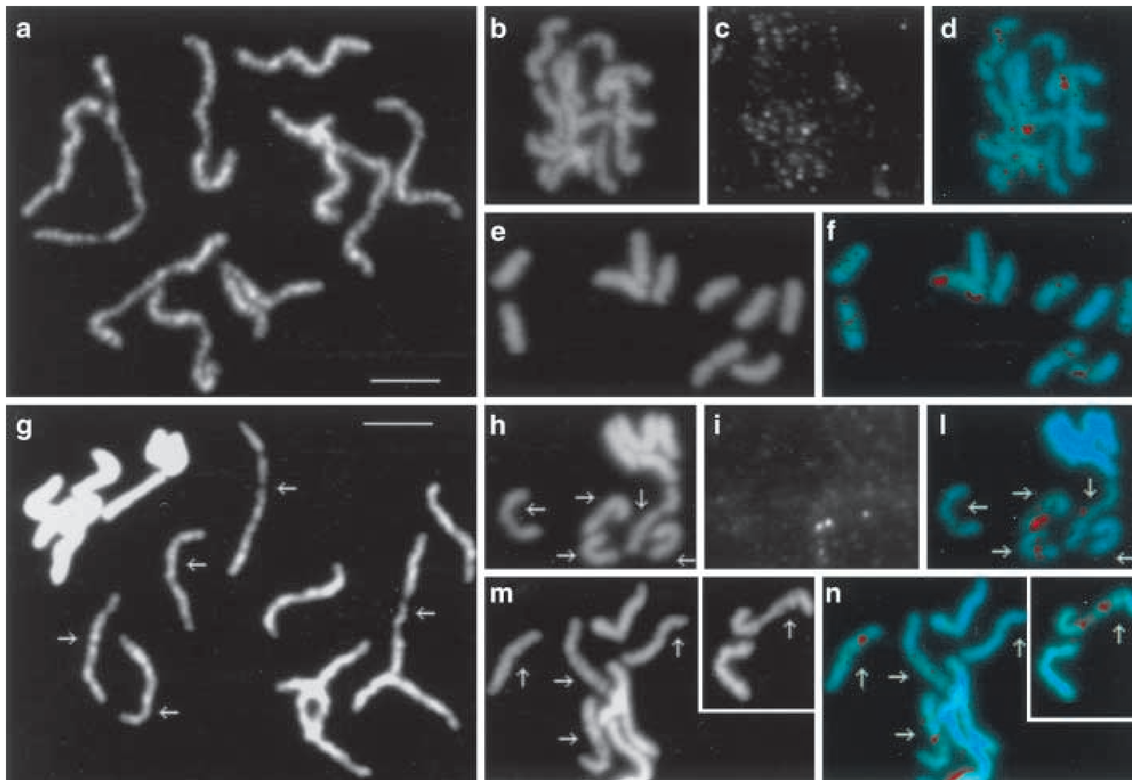

Fig. 6 Metaphase spreads, obtained from ovaries of gravid females, stained by Methyl green/DAPI only (**a, g**) or exposed to antibodies to acetylated histone H4 and counterstained by Methyl green/DAPI (**b–f, h–n**). Two types of metaphases are usually seen after Methyl green/DAPI staining: with all the chromosomes showing a similar level of intensity of fluorescence (**a, b, e**), or with five chromosomes less intensely stained (**g, h, m**; *arrows*). When exposed to antibodies to acetylated histone H4 the two types of metaphases show a different distribution of immunolabeling signals. In metaphases where all the chromosomes show the same intensity of fluorescence (**b, e**) all of them are decorated by immunolabeling signals. **d** and **f** are the merged images of metaphases **b** and **e** taken after pseudocoloring for Methyl green/DAPI (*blue*) and for acetylated H4 (*red*). Panels **h** and **m** show metaphases with five chromosomes less intensely stained (*arrowed*): these chromosomes are immunolabeled (**l, n**, *arrowed*) while the other five are usually devoid of signals. Pseudocoloring of **l** and **n** is as reported for **d** and **f**. In **c** and **i** is shown the immunolabeling obtained for metaphases shown in **b** and **h**, respectively. *Bar* represents 10 μm

chromatic paternal genome of interphase cells, in which less acetylation also resulted.

We would also like to note that Methyl green/DAPI staining results in a karyotype of *P. citri* very similar to that obtained after D287/170 staining (not shown).

Discussion

The modification of chromatin-related structural proteins is considered a way of controlling transcriptional activity. Histones undergo a variety of posttranslational modifications. In particular, histone acetylation, which is found throughout eukaryotes, from unicellular organisms to the most complex of higher plants and animals (for review see Jeppesen 1997), has been given particular attention. In fact, since its discovery more than three decades ago (Allfrey et al. 1964) ample evidence supporting the model that hyperacetylated chromatin is transcriptionally more active than hypoacetylated chromatin has been obtained at the cytological, genetic and biochemical levels (Durrin et al. 1991; Turner et al. 1992; Braunstein et al. 1993).

Planococcus citri is a mealybug where males are characterized by early heterochromatization of the genome of paternal origin (Brown and Nelson-Rees 1961). The assumption of the heterochromatic state brings about almost complete inactivation of this genome, as was later demonstrated by genetic analysis (Brown and Wiegmann 1969). Here we report data indicating the possible involvement of histone H4 acetylation in the control of the different activity states of the two genomes of different parental origin, evidenced at the cytological level by different chromatin states.

In male interphase cells where the two genomes are easily distinguishable by their different degrees of compaction, immunolabeling with antibodies specific for acetylated histone H4 indicates that the heterochromatic, mainly inactive, genome of paternal origin is clearly hypoacetylated with respect to the euchromatic, active, maternally derived one.

The possible involvement of histone H4 underacetylation in the transcriptional repression (through heterochromatization) of the paternal genome of *P. citri* males is strengthened by the results obtained from the experiments with the histone deacetylase inhibitor TSA. Actu-

ally, the exposure, before fixation, of ovaries dissected from gravid females to this microbial metabolite affects both the reactivity of the genome of paternal origin to antibodies to acetylated histone H4 (enhancing it) and its chromatin compaction (reducing it). It is worth remembering that, for *P. citri*, chromatin compaction has been the parameter used to judge the different activity states of the two genomes of different parental origin when distinguishable from each other.

The existence of multiprotein complexes that mediate changes in chromatin structure is supported by both genetic and biochemical data (for review see Kingstone et al. 1996). Moreover, it has recently been reported that treatment with TSA leads to an alteration of local chromatin structure (Minucci et al. 1997). The appearance, in male cells of *P. citri*, of a greater number of immunolabeling signals, together with modification of compaction of the heterochromatic genome of paternal origin once the cells are exposed to TSA therefore suggests that the heterochromatization-inactivation of this genome might indeed be mediated by the modification of a class of chromatin-related structural proteins such as histones.

In this context, one of the main questions is how chromatin-encoded information could be passed to progeny cells through metaphase. Several models have been proposed. One of these models entails an interaction between separated multipartite elements before they lose their epigenetically modified structure at the replication fork passage. Through a "looping-back" mechanism they would function as seeding elements for the reestablishment of the former chromatin state (Wolffe 1994). According to this model, one or more nucleoprotein complexes must be retained by metaphase chromosomes. Actually, protein-dependent conformational perturbations have been shown to persist at transcription start sites during mitosis in genes scheduled for reactivation, while these do not occur in repressed genes (Michelotti et al. 1997). The immunolabeling signals found along *P. citri* metaphase chromosomes provide evidence for the persistence, at metaphase, of DNA regions with modified H4 histones, indicative of a specific chromatin structure. These findings support the idea of the inheritance of information on a given activity state determined by chromatin organization, as well as that on a given chromatin state that may be self-templating.

As for metaphases showing homologous chromosomes with different immunolabeling intensities, it is tempting to speculate that metaphases where the two homologs of each pair show different levels of histone H4 acetylation could represent male cells where the two genomes of different parental origin are differentially active. Our conjecture is strengthened by data obtained by Methyl green/DAPI staining per se, showing the existence of metaphases where the two genomes are in different chromatin conformations. What is more, the two genomes also show that they belong to two differentially acetylated chromatin compartements, the less fluorescently stained, i.e. less condensed, chromosomes being the ones showing immunolabeling signals.

To summarize, our data show that in *P. citri* males the heterochromatic genome of paternal origin is underacetylated at histone H4 relative to the euchromatic maternally derived one. We know that after heterochromatization the paternal genome is mainly inactive (Brown and Wiegmann 1969). However, as the phenomenon of heterochromatization is the first sign that an embryo is male, we were unable to analyze the histone H4 acetylation level of the paternal genome before its heterochromatization. Nevertheless, simultaneous modification of the histone H4 acetylation level and of chromatin compaction was observed following TSA treatment. Moreover, when five out of the ten chromosomes of the karyotype stain differently with a compound thought to differentiate chromosomes on the basis of DNA conformation in addition to base-pair specificity, the five faintly staining chromosomes show immunolabeling by anti-acetylated histone H4, while the other five do not.

In conclusion, in our opinion the data reported in this paper support the idea that histone H4 acetylation is involved in the control, through the chromatin state, of the transcriptional activity, and, possibly, the imprinting of the paternal genome in *P. citri* males.

Finally, our data confirm that, in organisms lacking DNA methylation, histone H4 acetylation can well be regarded as representing an alternative mechanism for propagating the "cell memory" that is necessary for any type of epigenetic control of gene expression.

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