Facultative heterochromatization in parahaploid male mealybugs: involvement of a heterochromatin-associated protein

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SUMMARY

The behavior of chromosomes during development of the mealybug Planococcus citri provides one of the most dramatic examples of facultative heterochromatization. In male embryos, the entire haploid paternal chromosome set becomes heterochromatic at mid-cleavage. Male mealybugs are thus functionally haploid, owing to heterochromatization (parahaploidy). To understand the mechanisms underlying facultative heterochromatization in male mealybugs, we have investigated the possible involvement of an HP-1-like protein in this process. HP-1 is a conserved, nonhistone chromosomal protein with a proposed role in heterochromatinization in other species. It was first identified in Drosophila melanogaster as a protein enriched in the constitutive heterochromatin of polytene chromosome. Using a monoclonal antibody raised against the Drosophila HP-1 in immunoblot and immunocytological experiments, we provide evidence for the presence of an HP-1-like in Planococcus citri males and females. In males, the HP-1-like protein is preferentially associated with the male-specific heterochromatin. In the developing male embryos, its appearance precedes the onset of heterochromatization. In females, the HP-1-like protein displays a scattered but reproducible localization pattern along chromosomes. The results indicate a role for an HP-1-like protein in the facultative heterochromatization process.

Key words: Facultative heterochromatization, HP-1, Mealybug

INTRODUCTION

In the Lecanoid chromosome system of Coccids, or mealybugs, two important genetic phenomena are widely expressed: facultative heterochromatization and chromosome imprinting. The classical cytogenetic descriptions of Coccids (Hughes-Schrader, 1948; Brown and Nur, 1964) showed that both of these chromosomal processes are developmentally regulated. In the Lecanoid system, chromosomes are holocentric and there are no sex chromosomes. All embryos start development from fertilized eggs and both males and females begin with diploid euchromatic chromosome complements. The paternal and maternal chromosome sets remain euchromatic throughout ontogeny of females in the soma and the germline (Brown and Nur, 1964). By contrast, in embryos that are to become male, the entire paternal derived haploid chromosome set becomes heterochromatic at mid-cleavage. In subsequent cell divisions, the derivatives of the paternal chromosome set appear as chromatcenters in interphase nuclei in most tissues. The heterochromatic set retains the cytological properties of condensed metaphase chromosomes throughout the cell cycle. It is distinguishable from the euchromatic one until metaphase, when both sets reach the same degree of condensation. During spermatogenesis, the heterochromatic set is eliminated and only the maternally derived euchromatic chromosomes are included in the sperm. The inactivation of the haploid paternal set of chromosomes represents a dramatic example of facultative heterochromatization that renders male individuals functionally haploid (parahaploidy). Moreover it is evident that there is a mechanism for distinguishing between chromosome sets of maternal and paternal origins (genomic imprinting) in the developing male embryo (Nur, 1990; Bongiorni et al., 1999).

Our previous work on imprinting in Coccids (Bongiorni et al., 1999) suggested that the paternal and maternal chromosome sets are characterized by different levels of CpG methylation in embryos. Specifically, we provided cytological evidence for hypomethylation of the paternal set at a whole chromosomal level which might serve as a distinguishing mark of parental origin but it does not reflect overall transcriptional activity of the chromosome sets. We now further investigate the underlying molecular basis for imprinting by asking whether a chromosomal protein, HP-1, which is implicated in heterochromatization in other systems, is involved in imprinting and facultative heterochromatization in Coccids.

In 1928, Heitz defined the heterochromatin as regions of chromosomes that do not undergo cyclical changes in condensation during cell cycle as the other chromosome regions (euchromatin) do (Heitz, 1928). The condensed appearance of heterochromatin in higher eukaryotes is thought to be due to a specialized nucleoprotein structure. Thus far,
the best characterized candidate proteins involved in heterochromatization belong to a family of proteins containing a conserved motif known as the chromo domain (Singh et al., 1991). The founding member of this family is the Drosophila melanogaster HP-1 (Heterochromatic Protein 1), a non-histone chromosomal protein that binds preferentially to constitutive heterochromatin on polytene chromosomes (James and Elgin, 1986; James et al., 1989). HP-1 is a dose sensitive modifier of position effect variegation (Eisenberg et al., 1990). It is also required for the proper segregation of chromosomes in embryos (Kellum and Alberts, 1995), and has a telomere capping function that prevent telomere fusions (Fanti et al., 1998b).

Orthologs of Drosophila HP-1 have been found in many species, including mammals (Eisenberg and Elgin, 2000). HP-1-like proteins have two regions that show a high degree of amino acid sequence conservation (Singh et al., 1991; Clark and Elgin, 1992; Epstein et al., 1992). One region, termed chromo shadow domain, is in the C-terminal half of HP-1 and is necessary for nuclear targeting and heterochromatin localization (Powers and Eisenberg, 1993; Platero et al., 1995). The other highly conserved region is found within the N-terminal half of HP-1. This is the conserved, ~46 amino acid region called the chromo domain, whose amino acid sequence shares ~60% sequence identity with a motif found in the Polycomb protein, a silencer of homeotic genes in Drosophila melanogaster (Paro and Hogness, 1991). HP-1 chromo domain is sufficient for heterochromatin targeting (Platero et al., 1995). However, the intact protein is required for HP-1 binding to DNA in vitro (Zhao et al., 2000).

In 1992, Epstein and collaborators screened a cDNA library from the Coccid Planococcus citri, with a DNA probe corresponding to the Drosophila HP-1 chromo domain (Epstein et al., 1992). They isolated and sequenced two chromo domain containing sequences from P. citri, with 69.5% and 57% homology at the amino acid level to the Drosophila HP-1 chromo domain. The two sequences were called pchet1 and pchet2 for putative Coccid heterochromatin proteins 1 and 2 respectively. The authors produced polyclonal antibodies against the entire pchet1 protein expressed in bacteria. From immunoblotting analyses, PCHET1 appeared to be sex limited to males. However, using immunolocalization, the authors could not find a correlation between the distribution of anti-PCHET1 antibody signals and the heterochromatic chromosomes in male cells. They concluded that PCHET1 contained a chromodomain related to HP-1, but that it may differ from the Drosophila HP-1 in function (Epstein et al., 1992).

To further investigate the possible role of an HP-1 like protein in chromosome imprinting and heterochromatization in the mealybug, we have used the C1A9 monoclonal antibody raised against the Drosophila melanogaster HP-1 (James and Elgin, 1986), to probe the chromosomes of the mealybug Planococcus citri (Homoptera, Coccoidea). The results of western blotting and immunolocalization studies identified a cross-reactive protein whose properties suggest that a homolog of Drosophila HP-1 is present in this species. Moreover, by analyzing the distribution of this HP-1-like protein in male and female cells during the cell cycle and in the early embryogenesis, we show that this protein co-localizes with male specific heterochromatin. Our results suggest that an HP-1-like protein plays a role in the process of facultative heterochromatization in Coccids.

MATERIALS AND METHODS

Mealybug cultures

Planococcus citri (Homoptera, Coccoidea) were raised in our laboratory on sprouting potatoes at 26°C inside glass food containers covered with gauze. The potatoes were kept in the dark to sprout for 1 month before use.

Chromosome and whole embryo preparations

Chromosome spreads were obtained from embryos as previously described (Bongiorni et al., 1999). Gravid females were dissected and maintained in a solution of 0.8% sodium citrate. The embryos were then transferred to minitubes and centrifuged at 550 g for 2 minutes. Bradford-Carnoy fixative (chloroform:ethanol:acetic acid, 4:3:1) was added to the pellet. The pellet was dispersed by repeated passages through a 26 gage hypodermic needle attached to a 1 ml syringe until it was thoroughly homogenized. The suspension was centrifuged at 1250 g for 8 minutes. After resuspending the pellet in methanol:acetic acid (3:1), 60 µl of cellular suspension was dropped onto clean slides and air dried. Whole embryos were prepared as above, but the pellet after the first centrifugation was only gently dispersed. A gravid female contained eggs at different developmental stages (from unfertilized eggs to gastrula embryos). We staged the embryos by counting the number of cells they contained.

In situ HP-1 immunolocalization

Immediately after preparation, slides were washed three times in 1× phosphate-buffered saline (PBS) (10 minutes each), treated in 1× PBS + 1% Triton X-100 (10 minutes at room temperature) and washed again three times in 1× PBS. Blocking was performed with 3% nonfat dried milk in 1× PBS for 30 minutes. Slides were incubated overnight, at 4°C, with mouse anti-HP-1 antibody), diluted 1:10 in 1× PBS + 0.1% bovine serum albumin (BSA). The anti-HP-1 monoclonal antibody C1A9 was isolated by James and Elgin (James and Elgin, 1986) and provided by B. Wakimoto. After washing three times in 1× PBS, slides were incubated with the secondary antibody, diluted 1:100 in 1× PBS + 0.1% BSA for 1 hour, at room temperature. The secondary antibody was Alexa™ 488 goat anti-mouse IgG (H+L)-conjugated (Molecular Probes, Eugene, OR). After incubation the slides were washed three times in 1× PBS and counterstained with 0.2 µg/ml DAPI (4’,6-diamidino-2-phenilindole, Boehringer, Mannheim) in 2× saline sodium citrate (SSC) for 5 minutes. Finally, the slides were mounted in antifade medium (DABCO (Sigma, Milano) 23.3 mg/ml, 10 mM TRIS-HCl pH 7.5-8, 90% sterile glycerol). Negative controls were obtained by incubating slides with the secondary antibody only.

C-banding

Slides were incubated in 0.2 N HCl for 30 minutes, then washed in H2O and treated with a saturated solution of Ba(OH)2 for 15 minutes. After washing three times in 1× PBS and counterstained with 0.2 µg/ml DAPI (4’,6-diamidino-2-phenilindole, Boehringer, Mannheim) in 2× saline sodium citrate (SSC) for 5 minutes. Finally, the slides were mounted in antifade medium (DABCO (Sigma, Milano) 23.3 mg/ml, 10 mM TRIS-HCl pH 7.5-8, 90% sterile glycerol). Negative controls were obtained by incubating slides with the secondary antibody only.

Fluorescence microscopy

Immunofluorescent preparations were observed with a Zeiss Axioskop 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 Alexa™ 488 (450-490 nm excitation range). Fluorescent images were captured with a CCD camera (series 200; Photometrics, Tucson, AZ) using IPLab software (Signal Analytic, Vienna, VA) and processed with a Power Mac G4 (Apple, Cupertino, CA) using Adobe Photoshop software (Adobe Systems, Mountain View, CA).
Western blot analysis
Whole protein extracts were prepared from 25–30 fertilized adult females of Planococcus citri according to the methods of Platero et al. (Platero et al., 1995). Proteins were loaded onto 12% SDS-polyacrylamide gel and run at 60 mA for 4 hours, by using the Mini-Protein II system (BioRad, Richmond, CA). Proteins were electrically transferred to a nitrocellulose membrane at 280 mA for 1 hour, by using a semidy electroblotting apparatus (BioRad). After transfer, the filter was blocked overnight at 4°C with 5% nonfat dried milk in TBST (0.05% Tween 20 + 1× TBS (10 mM Tris-HCl, pH 8.0, 150 mM NaCl)). The filter was incubated with mouse anti-HP-1 antibody (C1A9 monoclonal), 1:500 in TBST, for 1 hour at room temperature, washed in TBST three times for 15 minutes each and then incubated 1 hour with a 1:2000 dilution in TBST of goat anti-mouse IgG-HRP (horseradish peroxidase)-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). After three additional washes of 15 minutes each in TBST, the filter was incubated with the luminol reagent (Santa Cruz Biotechnology). Chemiluminescent bands were detected by exposing the filter to an X-ray film (Kodak, Rochester, NY) for times ranging from 1 to 5 minutes, Cruz markers (Santa Cruz Biotechnology) were used as internal molecular weight standards. Negative controls were performed by incubating protein samples with secondary antibody only.

RESULTS

Immunoblotting detection of a Planococcus citri protein by an anti-HP-1 antibody
As preliminary step, we used an antibody that recognizes the Drosophila HP-1 protein to assess whether the mealybug Planococcus citri might contain a cross-reactive protein species. Accordingly, western blot analysis was performed on whole extracts of fertilized adult females of Planococcus citri. Adult gravid females contain about 400 embryos of both sexes. Protein samples were probed with the mouse monoclonal anti-HP-1 antibody C1A9 raised against Drosophila melanogaster HP-1 (James and Elgin, 1986).

In the lane containing protein extracts from Drosophila melanogaster, the HP-1 antibody recognized an antigen of 34 kDa as expected from previous studies (James and Elgin, 1986). In the lanes containing P. citri extracts, two antigens were detected, one migrating at 29 kDa and the other at 46 kDa. We attribute the detection of the 46 kDa species to reactivity with the secondary antibody, rather than the anti-HP-1 antibody, as it is detected on western blots probed with the secondary antibody alone (Fig. 1, lane 6). This reactivity was absent in immunocytochemical experiments, in which secondary antibody alone never produced fluorescent labeling over nuclei (Fig. 1B). From these data, we conclude that the HP-1 antibody recognizes a single cross-reactive protein band in P. citri. Its apparent molecular weight of 29 kDa is only indicative because it is known that the D. melanogaster HP-1, like other chromosomal proteins, migrates aberrantly on SDS-polyacrylamide gels that use discontinuous buffer system (James and Elgin, 1986).

Chromosomal localization of a P. citri antigen by the anti-HP-1 antibody in male and female cells
To determine if the anti-HP-1 reactive signal we detected in P. citri extracts by western blotting might correspond to an HP-1-like protein, we used the monoclonal anti-HP-1 antibody on cytological preparations. Cellular suspensions of embryonic tissues were prepared by dissecting gravid females. Both male and female embryonic tissues were simultaneously present in the same preparation (see Materials and Methods), allowing comparisons within the same experiment. Male and female tissue patches can be easily distinguished after DAPI staining (Fig. 2). In DAPI-stained male cells, the heterochromatic haploid set formed a conspicuous, brightly stained chromocenter in interphase nuclei (Fig. 2B,C). In some cases, the chromocenter appeared split in two masses (Fig. 2B). The morphological distinction between the two sets is still apparent at early and mid-metaphase (Fig. 3B), but at late metaphase both euchromatic and heterochromatic chromosomes reach the same degree of condensation (Fig. 3C).

In female cells, the HP-1 antibody detected a nuclear antigen that was widespread in all interphase nuclei (Fig. 2A). In male cells, the antigen was strictly associated with male-specific heterochromatin, forming a conspicuous fluorescent mass that co-localizes with the chromocenter (Fig. 2B,C). The HP-1 antibody signal and the DAPI-bright chromocenter are overlapping, even though not fully co-extensive. In male nuclei, fluorescent spots were only rarely seen over the euchromatin.

In condensed mitotic chromosomes of females, the anti-HP-1 fluorescent signals were scattered along all of the chromosomes (Fig. 3A). The analysis of the HP-1 antibody distribution revealed a reproducible banding pattern. Bright staining was often observed at subtelomeric regions of the chromosome. Remarkably, the HP-1 staining patterns allowed us to identify homologous pairs of chromosome and construct...
a karyotype for the five pairs of euchromatic chromosomes (Fig. 4).

In male cells, the co-localization of the HP-1 antibody signal and the male-specific heterochromatin persists throughout the entire cell cycle. At early metaphase, the HP-1 signal was present only on the five heterochromatic chromosomes (Fig. 3B). This exclusive heterochromatic localization was maintained even when the five euchromatic chromosomes reached a high degree of condensation and the differentiation between heterochromatic and euchromatic chromosomes by DAPI staining alone was less apparent (Fig. 3C).

C-banding is a chromosome banding technique that is commonly used to define constitutive heterochromatin (Sumner, 1972). To determine if the intense HP-1-binding sites we observed on the chromosomes from female cells corresponded to constitutive heterochromatin, we prepared chromosomes for simultaneous HP-1 immunolocalization and C-banding. As shown in Fig. 5, some HP-1 sites and C bands overlapped but many did not. Moreover, even within the regions of overlapping, the HP-1 signal and the C-band are only rarely co-extensive. HP-1, in fact, often occupies only a small portion of the C-band. This analysis is hampered by the fluctuant behavior of C-bands in holocentric chromosomes, by which splitting and fusion of interspersed C-bands are frequently observed (Collet and Westerman, 1984; Manicardi et al., 1996). Moreover, holocentric chromosomes lack any cytological landmarks such as the primary constriction. However, from the analysis of 30 female metaphases we concluded that there was not a strict correlation between HP-1 localization and constitutive heterochromatin, as defined by C-banding.

We infer from these immunolocalization studies that the P. citri epitope recognized by the HP-1 antibody has features expected of an HP-1-like protein. These features include a chromosomal localization and, in male chromosomes, a specific targeting to heterochromatin. In the case of P. citri, the
heterochromatic localization of the HP-1-like epitope corresponds to facultative heterochromatin.

**Developmental profile of HP-1 staining and facultative heterochromatization in male embryos**

The results above show a striking correlation between chromosomal regions showing HP-1 staining and those that undergo facultative heterochromatization in male embryos. To examine if HP-1 staining is coincident with the process of heterochromatization of the paternal chromosome set in males, or whether it occurs well before or after, we compared the HP-1 immunoreactivity of different developmental stages and tissues.

Schrader (Schrader, 1923) suggested from his studies that the paternal chromosome set becomes heterochromatic during blastoderm formation, after fifth-cleavage nuclei migrate from the center of the egg to the surface. However, we did not observe heterochromatization or HP-1 staining in nuclei of embryos before the seventh cleavage division. At these early stages, the male and female embryos could not be discriminated. However, during the seventh cleavage division, some embryos exhibited a very faint differentiation of chromatin mass in a few of their nuclei. In these nuclei, small DAPI-positive spots were visible, generally at the edge of the nuclei (Fig. 6, insert). These DAPI-positive spots are also positive for HP-1 antibody staining. After the completion of the seventh nuclear division, two classes of embryos could be scored: embryos with a fractions of nuclei showing a chromocenter, and embryos with all nuclei lacking a chromocenter (Fig. 7). We assumed the first class represented male embryos, and the second one female embryos. We observed that heterochromatization does not simultaneously occur in all nuclei of a male embryo, but instead, it occurs in a wave from one pole of the embryo toward the other. Thus, in blastoderm stage male embryos (at the 128 or 256 nuclei stage) it is possible to observe nuclei still lacking a chromocenter and patches of nuclei with a fully developed chromocenter (Fig. 7, left). Both categories of nuclei showed the HP-1 antibody signal. HP-1 staining colocalized with the chromocenter in heterochromatin-positive nuclei (Fig. 7B) and, interestingly, it often formed conspicuous, intense spots even in nuclei with no cytologically apparent heterochromatin (Fig. 7A). Female embryos at this stage also have nuclei that stain with the HP-1 antibody, but the signal is scattered over the chromatin and never formed large spots (Fig. 7, right). In older male embryos, when all the nuclei had undergone heterochromatization, HP-1-staining is present in all nuclei colocalized with the chromocenter (Figs 8, 9).

We also examined HP-1 staining during blastoderm formation. During this process, some nuclei at the surface of the egg are destined to become the outer embryonic membrane, the serosa (Nur, 1967; this paper). The future serosa cells are identified by their large nuclei and are situated about midway between the anterior and posterior poles (Fig. 8, DAPI staining). Later, in an advanced blastoderm stage, these cells move together along the surface of the egg toward the anterior pole to form a cap over that pole (Nur, 1967; present paper Fig. 9, DAPI staining). At the time of gastrulation, the serosa expands and covers the entire surface of the embryo (Fig. 10, DAPI staining).
In *P. citri* and several other species of Coccids, heterochromatinization of the serosa cells is delayed relative to other cells in the embryo. This difference is clear at the mid-cleavage state where facultative heterochromatin is cytologically visible in many male blastoderm nuclei but not in the serosa nuclei (Fig. 8). We used CIA9 antibody to ask if the cell type differences could also be observed with HP-1 immunoreactivity. As shown in Fig. 8, in all nuclei of the embryonic body that had already heterochromatized, the HP-1 antibody signal was present on the chromocenter. Conversely, at this stage the serosa nuclei lacked both the chromocenter and the HP-1 antibody signal (Fig. 8). In later male embryos (Fig. 9), a bright HP-1 antibody signal appeared in the serosa nuclei and colocalized with a differentiated chromatin mass not yet showing the characteristic, bright DAPI-staining of the chromocenter (Fig. 9B).

During gastrulation, the heterochromatization process was completed in all cells, including the serosa cells. At this stage, the HP-1 staining colocalizes with a bright DAPI-stained chromocenter (Fig. 10).

Taken together, these data indicate that the appearance of the HP-1 epitope in male embryos precedes the appearance of a cytologically differentiated chromocenter.

**DISCUSSION**

The study of heterochromatization in mealybugs began in 1921 with Schrader’s pioneering work (Schrader, 1921), and was extended by the work of Hughes-Schrader. These early studies led to a cytological description of the lecanoid chromosome
cycle (Hughes-Schrader, 1948) and a clear demonstration that facultative heterochromatization occurs and is developmentally significant in mealybugs. Unfortunately, further studies of the underlying mechanisms of this interesting chromosomal phenomenon have been hampered by the lack of molecular and genetic probes. To overcome this limitation, we took advantage of a tool commonly used to study heterochromatin in *Drosophila* and applied it to the Coccid system. This approach allowed us to investigate possible parallels between facultative heterochromatization in Coccids and constitutive heterochromatization in *Drosophila melanogaster*.

Following a similar rationale, Epstein and collaborators (Epstein et al., 1992) isolated two *P. citri* genes closely related to *Hp-1*. As mentioned in the introduction, these authors raised an antibody against the product of one of these genes, *pchet1*. However, the antibody pattern did not coincide with that of male-specific heterochromatin. Our results differ from those of Epstein et al., in showing that mealybugs contain a protein that contains an HP-1 cross-reactive epitope and is closely associated with the male-specific heterochromatin. We also showed that this protein is present in female cell nuclei. In our experiments, the simultaneous presence of both male and female tissue patches in the same cytological preparation eliminated the possibility of differential staining, as caused by fixation artifacts. Although our fixation procedures differed from those used by Epstein et al., we think that the sum total of differences make it unlikely that the protein we detected with the Drosophila HP-1 antibody corresponds to PCHET1. Whether the HP-1-like protein we describe corresponds to PCHET2 cannot be determined without further definitive identification and localization of PCHET2 (Epstein et al., 1992).

We show that the monoclonal antibody C1A9, which is specific to the *Drosophila melanogaster* heterochromatin-specific protein HP1 (James and Elgin, 1986), can be used as an effective tool with which to study the switch from the euchromatic to the heterochromatic state in Coccids. We first showed by immunoblotting that the monoclonal antibody raised against the *D. melanogaster* HP-1 (HP-1 antibody)
recognizes a cross-reactive protein band in *P. citri* protein extracts with an apparent molecular weight of 29 kDa. This indicates that *P. citri* contains at least one species of protein that shares the epitope recognized by the monoclonal C1A9 antibody.

Our immunolocalization studies provide compelling evidence that the *P. citri* epitope recognized by the antibody directed against *Drosophila* HP-1 is an HP-1-like protein. The similarities between the Coccid protein containing the anti-HP-1 reactive epitope and *Drosophila* HP-1 include a chromosomal localization, with preferential binding to domains including telomeres, certain euchromatic regions, and most strikingly to heterochromatin. All of these localizations are apparent in the staining of polytene or mitotic chromosomes of *Drosophila* (James and Elgin, 1986; James et al., 1989; Fantini et al., 1998a; Fantini et al., 1998b). Interestingly and most importantly, in the case of *P. citri*, the heterochromatic region corresponds to facultative heterochromatin, while in *Drosophila* the heterochromatic region is considered to be constitutively heterochromatic. Because of these parallels, we refer to the *P. citri* protein identified in these studies as *P. citri* HP-1-like.

Our studies reveal developmental and sex-specific regulation of the distribution of *P. citri* HP-1-like. In the Coccids, the heterochromatic haploid set in male cells was heavily decorated by the HP-1 antibody throughout the cell cycle. In fact, both the chromocenter in interphase nuclei and the five heterochromatic chromosomes in mitotic nuclei are the nearly exclusive site of anti-HP-1 staining, whereas the euchromatin lacks any detectable HP-1 signal. By contrast, interphase nuclei and mitotic chromosomes of female cells showed a scattered distribution of the HP-1-like protein (see below). The bright fluorescence of the male heterochromatic chromosomes after immunolabeling with the HP-1 antibody cannot be ascribed to their higher condensation, when compared with the euchromatic ones. The strong difference in immunofluorescence between the two sets still persists in late metaphase when they achieved nearly the same degree of condensation as estimated by their staining with the DNA-binding stain DAPI. Our results strongly suggest that in male mealybugs, HP-1-like is correlated with the inactivation of the hypomethylated paternally derived haploid chromosome set in only male individuals. The presence of *P. citri* HP-1-like in embryos of both sexes led us to exclude this protein as the factor involved in the recognition of the imprint signal, suggesting that at least another factor is necessarily involved in the induction of facultative heterochromatization and that this factor should be male-limited.

The female mitotic chromosomes displayed reproducible pattern of HP-1 staining. The comparison of the HP-1 antibody pattern with the C-banding showed that many HP-1-like sites did not coincide with constitutive heterochromatin as defined by the C-banding criteria. Therefore, in female cells, HP-1-like appears able to bind both constitutive heterochromatin and euchromatin. This is in contrast to male cells, in which, as discussed above, HP-1-like binds almost exclusively to heterochromatic chromosomes. We suggest that the regulated amount of HP-1-like that normally enters the male nuclei is recruited by chromosomes destined to become heterochromatic. This implies that mealybug HP-1-like preferentially binds to potential heterochromatin and that its cellular amount is strictly regulated. Both these characteristics have been postulated for HP-1 to explain its dose-dependent effect on PEV-mediated gene silencing in *Drosophila* (Eissenberg et al., 1992), as well in mammals (Festenstein et al., 2000). In this regard, it is interesting to note that the HP-1 antibody recognizes a striking pattern of staining of female chromosomes. The staining appears at discrete sites in the euchromatin of all the chromosomes and is entirely distinct from the male staining pattern. This observation leads us to speculate a possible role for HP-1-like in dosage compensation. In Coccids, dosage compensation is expected to equalize the gene products produced by diploid females and parahaploid males.

The results presented here clearly show that Coccid chromosome system offers a powerful tool for gaining insights into the structure of facultative heterochromatin, and into the
genetic and molecular mechanisms of its developmentally regulated formation.

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