

# Genomic imprinting in the mealybugs

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**Abstract.** The coccid insects (Hemiptera; Sternorrhyncha; Aphidiformes; Coccoidea; Pseudococcidae) are well suited to study not only the mechanisms of genomic imprinting but also facultative heterochromatization, a phenomenon well exemplified by inactivation of the X chromosome in female mammals. Coccids show sex-specific heterochromatization of an entire set of chromosomes and transcriptional silencing of all the paternally contributed chromosomes in males. Thus, genomic imprinting and the resultant differential regulation operate on 50% of the genome in contrast to the single X chromosome in female mammals. A significant insight into the

phenomenon of genomic imprinting has come from very elegant cytological analysis of the coccid system. Recently, efforts have been made to dissect out at the molecular level the phenomenon of genomic imprinting in these insects. The present review summarizes both of these aspects. In light of the accruing experimental evidence for chromatin-based differences in the maternal and paternal genomes, it appears that the mealybug system may provide evidence for stable maintenance of chromatin code not only through mitosis but also through meiosis.

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The concept of genomic imprinting can be traced back to Metz (1938) and Helen Crouse (1960), who coined the term in the context of the unique inheritance of sex chromosomes in the dipteran insect *Sciara coprophila*. Crouse described it as a process wherein 'a chromosome which passes through the male germ line acquires an 'imprint' which will result in behaviour exactly opposite to the 'imprint' conferred on the same chromosome by the female germ line. In other words, the 'imprint' a chromosome bears is unrelated to the genic

constitution of the chromosome and is determined only by the sex of the germ line through which the chromosome has been inherited' (Crouse, 1960). Thus the phenomenon of genomic imprinting, which violates the Mendelian principles of equivalence of genetic information in reciprocal crosses, was first recognized in a modest insect system.

Genomic imprinting, a process that reversibly marks one of the two parental contributions and can function at the level of homologous loci, chromosomes or chromosomal sets during development, results in the functional non-equivalence of genes (Chandra and Nanjundiah, 1990). There are numerous examples of imprinting operating at the level of genes and segments of chromosomes in mammals that are discussed in this issue. The inactivation of paternal X chromosomes in the extra embryonic tissue of mice serves as an example of parental origin effect on whole chromosomes while the inactivation of the paternal set of chromosomes in male mealybugs serves as a unique example of the whole haploid genome being subjected to genomic imprinting (Schrader and Hughes-Schrader, 1931; Brown and Chandra, 1977). Imprinting involves not only a mechanism to mark the con-

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cerned genes or genetic loci depending on their parental origin but also mechanisms which can recognize, maintain and then erase the imprint to allow the system to remark it on the basis of sex of the progeny.

The phenomenon of genomic imprinting is particularly well studied in mammals. In insects, in addition to *Sciara*, studies on coccids, the mealybugs also point to similar non-equivalence of parental genomes (Brown and Nur, 1964). In the present review we discuss the correlation of genomic imprinting with sex determination along with the known molecular attributes associated with genomic imprinting in coccid insects.

### Coccids – the sedentary plant parasites

The coccids constitute a relatively small group of insects, placed under the order Hemiptera (Insecta; Dicondylia; Pterygota; Neoptera; Paraneoptera; Hemiptera; Sternorrhyncha; Aphidiformes; Coccoidea; Pseudococcidae). Taxonomically, some of its close relatives are the cicades, leafhoppers, sharpshooters and aphids (Brown and Chandra, 1977). Coccids are pests of citrus fruit plants and ornamental plants. The males and females in this group are morphologically distinct and can be mistaken for individuals of different species (Fig. 1). The females retain their larval morphology even after attaining adulthood, whereas the males undergo several metamorphic moultings before they finally emerge as winged adults in most species. Females are largely sedentary, feeding on the sap from fruits and tender portions of the shoot. Males, in contrast, are more active, mate several times but do not feed. They die within a few days after emerging as adults from the last molt.

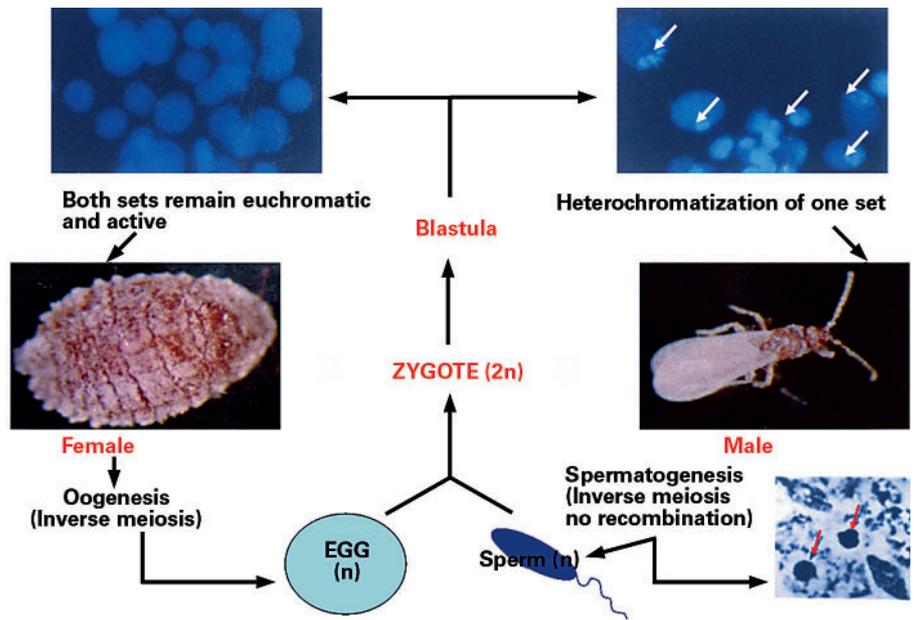
Schrader (1921) observed that males of the mealybug species *Pseudococcus nipae* had a rather unorthodox chromosome system. The diploid number of chromosomes in most mealybugs is ten. He found that unlike females who had ten euchromatic chromosomes, males had five euchromatic and five heterochromatic chromosomes (the terms euchromatin and heterochromatin were coined by Heitz in 1928; these terms have been used here for convenience). This rather small group of insects has diverse chromosomal systems, and at least nine sexual systems have been described (Brown and Chandra, 1977). However, for simplicity coccids have been divided into three subgroups: (i) the Primitive coccids, including Iceryine coccids and *Stictococcus*; (ii) the Lecanoids, including mealybugs and soft scales; and (iii) the Specialized coccids (Diaspidids), including armored scale insects and palm scales. The primitive coccids have the XX (females) – XO (males) sex determining mechanism. Male haploidy is seen in Iceryine coccids. Male haploidy with functional hermaphroditism is seen in some of the Iceryine species. A detailed discussion of the various genetic systems found among coccids including the evolutionary aspects can be found in the review by Brown and Chandra (1977). In the present review we focus on the attributes of the differential organization of homologous chromosomes and genomic imprinting observed in the lecanoid group of coccids.

### Facultative chromosome behaviour in coccids

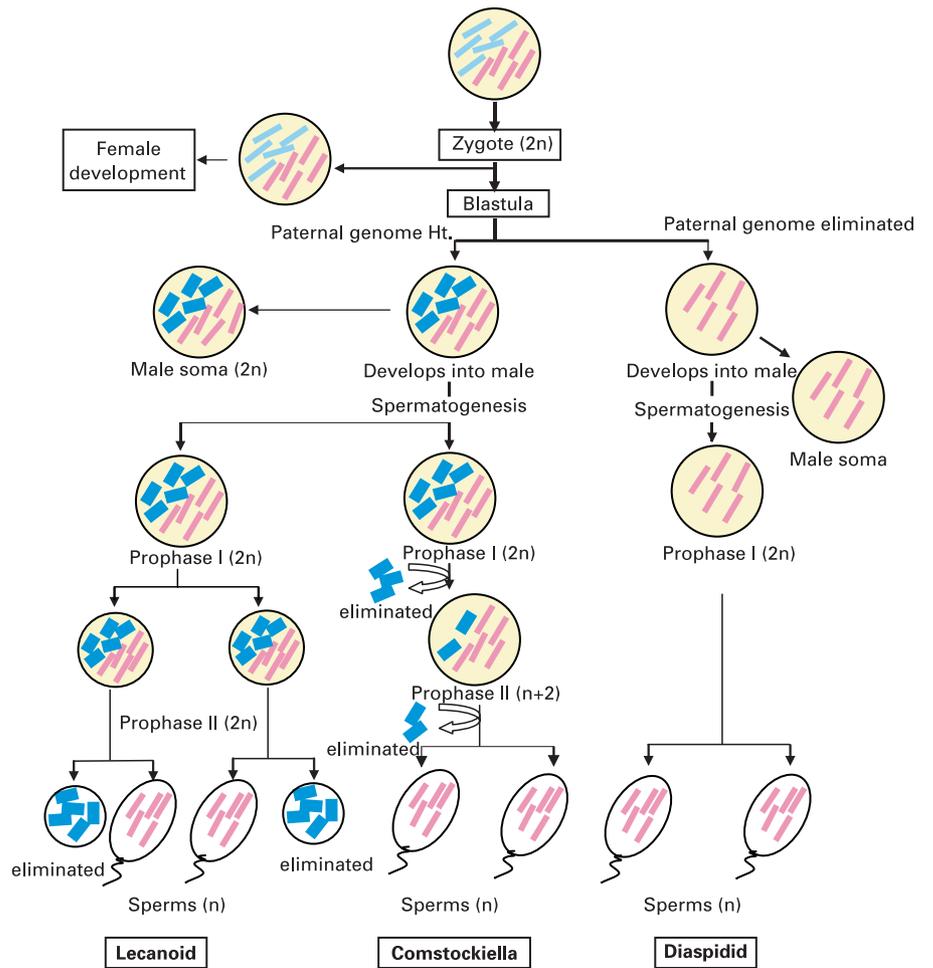
Sex determination in the lecanoids and the diaspidids seem to be dependent on the behaviour of a set of chromosomes and not a single chromosome. A set of chromosomes is either heterochromatized as in lecanoids or eliminated as in diaspidids (Fig. 2). Insects from both these coccid groups have zygotes with the same chromosomal constitution and have no sex chromosomes. During embryogenesis of lecanoid coccids, in some embryos heterochromatization of an entire set of chromosomes takes place during the cleavage stage. These embryos develop into males and the others develop into females. In diaspidids, represented by armored scales, an entire set of chromosomes is eliminated during cleavage stages from some of the embryos and these develop into males. This elimination is accomplished by anaphase lagging. A combination of the above two modes of chromosome behaviour is seen in the *Comstockiella* group of coccids. In this group one set of chromosomes is heterochromatized during cleavage divisions. Groups of these heterochromatized chromosomes are eliminated during prophase I and II of spermatogenesis by anaphase lagging (Brown and Bennet, 1957; Bennet and Brown, 1958; Brown, 1959, 1965, 1966).

As mentioned earlier, the lecanoid genetic system is characterized by the selective inactivation of the paternal chromosomes in males and the subsequent loss of paternal chromosomes during spermatogenesis leading to the exclusion of the paternal set from genetic continuum through males. Commenting that the lecanoid system leading to the inactivation/heterochromatization is not an anomaly, Brown and Nelson-Rees observed that, 'although a striking departure from the more conventional forms of chromosomal behaviour, the lecanoid system is not a passing accident but has sustained quite considerable evolution. It occurs throughout a series of diverse families including the primitive mealybugs (Pseudococcidae), the cochineal dye insect, the lac insects, and the soft scales (Lecaniidae)' (Brown and Nelson-Rees, 1961). Thus, the mechanisms that are adopted to achieve this differential regulation of homologous chromosomes across the systems exhibiting lecanoid behaviour of chromosomes are likely to retain several common themes.

The phenomenon of selective inactivation of one set of chromosomes observed in mealybugs can be closely paralleled with the differential regulation of homologous X chromosomes observed in mammalian females. However, the inactivation of the male genome in male mealybugs is a non-random process and depends on signals carried through meiosis unlike the random inactivation of the X chromosomes that occurs in somatic cells of female mammals, i.e. signals for inactivation being stably transmitted through mitosis. A detailed analysis of the imprinting mechanism in mealybugs is required before we can make a comparison of the mechanisms involved in differential regulation of homologous chromosomes in mealybugs and female mammals.



**Fig. 1.** Life cycle of a mealybug. The photographs on the top show interphase nuclei from female (left panel) and male (right panel). The heterochromatic set of chromosomes for male nuclei is indicated by white arrows. The bottom right panel shows sperm bundles (red arrows indicate degenerating heterochromatic set of chromosomes; photograph taken from Nelson-Rees, 1963).



**Fig. 2.** A diagrammatic representation of the various patterns of sex-specific behaviour of chromosomes in coccids. The fate of paternal and maternal genomes in males is indicated. Blue lines represent paternal and pink lines maternal chromosomes; thin and thick lines for euchromatin and heterochromatin respectively.

## Heterochromatic chromosomes of mealybugs

The diploid number of chromosomes in most mealybugs is ten. Mealybugs follow the lecanoid system of chromosome behaviour, which is characterized by the heterochromatization of an entire set of chromosomes in males. Presented below is the fate of these chromosomes during different developmental stages in mealybugs (Fig. 1). The observations presented here are almost entirely based on cytological analysis. Heterochromatin and euchromatin are distinguished by their cytological appearance rather than any other biochemical or molecular parameters.

It was observed that all the chromosomes in the mealybug embryo appear to be euchromatic from the zygotic stage through the cleavage divisions immediately following fertilization. However, during late cleavage or early blastula, half the number of chromosomes becomes heterochromatized in some of the embryos. These embryos develop into males while the rest of the embryos develop into females. Thus, male and female mealybugs are distinguished cytologically by the heterochromatized set of chromosomes. Exhaustive study of the chromosomal system in various species of mealybug and other related coccids is documented by Schrader (1921, 1929), Schrader and Hughes-Schrader (1931), Hughes-Schrader (1948), and is reviewed in Brown and Nur (1964) and Brown and Chandra (1977). Heterochromatization that occurs around the blastula stage is maintained during subsequent development, except for cells in a few tissues which show reversal of heterochromatization (Nur, 1967).

## Gametogenesis in mealybugs

Mealybugs like other Hemipterans exhibit 'inverse meiosis'. In this unorthodox meiosis, the first division is equational with chromatids and not chromosomes separating from each other and moving to the opposite poles at anaphase I. Two chromatids from each bivalent pair at telophase I form a dyad. The second meiotic division is reductional, the dyad chromatids separate and move to opposite poles at anaphase II, resulting in the formation of haploid nuclei (Fig. 1, Hughes-Schrader, 1944, 1948).

Spermatogenesis in male mealybugs is unusual. At prophase in the primary spermatocytes, the heterochromatic mass separates into individual chromosomes, which remain condensed, followed by the condensation of the euchromatic chromosomes, which is completed by metaphase I. Through the entire process, the heterochromatic and euchromatic chromosomes seem to remain in distinct groups, the heterochromatic set forming a tighter group than the euchromatic set. Thus, at the end of meiosis there are four haploid nuclei, two of which exclusively contain the euchromatic and the other two heterochromatic chromosomes. During the whole process neither pairing nor recombination occurs (Fig. 1, Brown and Nur, 1964). The meiotic division is not followed by cytoplasmic division and the nuclei remain embedded in cysts and formation of a quadrinucleate spermatid is observed (Hughes-Schrader, 1935; Nelson-Rees, 1963).

However, the most important observation that was made regarding spermatogenesis was that the condensed chromosome set slowly disintegrates in the quadrinucleate spermatid and does not form the sperm. Thus, only the euchromatic nuclei in these cysts form the genetic continuum through the males (Hughes-Schrader, 1935, 1948; Nelson-Rees, 1963; Brown and Nur, 1964 [review]). Inverse meiosis is also observed in female mealybugs (Brown and Chandra, 1977).

## Correlation of heterochromatization with sex determination and genomic imprinting

In order to explain the unusual chromosomal behaviour in mealybugs, Schrader and Hughes-Schrader proposed that: (a) the heterochromatic set in males is genetically inert and thus male mealybugs are physiologically haploid (Schrader and Hughes-Schrader, 1931) and (b) the heterochromatic set of chromosomes in the males is of paternal origin (Hughes-Schrader, 1948).

Brown and co-workers later confirmed these hypotheses. They made use of the fact that coccid chromosomes are holokinetic i.e. they have diffuse centromeres and are attached to the mitotic spindle along their entire length. Fragmented chromosomes of mealybugs do not lag behind at metaphase and are mitotically stable (Hughes-Schrader and Ris, 1941). Brown and co-workers irradiated male and female mealybugs and mated them with unirradiated counterparts. The progeny obtained was then analysed cytologically.

It was found that when mothers were irradiated, the euchromatic set of chromosomes in the male progeny was affected. It was the heterochromatic chromosomes in the male embryos that showed aberrations when fathers were irradiated (Brown and Nelson-Rees, 1961). This clearly indicates that it is the paternally derived chromosomes that get heterochromatized in the male progeny, as Schrader and Hughes-Schrader (1931) had hypothesized. A corollary of this experiment was the observation that even the smallest of the chromosomal fragments when derived from an irradiated father was heterochromatized in his male progeny. In the light of these results it was observed that the ability to become heterochromatic is probably dispersed along the entire length of the chromosomes similar to the centromeric property (Brown and Chandra, 1977; Khosla et al., 1999). This is in contrast to what is known of a single inactivation centre on the mammalian X-chromosome (Cattanach, 1975; Lyon, 1995; Avner and Heard, 2001; Brockdorff, 2002). This inference, along with the previous observation that the heterochromatic set of chromosomes degenerates after spermiogenesis, would mean that the maternally derived euchromatic chromosomes of the father become heterochromatic in the sons (Brown and Nur, 1964). Thus implying that the signals for heterochromatization in cleavage stages which is present on the paternally contributed genome and absent on the maternally inherited genome of the males, are acquired by the maternally derived euchromatic chromosomes during spermatogenesis (Khosla et al., 1996).

The heterochromatic set of chromosomes in males has been demonstrated to be inert by irradiation experiments. Irradiation of males at high doses of X-ray was lethal only to their female progeny but not the male progeny (Brown and Nelson-Rees, 1961). The transcriptional silencing of the heterochromatic chromosomes was shown by monitoring RNA synthesis *in situ* (Berlowitz, 1965). The inertness of the paternal chromosomes in males was also evident by the patterns of inheritance of genetic markers known in mealybugs, like salmon-eye, an eye colour mutation, and wing morphology (Brown and Wiegmann, 1969).

Male and female mealybugs are strikingly different morphologically though as zygotes they are similar. Even during embryogenesis the only cytological difference is the heterochromatization of a set of chromosomes, which is apparent only by the blastula stage of development. Thus, heterochromatization seems to be the point of divergence between the male and female developmental pathways. Whether it is a cause or an effect of the sex determination is not clear but it does seem to affect the developmental pathways of the two sexes.

### **Is facultative heterochromatin in male mealybugs dispensable?**

The heterochromatic chromosomes are genetically inactive and therefore what is the need of retaining such a haploid set, or in other words why did mealybugs not adopt true male haploidy instead of functional haploidy as a means of sex determination? The answer is not straightforward and the reports suggesting the need for persistence of heterochromatic chromosomes are very few and not conclusive.

Nelson-Rees (1962) during irradiation studies found that after severe irradiation of fathers (60,000–90,000 rep), only a few sons survived. In all these survivors, no matter how badly damaged or rearranged the heterochromatic set was, the amount of the heterochromatic material was estimated to be similar to the amount in untreated controls. In this experiment Nelson-Rees also observed sterility in a large proportion of sons of irradiated fathers and this fraction increased with dose of irradiation (Nelson-Rees, 1962; Brown and Nur, 1964).

Nur and Chandra (1963) showed that a heterochromatic set from one species of mealybugs can not be substituted for that of another. The rationale was that heterochromatic chromosomes being genetically inactive, in some of the interspecific crosses at least the male offsprings would survive, if not the females. However, they found that no offspring of either sex survived beyond the first instar stage in interspecific crosses.

These observations suggesting a need to retain the heterochromatic set were challenged by the findings of Nur (1967). He reported reversal of heterochromatization in some tissues of male mealybugs. In *Planococcus citri* as well as other species the following tissues did not show the presence of heterochromatic set (Nur, 1967): the yolk cells in the embryo; the mycetocytes; some of the oenocytes; some skeletal muscle cells; cyst wall cells of the testes; cells of the intestinal tract and cells of the malpighian tubules.

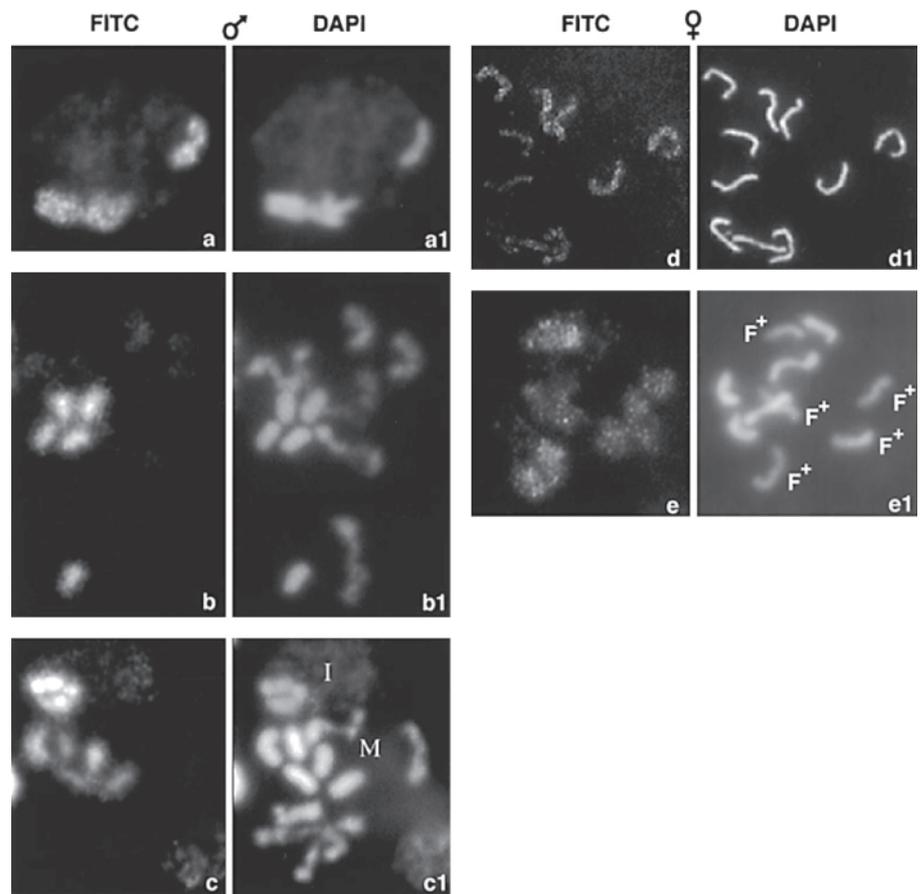
Nur (1967) then repeated the irradiation experiments of Nelson-Rees (1962) and interspecies crosses of Nur and Chandra (1963). He observed the reversal of heterochromatization in the cells of malpighian tubules and gross modification of the tissue in the sons of irradiated fathers. He pointed out that male sterility was due to the fact that the tissues where reversal occurred did not develop properly, causing sterility or lethality and not because of bulk requirement of the heterochromatic set of chromosomes as was earlier postulated. Similarly, in the case of the interspecific cross (*Pseudococcus obscurus* females crossed with *Pseudococcus gahani* males); female hybrid embryos did not survive beyond the blastoderm, whereas some male hybrid embryos survived only till the first instar stage. In these males the tissues which normally show reversal of heterochromatization showed incomplete reversal. Most of the hybrid males lacked any structure which could be considered as malpighian tubules and wherever distinguishable they were poorly developed. However, these analyses by Nur lacked the comprehensive study of other tissues where reversal of heterochromatization does not occur and the results failed to provide a satisfactory explanation for the need to retain highly fragmented but equivalent amounts of heterochromatin in males irradiated at very high doses.

In a later study on males that were irradiated during early stages of their development, Nur (1970) found some spermatogonia where both sets of chromosomes were euchromatic. In these spermatogonia, the second meiotic division was disrupted and diploid spermatids were formed. This would suggest that heterochromatization of a haploid set is necessary for normal meiosis in males. It is possible that the genes involved in pairing and recombination are silent in males but become active when both chromosome sets are active in males. Defective resolution of synaptonemal complexes may also disrupt meiosis and lead to sterility as in human males. Even earlier observations by Hughes-Schrader (1935) and Nelson-Rees (1963) pointed to the fact that the movement of heterochromatic chromosomes to the poles during anaphase II precedes the movement of the euchromatic set to the opposite pole. As apparent from the above discussion, the role of heterochromatic chromosomes in mealybugs is debatable and inconclusive and needs to be substantiated.

### **Models for imprinting in mealybugs**

The phenomenon of parent-specific marking of the epigenetic modification of genes/chromosomes demands that any mechanism of imprinting should include reversibility, modulation of expression *in cis* without altering the primary sequence of DNA and clonal inheritability.

Nur described parthenogenesis in mealybugs of the species *Pulvanaria hydrangea*. In this species, the haploid egg pronucleus first divides and its products fuse to form a diploid zygote substitute. The progeny produced are females and no adult males are observed. However, he found some embryos in a particular population of this species which showed heterochromatization of a set of chromosomes but these did not



**Fig. 3.** Hypomethylation of paternal genome. Biotinylated dUTP incorporated by nick translation is detected by FITC-labeled anti-biotin antibodies. The metaphase plates are treated with *Hpa*II restriction endonuclease before nick translation. Nuclei from males (**a–c**) show intense staining with FITC on heterochromatic chromosomes. (**d–e**) Nuclei from females show non-uniform staining with FITC. F+ indicates the chromosomes that are labeled by FITC in nuclei from females shown in frame **e**. Reproduced with authors' permission. (Please see Bongiorno et al., 1999.)

develop to term. Such embryos were produced only by a small number of females. Since in other species of *Pulvanaria* with a sexual mode of reproduction, heterochromatization is linked to male development, Nur (1963) proposed that these embryos would have subsequently developed into males. In 1972 Nur studied three more parthenogenetic species of coccids and found that they produce either only male or female progeny parthenogenetically, depending on the species. In *Lecanium putamani*, a species of mealybugs in which males are produced parthenogenetically, female progeny develop from fertilized eggs. In all these species males had one set of chromosomes heterochromatized.

To explain the heterochromatization of a set of chromosomes in parthenogenetically produced males, Nur proposed that the daughter pronucleus in *Pulvanaria hydrangea* and other parthenogenetic species of mealybugs, after division, are separated in space and this spatial distribution causes one to become different from the other. He compared this with the separation of polar body I and II from the egg which makes them different from the egg nucleus even though the genomic complements in them are identical (Nur, 1963, 1990).

Based on the observations of Nur, two models were proposed to explain differential behaviour of homologous chromosomes (Chandra and Brown, 1975; Sager and Kitchin, 1975). Both the models suggest the place of imprinting to be the egg, where imprinting occurs just after fertilization. Sager and Kitchin addressed the molecular nature of imprinting

and suggested that the oocyte genome is modified before fertilization and the divergence of male and female development is decided by the state of modification of the sperm genome after fertilization. This is based on the principles of the restriction-modification system known in prokaryotes. However, Chandra and Brown (1975) suggested variability in extent of the imprinting region within the egg and also total absence of such a region in some eggs. They proposed that the egg and polar body nuclei escape imprinting.

It is important to distinguish between primary imprint and the manifestation of the imprint as separate events brought about at different stages in development. However, this distinction is not apparent in the above mentioned models. The Sager and Kitchin model (1975) failed to make impact since neither have restriction enzymes been reported in higher eukaryotes nor does the model explain the reversal of heterochromatization in some tissues of mealybugs in a satisfactory manner.

#### DNA methylation as a molecular correlate of imprinting in mealybugs

Deobagkar et al. (1982) examined the methylation status of the mealybug genome. Significant amounts of 5-methylcytosine in the mealybug genome not only in the dinucleotide (CpG) but also in (CpA), (CpC) and (CpT) were found. Also

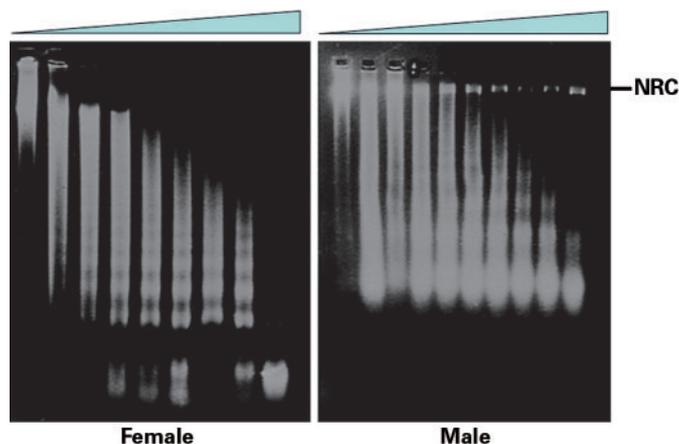
reported were high amounts of 6-methyladenosine and 7-methylguanosine which are rarely found in DNA of higher eukaryotes. This was later confirmed in the same species by Achwal et al. (1983) using a highly sensitive immunochemical approach. Devajyothi and Brahmachari (1992) reported a CpA methylase from the mealybug *Planococcus lilacinus* which could methylate both (CpG) and (CpA) dinucleotides. They also reported modulation of DNA methyltransferase activity during the life cycle of *P. lilacinus*, the enzyme activity being higher in third instar females when gametogenesis, fertilization and subsequent development are initiated (Devajyoti and Brahmachari, 1989). Based on sequencing of random stretches of DNA from the mealybug, *P. lilacinus*, Mohan et al. (2002), inferred that repetitive DNA content in the mealybug genome is higher than that of *Drosophila* while GC content is less. This might influence the CpG dinucleotide frequency in the mealybug genome.

Scarborough et al. (1984) did not find any modified bases other than 5-methylcytosine in two other species of mealybugs, namely *Pseudococcus obscurus* and *Pseudococcus calceolariae*, though they found somewhat higher levels of 5-methylcytosine in males. This difference, however, was found not to be very significant. Later Bongiorni et al. (1999) reported hypomethylation of the paternal genome in both males and females using a combination of restriction endonuclease treatment followed by nick translation with fluorescence labelled precursors. The incorporation of biotinylated dUTP during in situ nick translation varied based on susceptibility of the chromatin to methylation-sensitive restriction enzymes, *MspI* and *HpaII*. These results showed that the paternal genome is hypomethylated as compared to the maternal set in both males and females (Fig. 3, Bongiorni et al., 1999). However, in this approach only methylation at CCGG sequences is assessed.

### Chromatin organization as a correlate of imprinting in mealybugs

While distinguishing the primary signal of imprinting from the manifestation of imprinting, Khosla et al. (1996) examined the chromatin organization of mealybugs, *P. lilacinus*. They observed that approximately 10% of the genome in male mealybugs was organized into a nuclease-resistant chromatin (NRC) only in the males and not in females, thus correlating it with heterochromatinization of the paternal genome in males (Fig. 4, Khosla et al., 1996). They demonstrated additional attributes of heterochromatin in mealybugs in the form of nuclease resistance and matrix association (Khosla et al., 1996, and unpublished data). These parameters can serve as novel and easily assayable readouts for heterochromatinization in mealybugs.

Arguing that the nuclease resistant property if attributed to all the heterochromatin present in males, NRC should have been nearly 50% and not 10% of the genome as observed; they hypothesized that this fraction contains sequences that are at the core of the heterochromatin perhaps containing the putative centres of inactivation as a subset of sequences within the

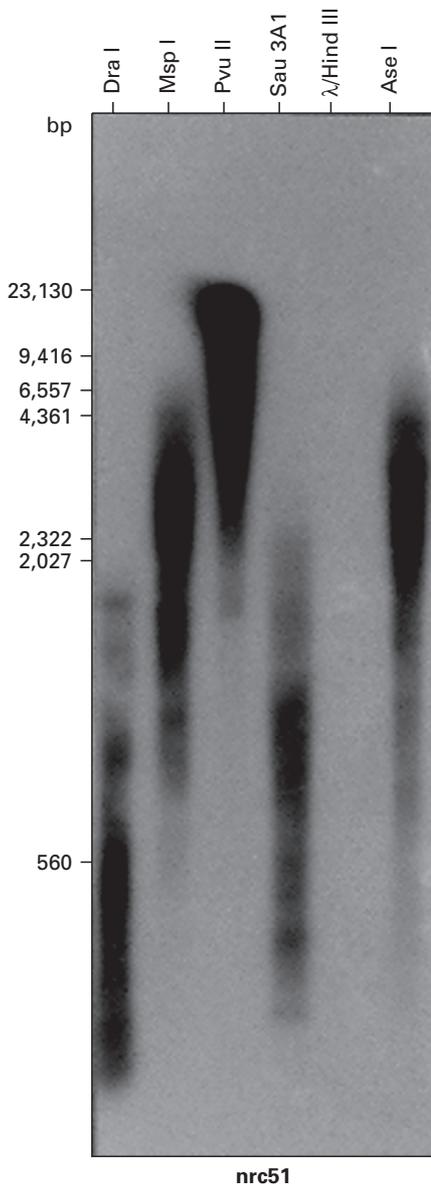


**Fig. 4.** Differential sensitivity of male and female nuclei to micrococcal nuclease. Nuclease Resistant Chromatin (NRC) is present exclusively in male mealybugs. Nuclei from male and female mealybugs were incubated for increasing time (represented by triangles above the panels) with MNase. (Please see Khosla et al., 1996 for details.)

NRC (Khosla et al., 1996). The multiple centres of inactivation that were indicated by the irradiation experiments further imply that any sequence that can serve as a centre of inactivation in mealybugs should be present in multiple copies, dispersed within the genome. Khosla et al. identified one such middle repetitive DNA sequence (*nrc51*) which was part of the unusually organized genome in the male (Fig. 5, Khosla et al., 1999). In conjunction with the hypomethylation of paternal genome in males observed by Bongiorni et al. (1999), this specialized packaging of the chromatin in male mealybugs occurs on the paternal heterochromatinized chromosomes. This mutual exclusiveness between DNA methylation and specialized chromatin organization is also observed for several imprinted loci in mammals (Feil and Khosla, 1999).

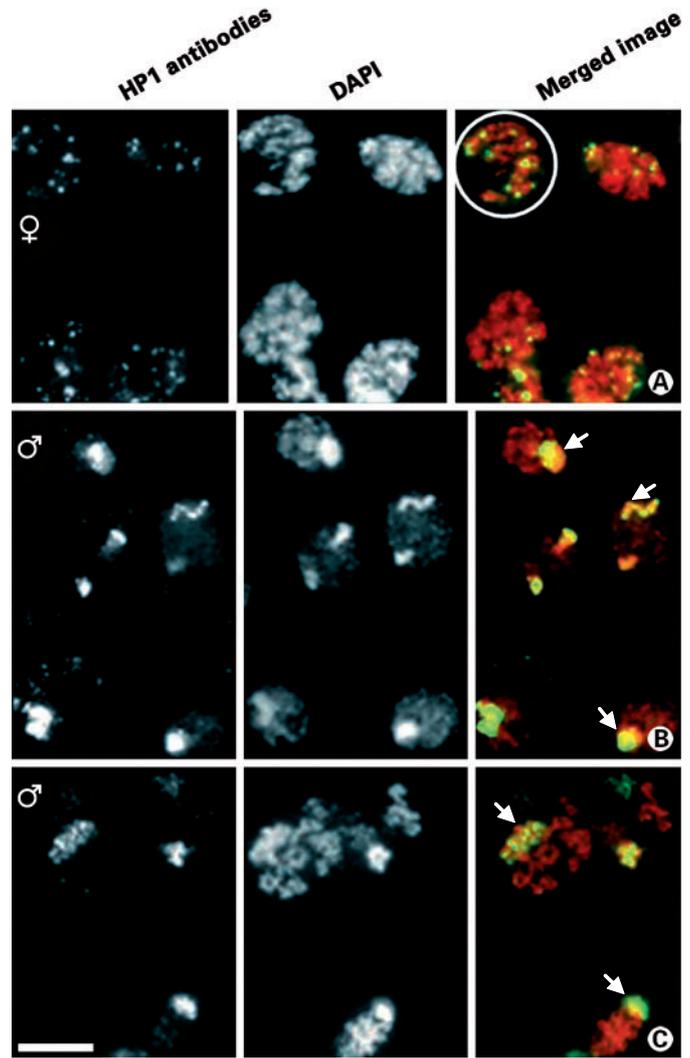
Investigating the possible involvement of HP1-like protein in heterochromatinization in mealybugs, Bongiorni et al. demonstrated the differential distribution and co-localization of HP1-like protein with heterochromatin in male mealybugs (Fig. 6, Bongiorni et al., 2001). The authors used monoclonal antibodies raised against HP1 protein of *Drosophila melanogaster* for immunolocalization as well as Western blotting in *Planococcus citri*. HP1-like protein was detected in both the sexes but it is scattered over the whole genome in females while in males it is localised to the heterochromatic genome. This exclusive localization of HP1 protein to heterochromatin was maintained throughout the cell cycle. Thus HP1 protein in mealybug distinguishes between metaphase condensation and the condensation of the paternal set in males.

Bongiorni et al. (2001) also provide evidence of appearance of this protein preceding the onset of heterochromatinization in developing males. They observed the presence of HP1-like protein in embryos at seventh cleavage, while Schrader had proposed that heterochromatinization of the paternal genome in male occurs at fifth cleavage. The appearance of a chromocenter representing heterochromatin occurs in a wave



**Fig. 5.** Middle-repetitive sequences are a part of NRC. Mealybug genomic DNA digested with different restriction enzymes (as indicated above each lane) and electrophoresed on 1.1% agarose gel was Southern blotted and probed with  $^{32}\text{P}$ -labelled DNA from *nrc51* (an NRC-specific clone) was found to be a middle repetitive sequence as it hybridizes at several loci in the mealybug genome. (Please see Khosla et al., 1999 for details.)

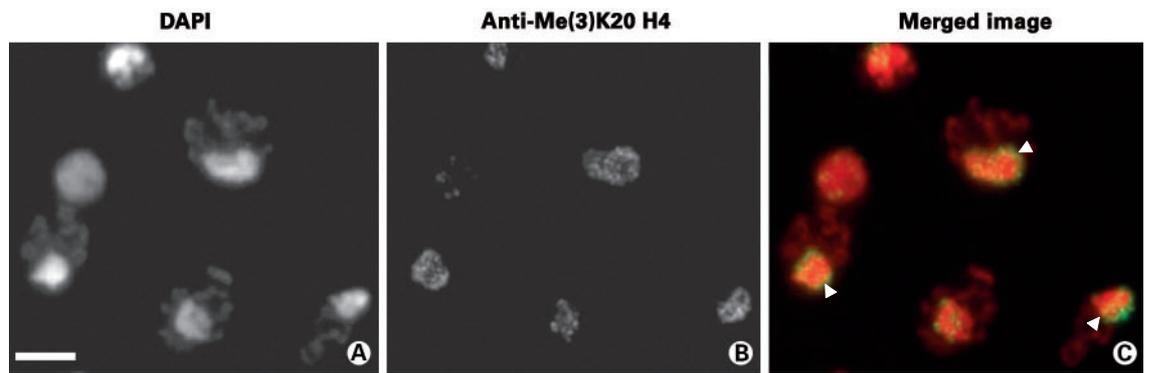
from one pole of the embryo towards the other and not simultaneously in all the nuclei (Bongiorni et al., 2001). These results are different from those reported by Epstein et al. (1992), in which the authors used antibodies directed against the entire protein from a gene (*pchet 1*) having homology with the chromodomain of HP1 of *Drosophila*. They failed to correlate the localization of the signals with the presence of heterochromatin (Epstein et al., 1992). In these two instances the authors may be dealing with different proteins, as it is not clear if *pchet 1* protein shares only the chromodomain or the other regions also with HP1 protein. In *Drosophila*, HP1



**Fig. 6.** Co-localization of HP1-like protein and the heterochromatin in male mealybugs. The frames from left to right represent the immunostaining/DNA staining as indicated above the panels. Monoclonal antibodies directed against HP1 protein from *D. melanogaster* were used for immunostaining. Punctate staining on all chromosomes of female (shown in the encircled area in **A**), chromosomes intensely stained with DAPI are stained with anti-HP1 antibody in nuclei from males (shown by arrows in **B** and **C**). **C** shows prometaphase cell (top right). Scale bar: 10  $\mu\text{m}$ . Figure reproduced with authors' permission. (Bongiorni et al., 2001.)

is associated with constitutive heterochromatin, the observations of Bongiorni et al. (2001) suggest an underlying similarity between constitutive and facultative heterochromatization processes and illustrate that these two descriptions of heterochromatin may not necessarily indicate mechanistic differences in achieving the compromised functional state of chromatin.

There are a number of covalent modifications of histones that correlate with active and inactive chromatin organization. Acetylation of histone H4 is an activating modification while methylation of lysine 9 of histone H3 is an inactivating modi-



**Fig. 7.** Detection of H4 trimethylated at lysine 20 (Me(3)K20H4) in male mealybugs. Prometaphase male mealybug nuclei stained with DAPI (**A**) show the intensely stained heterochromatin, the same fraction stains with antibodies against Me(3)K20H4 (arrowheads in **B**). (**C**) Merged image with DAPI pseudocoloured in red. Scale bar 5  $\mu\text{m}$ . Figure reproduced with authors' permission. (Kourmouli et al., 2004.)

fication (Li, 2002). Association of these modifications with differentially regulated homologous chromosomes in mealybugs has been examined (Ferraro et al., 2001; Cowell et al., 2002). A difference in histone H4 acetylation between the paternally and maternally inherited genomes was reported based on immunolocalization using antibodies raised against histone H4 acetylated on all four lysine residues (Ferraro et al., 2001). They further observed the retention of acetylation on metaphase chromosomes, suggesting that such a modification could be a part of a cellular memory mechanism during mitosis.

Cowell et al. (2002) demonstrated that histone H3 methylated at lysine 9 (Me9H3) is associated with both constitutive and facultative heterochromatin in different animal species. In mealybugs, they observed co-localization of Me9H3 and HP1 proteins (Cowell et al., 2002). They used antibodies raised against synthetic peptide derived from histone H3 trimethylated at lysine while the HP1 antibody was the same as that used by Bongiorno et al. (2001). In case of both HP1 and Me9H3 a difference in the nature of chromatin localization between male and female mealybugs is reported rather than differences in the presence/absence of the protein or the modification (Bongiorno et al., 2001; Cowell et al., 2002). This can be explained in terms of association of these factors not only with facultative but also constitutive heterochromatin.

More recently, trimethylated lysine 20 of histone H4 (Me(3)K20H4) has emerged as a robust marker for constitutive heterochromatin in murine interphase and metaphase cells (Kourmouli et al., 2004). Studying the association of this modification with heterochromatin in different contexts, Kourmouli et al. (2004) reported the presence of Me(3)K20H4 on facultative heterochromatin in mealybug males while in females it was scattered throughout the chromosomes and no difference was seen between the homologues (Fig. 7). However, they did not find any association of Me(3)K20H4 with the inactive X chromosome in female mammals.

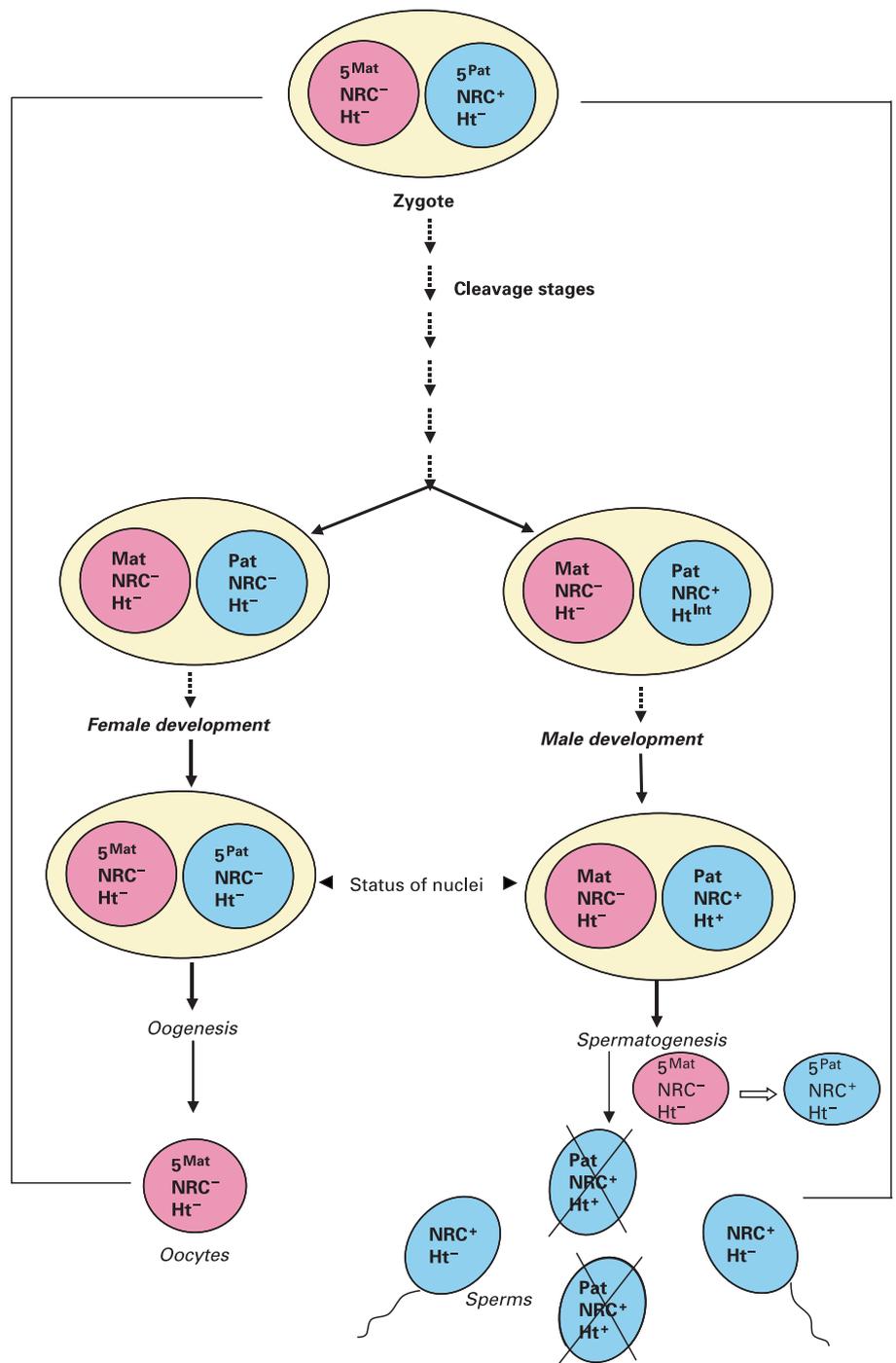
The data available on molecular dissection of parental-origin specific heterochromatin in mealybugs suggests a major role for chromatin-associated factors rather than DNA meth-

ylation in this phenomenon. Except HP1, which appears before heterochromatin in developing embryos, the other modifications of histones are associated with heterochromatin. Khosla et al. (1996) have shown association of nuclease-resistant chromatin not only in somatic nuclei but also in nuclear preparations enriched with sperm-derived nuclei. In the light of these reports, a chromatin-based imprinting mechanism appears as a distinct possibility in mealybugs.

#### Differential chromatin organization as a mechanism of genomic imprinting in mealybugs

In this proposal, we distinguish between heterochromatin as seen in somatic nuclei and the status of the paternal genome in sperms. Heterochromatin is an end point in a sequence of events that begins during spermatogenesis in males. The early events in this sequence do not result in conferring all the attributes known for facultative heterochromatin. In the present context considering the experimental evidence known so far, we assume Nuclease Resistant Chromatin (NRC) as a distinguishable organization of the paternal genome that can be assayed by biochemical and/or molecular methods and consider it as the primary event. This is analogous to the 'seeding elements' that Wolffe (1944) proposed in the context of maintenance of cellular memory factors through mitosis. Thus, NRC is not equivalent to heterochromatin but is a step towards achieving facultative heterochromatinization. One of the features of spermatogenesis in male mealybugs that was discussed earlier and is relevant here is the complete segregation of paternal and maternal genomes from each other during spermatogenesis and the subsequent disintegration of the nuclei containing the heterochromatinized paternal genome (Brown and Nur, 1964). Thus, it is to be noted that the maternal euchromatic genome is packaged into functional sperms and they are received in the oocytes as the paternal genome.

Therefore, we propose that following fertilization the zygote has the paternal genome in NRC positive and hetero-



**Fig. 8.** A diagrammatic representation of the model proposed for altered chromatin as a mechanism of genomic imprinting in mealybugs. NRC<sup>-</sup> Nuclease resistant chromatin, NRC<sup>+</sup> state is assumed as an epigenetic mark in mealybugs in this model. Mat-Maternal genome; Pat-Paternal genome; Ht<sup>int</sup> is an intermediate state of heterochromatin which is NRC<sup>+</sup> but not cytologically heterochromatic, Ht<sup>+</sup> is facultatively heterochromatized genome, Ht<sup>-</sup> is genome without facultative heterochromatin i.e. euchromatic genome. The crossed ovals represent the haploid nuclei containing the paternal heterochromatized genome that disintegrate during spermatogenesis.

chromatin negative state (Fig. 8). Only after the sixth cleavage the NRC positive paternal genome acquires a heterochromatinized state as observed by cytological parameters. Considering the factor(s) contributing to NRC formation, we can attribute certain properties to these: (i) they can be proteins or RNA, (ii) they exhibit co-operativity in binding, (iii) one or more of such factors should have male-specific expression during spermatogenesis, (iv) they should be auto-regulatory in nature, (v) their expression would be sensitive to environmental cues either directly or indirectly, (vi) they function as

recruiting factors for heterochromatin mediating proteins. At the cleavage stages when the developmental decision has to be made there will be a choice to be executed between maintenance or loss of the NRC positive state concurrent with the choice of a male or female developmental pathway.

The interactions between opposing influences to direct a female or a male developmental pathway would be similar to the theme of competitive binding with *cis-trans*-acting factors that are so often encountered in developmental systems starting from the lytic versus lysogenic pathway in lambda

phage. Auto-regulation of critical factors is once again a familiar strategy seen in the sex determination cascade of well-studied systems like *Drosophila melanogaster* (Black, 2003). The assigned properties of factors required to be a part of the chromatin-based imprinting mechanism in mealybugs can address most of the interesting observations made over the years in relation to sex determination and genomic imprinting in mealybugs (Brown and Chandra, 1977).

The importance of chromatin remodeling and covalent modification of histones resulting in unique 'histone code' in maintaining the developmental fate of cellular lineages is being increasingly recognized (Strahl and Allis, 2000; Jenuwein and Allis, 2001). The role of chromatin in initiation and maintenance of X chromosome inactivation in female mammals is also established. The transcript from the *Tsix* gene acts as anti-sense for *Xist* in *cis* in the initiation of X chromosome inactivation. Recently, it has been shown that the transcription inhibition of *Xist* is mediated through modification of chromatin structure (Sado et al., 2005). Further in the main-

tenance of inactivation the involvement of Polycomb repressive complex (PRC) has been demonstrated. The initial labile state of inactivation with PRC is reinforced by DNA methylation, recruitment of MACROH2A and hypoacetylation of histone (Hernandez-Munoz et al., 2005). Thus, DNA methylation is a late event in X chromosome inactivation as well while modification of chromatin proteins occurs during initiation even during the inactivation of the paternal X chromosome in the extra-embryonic tissues of mouse.

Most of the known examples demonstrating chromatin as a key determinant in global regulation allude to the stable maintenance of chromatin code during mitosis, the mealybug system may provide an example of stable maintenance of chromatin code through meiosis.

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