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Sex-specific organisation of middle repetitive DNA sequences in the mealybug *Planococcus lilacinus*

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ABSTRACT

Differential organisation of homologous chromosomes is related to both sex determination and genomic imprinting in coccid insects, the mealybugs. We report here the identification of two middle repetitive sequences that are differentially organised between the two sexes and also within the same diploid nucleus. These two sequences form a part of the male-specific nuclease-resistant chromatin (NRC) fraction of a mealybug *Planococcus lilacinus*. To understand the phenomenon of differential organisation we have analysed the components of NRC by cloning the DNA sequences present, deciphering their primary sequence, nucleosomal organisation, genomic distribution and cytological localisation. Our observations suggest that the middle repetitive sequences within NRC are functionally significant and we discuss their probable involvement in male-specific chromatin organisation.

INTRODUCTION

Genomic imprinting involves epigenetic markings that are reversible through gametogenesis (1). The involvement of DNA methylation in imprinting and epigenetic inheritance is known for several genes (2). In a species of mealybug (Insecta; Homoptera: Coccoidea), *Planococcus lilacinus*, genomic imprinting operates on 50% of the genome resulting in heterochromatisation of the entire paternal set of chromosomes in males but not in females. High levels of DNA methylation, cytosine methylation in unusual dinucleotides like CpA and CpT and the presence of a DNA methylase with *de novo* methylation activity *in vitro* on both CpG- and CpA-containing polymers has been demonstrated (3–7). However, no significant differences in DNA methylation levels between the two sexes or at the single gene level have been identified (4,5).

Apart from DNA methylation, differential organisation of chromatin either by recruiting new protein components or by modification of existing proteins can also create epigenetic marking (8,9). On this count mealybugs serve as a robust example where heterochromatisation is an overt manifestation of genomic imprinting.

In mealybugs and other lecanoid coccids all embryos begin development with a diploid set of chromosomes, which is 10 in the majority of the species. There are no sex chromosomes. Both the sexes have identical chromosome complements. These chromosomally identical embryos can develop either into males or females depending on whether or not a set of chromosomes is inactivated (10–12). The entire haploid set of paternally derived chromosomes is inactivated in sons but not in daughters (13), thus manifesting differential regulation of homologous chromosomes as an example of genomic imprinting (11). As in the case of mammalian X chromosomes, the major consequence of imprinting is transcriptional inactivation (or heterochromatisation) of the vast majority of the genes within these chromosomes. At the level of resolution permitted by cytogenetic methods, the inactivated paternal chromosomes in the mealybug do not appear to contain regions escaping inactivation. Another unusual feature of mealybug chromosomes is that radiation-induced chromosome fragments, however small, can become heterochromatic and genetically inactive (13,14). Since minute chromosome fragments of the male parent induced by γ irradiation [up to 120 000 rep (roentgen equivalent, physical)] were capable of mitotic activity and, in particular, anaphase movement, the conclusion could be drawn that the centromere is diffuse (14). Since these small fragments also underwent heterchromatisation, the signals controlling heterochromatisation must also be dispersed along the length of each chromosome. These observations, and those of Nur (15) on a facultatively parthenogenetic coccid, suggested that in mealybugs genomic imprinting and sex determination are closely related.

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[+]U68053, U68054, U68042 and U27458

During an analysis of chromatin organisation in *P.lilacinus*, digestion with micrococcal nuclease showed that a fraction of the male genome is resistant to the enzyme (16). This nuclease-resistant chromatin (NRC) is associated with the nuclear matrix as defined by biochemical criteria (16). Here we report the results of further molecular analysis of the NRC fraction that has led us to identify middle repetitive DNA sequences contained therein, which show sex-specific differential organisation. The probable functional significance of these sequences is discussed.

MATERIALS AND METHODS

Cultures of a species of mealybug, *P.lilanicus*, were maintained on pumpkins at room temperature. All enzymes used were either from Amersham (Aylesbury, UK) or New England Biolabs (Beverley, MA). Hybond N+ from Amersham was used for Southern blotting. Nick translation kit from Boehringer Mannheim was used for labeling DNA for fluorescence *in situ* hybridisation (FISH) probes. Analytical reagents were used for all experiments. The sequences of the oligos used were: A2, GACCACAAAAAGTAGGTACAACCGCTACGCA; A3, ACGTCCACGTCCA-CGTCCACGTCCACGTCCACGTCC

Preparation of nuclei and DNA isolation

Nuclei were isolated from mealybugs as described previously (16). For DNA isolation, nuclei were resuspended in a buffer containing 0.8 M guanidine hydrochloride, 30 mM EDTA, 30 mM Tris–HCl, 5% Tween-20, 0.5% Triton X-100, pH 8.0 and treated with Protease (type XV; Sigma) at a final concentration of 1 mg/ml, for 2 h at 50°C. The suspension was then extracted once with phenol:chloroform (1:1) and precipitated with ethanol.

Restriction enzyme digestion, DNA electrophoresis, Southern blotting and hybridisation were performed according to standard protocols (17). Sequencing of the NRC-specific clones was carried out on either double-stranded plasmid clones denatured by NaOH or single-stranded DNA generated by asymmetric PCR (18). Sequencing was carried out by the dideoxy method using Sequenase (USB-Amersham Plc.) according to supplier's specifications. Densitometric scanning was performed using the I.D. Image Analysis Software of Kodak Digital Science gel documentation system.

Hybridisation was carried out at 42°C in the presence of 50% formamide in 6× SSPE, containing sodium chloride, sodium phosphate and EDTA. Blots were washed with 0.1× SSPE and 0.5% SDS at 65°C (16).

In situ hybridisation

The protocol for preparation of interphase and mitotic cells was according to Kantheti (19). Briefly, embryos were released from gravid females, washed with insect Ringer's and a cell suspension was prepared by gentle homogenisation. The pellet recovered after centrifugation was suspended in a hypotonic solution (0.9% w/v tri-sodium citrate), incubated at room temperature for 20 min and fixed in acetic acid:methanol (1:3 v/v) for 30 min. Fixed cells were dropped on cold wet slides. The slides were pretreated with RNase at 37°C for 1 h, washed with 2× SSC and dehydrated in alcohol. Before hybridisation the slides were treated with 70% formamide in 2× SSC at 65°C for 3 min and immediately dehydrated in cold ethanol. *In situ* hybridisation was carried out according to Johnson *et al.* (20). Probes used in FISH were total NRC DNA (2–100 kb) and inserts from clones nrc8 (187 bp), nrc50 (375 bp) and nrc51 (420 bp) which were labelled with digoxigenin 11-dUTP or biotin 14-dATP by nick translation. Hybridisation was carried out overnight in 50% formamide in 2× SSC at 37°C, washed with 50% formamide in 2× SSC followed by 2× SSC at room temperature for 30 min each. Detection was carried out using rhodamine-conjugated antidigoxigenin or FITC-conjugated avidin (Boehringer Manheim). Nuclei were counterstained with DAPI. Colour digital images were acquired using a triple-bandpass filter set (Chroma Technology, Inc., Brattleboro, VT) in combination with a charge coupled device camera (Photometrics, Inc., Tucson, AZ) and variable excitation wavelength filters (21). Images were analysed using the ISEE software package (Inovision Corp., Durham, NC).

RESULTS

Genomic distribution of NRC sequences

NRC DNA was cloned in the phagemid vector bluescript pSK(II)$^+$ and NRC-specific clones were selected as those which hybridised only to NRC DNA and not to the mononucleosomal DNA (16). It was observed that most NRC DNA clones were unstable in Sure™ (Stratagene) strain of *Escherichia coli*. About 50 clones were obtained with insert sizes ranging from 50 to 700 bp. The ones we have used in the present study are nrc7 (nrc8), nrc50 and nrc51. nrc7 and 8 are two independently isolated clones having the same DNA sequence (16). The genomic distribution of these clones was studied by Southern hybridisation. Restriction-enzyme-digested mealybug genomic DNA was probed with labelled DNA from three NRC-specific clones. The pattern of hybridisation obtained with nrc7 suggests that it is a single copy unique sequence (Fig. 1A) present at a single location in the genome, whereas that obtained with clones nrc50 and nrc51 suggests that they occur at multiple locations within the mealybug genome (Fig. 1B and C).

FISH

The sizes of the probes used were as follows: total NRC DNA (isolated after MNase digestion) ranged in size from 2.0 to >100 kb, nrc8 is 187 bp, nrc50 is 375 bp and nrc51 is 420 bp long. Representative images are shown in Figure 2. In Figure 2A and B, dividing nuclei are hybridised with total NRC DNA labelled with biotin 14-dATP and detected by FITC-conjugated avidin, and nrc8 labelled with digoxigenin 11-dUTP detected by rhodamine-conjugated anti-digoxigenin antibody. Figure 2C and D shows the hybridisation pattern of biotin 14-dATP-labelled nrc50 and digoxigenin 11-dUTP-labelled nrc51. Figure 2C shows male nuclei with darkly staining heterochromatic region in the black and white frame. Figure 2A–C suggests that there is a difference in accessibility of the euchromatic and the heterochromatic genomes to the probes. In Figure 2C, the heterochromatic block, darkly stained as seen in the black and white frame, is not accessible for hybridisation. However, in completely euchromatic nuclei from females (Fig. 2D) nrc50 and nrc51 hybridise all over the genome. In all instances total NRC DNA and DNA from nrc50 and nrc51 show hybridisation all over the accessible regions of the genome while nrc8 shows localised hybridisation shown as punctate signals (Fig. 2B). The nuclei

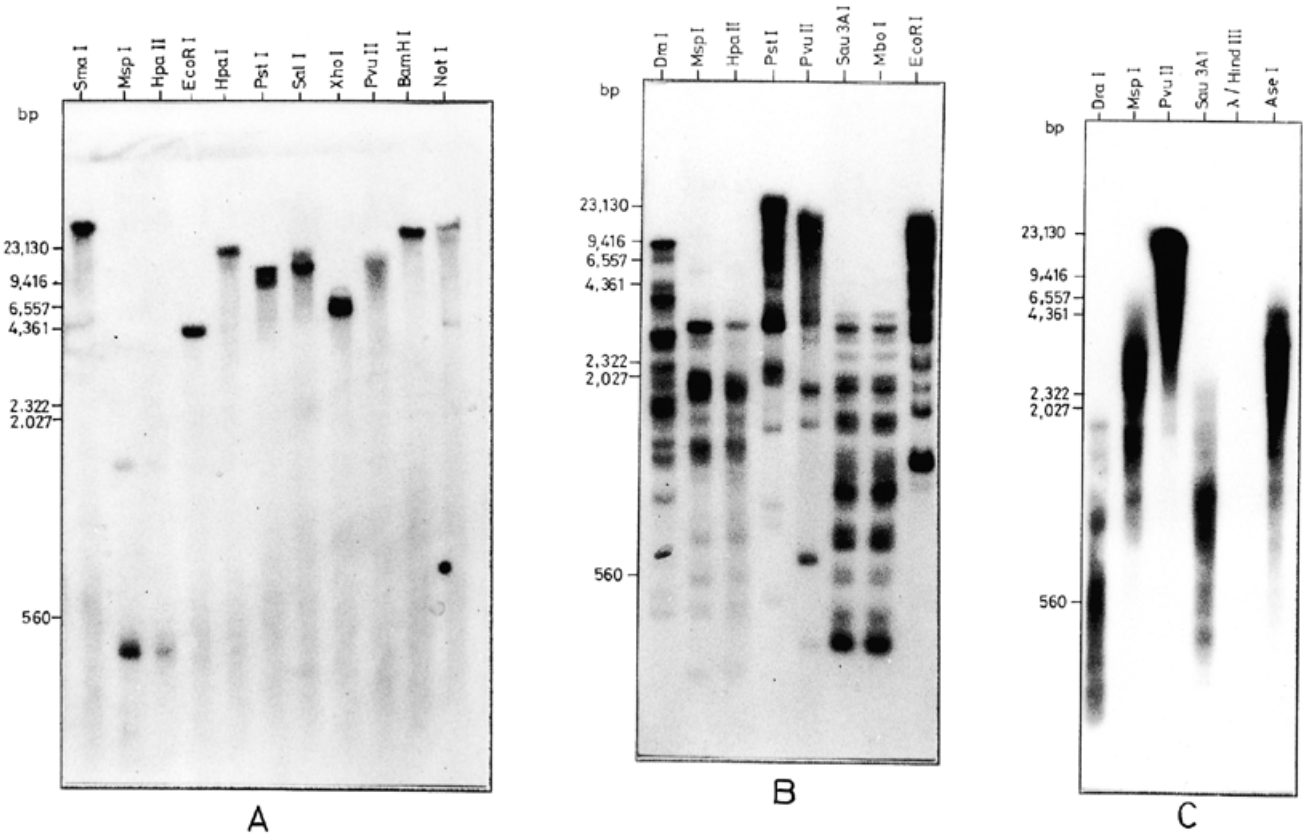

Figure 1. Genomic distribution of NRC-specific sequences. Mealybug genomic DNA digested with different restriction enzymes and electrophoresed on 1.1% agarose gel was Southern blotted and probed with ^{32}P-labelled DNA from different NRC-specific clones: (**A**) nrc7, (**B**) nrc50 and (**C**) nrc51.

were obtained from a mixture of male and female embryos present in the abdomen of female insects. The nucleus shown in Figure 2C is derived from a male embryo as the morphology suggests. On comparison of the results from Figure 2A–C it is suggested that the dividing nuclei represented in Figure 2A and B are derived from male embryos. These were difficult *in situ* experiments as the sizes of the probes were not very large. These results substantiate the results of Southern hybridisation with reference to the genomic distribution of NRC sequences.

Sequence analysis of NRC clones

Inserts from 15 clones have been sequenced. Most sequences have tandem stretches of purines and pyrimidines. The nucleotide sequences of the clones can be accessed from GenBank (accession nos U68042 and U27458). None of the sequences has shown any significant overlap with the nucleotide sequences in the database (GenBank, available in the public domain) except for certain motifs (16). Clone nrc51 is characterised by long stretches of adenines and thymidines. Clone nrc50 has five tandem repeats of a pentanucleotide (CACTA) and six tandem repeats of a hexanucleotide (ACGTCC). These hexanucleotide and pentanucleotide repeats, for which no functional assignment has been made, are present in several sequences in the database.

Localisation of the repeat unit

The restriction maps and nucleotide sequences of nrc50 and nrc51 were utilised to generate different fragments from the inserts and to analyse their pattern of distribution in the genome. nrc50 was restricted with *Pvu*II to generate two fragments, 'A' and 'B' [Figure 3(i)], each fragment was then used individually as a probe on genomic Southerns. The results are shown in Figure 3(ii). nrc50 includes a unique sequence 'B' along with a repeating unit 'A'. To identify the repeat unit within A, a 109 bp fragment at the 5' end of A, designated A1, was obtained by digesting the fragment A with *Acc*I and oligonucleotides designated fragment A2 (31 nucleotides) and A3 (36 nucleotides) were synthesised [Figure 3(i)]. Southern hybridisation performed with these fragments shows that only fragment A3 $(ACGTCC)_6$ is present at multiple locations in the genome. A3 has six repeats of the hexamer (ACGTCC) as revealed by sequencing.

A similar approach was followed for nrc51. Using the enzymes *Pvu*II and *Afl*II, nrc51 was divided into three regions. The pattern of hybridisation of the three fragments of nrc51 with mealybug genomic DNA suggested that they occur at multiple locations in the genome (Figure 4). The pattern of hybridisation of *Ase*I-digested genomic DNA with the three fragments A, B and C as probes is nearly identical. nrc51 has no site for *Ase*I, but has sites for other enzymes (*Dra*I, *Sau*3AI, *Msp*I and *Pvu*II) which have been used in genomic DNA digestion. Therefore, in addition to similarities there are some differences in the pattern of hybridisation of fragments A, B and C with genomic DNA digests. This may suggest the occurrence of nrc51 in different sequence contexts within the genome as against a tandem array at one locus.

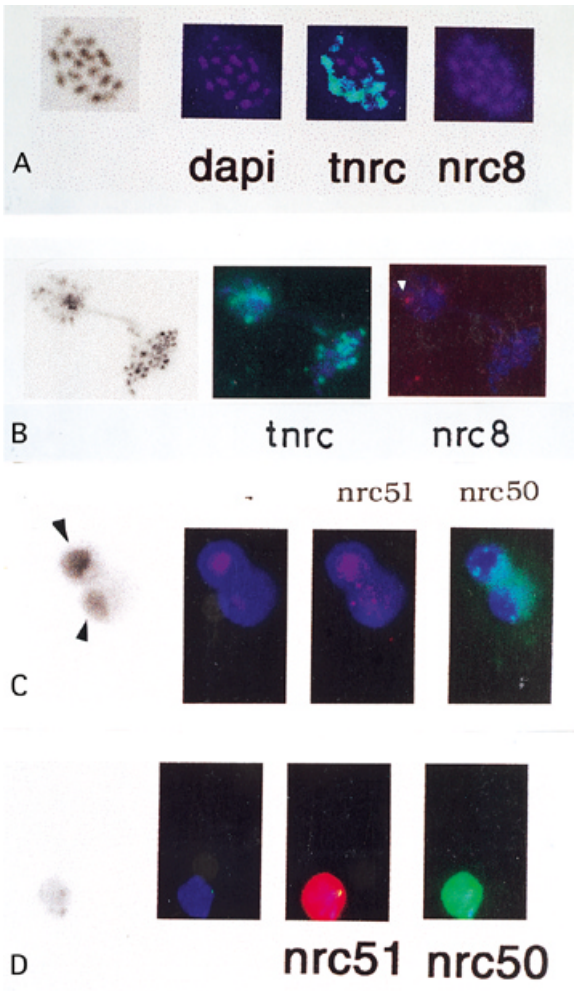

Figure 2. FISH was carried out using nuclei obtained from a mixture of male and female embryos derived from gravid females and specific DNA probes labelled with either biotin 14-dATP or digoxigenin 11-dUTP and detected using FITC-conjugated avidin or rhodamine-conjugated anti-digoxigenin respectively. (**A**) *In situ* hybridisation of a dividing nucleus with total NRC DNA (tnrc) labelled with biotin 14-dATP and nrc8 labelled with digoxigenin 11-dUTP. The figure shows, from left to right, a black and white picture of a DAP1-stained nucleus, DAPI staining, biotin-labelled total NRC DNA detected with FITC, and digoxigenin-labelled nrc8 detected with rhodamine (63×). There are some chromosomes not accessible to the probe tnrc. (**B**) Sequential acquisition of DAPI (black and white image), biotin-labelled total NRC and digoxigenin-labelled nrc8 (63×). This figure is the same as that shown in (A), another region is taken to show the localised hybridisation of nrc8 (marked by an arrow). (**C**) Nuclei hybridised with nrc50 labelled with biotin14-dATP and nrc51 labelled with digoxigenin 11-dUTP. The darkly stained region represents heterochromatic chromosomes (marked by arrows). (**D**) Nucleus hybridised with nrc50 and nrc51 as in (C). The nucleus has only enchromatic genomes.

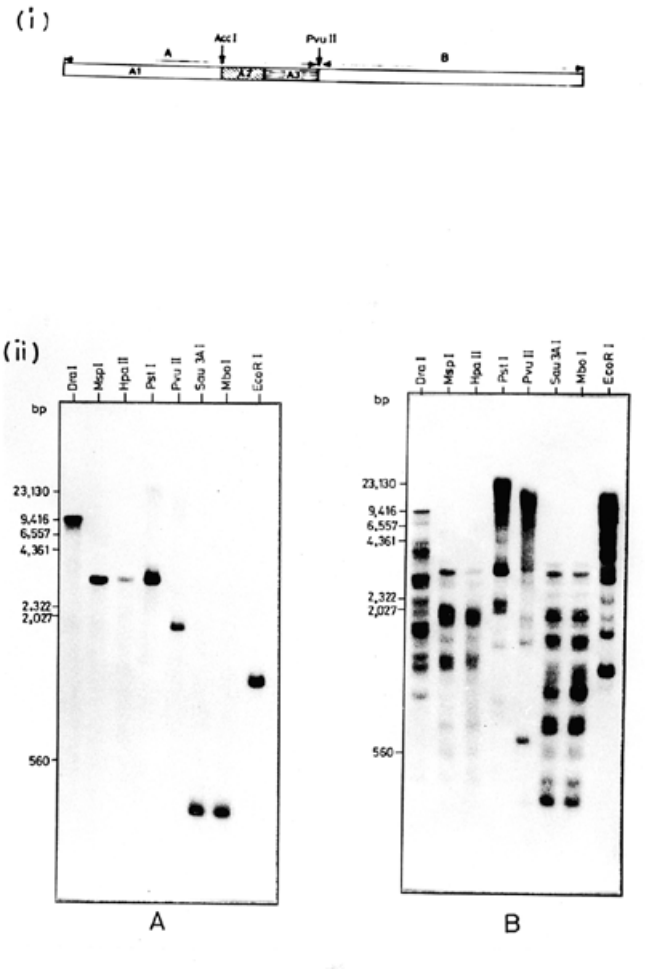

Figure 3. Identification of the repeat unit in nrc50. Restriction-enzyme-digested genomic DNA was Southern blotted after electrophoresis on 1.1% agarose gel and probed with DNA from different regions of clone nrc50 as indicated. (i) Representation of the insert in nrc50, showing the appropriate restriction sites and the nomenclature used for different regions. (ii) Autoradiograms of Southern hybridisations with different regions of nrc50 as indicated (A and B).

Organisation of middle repetitive sequences into chromatin

In order to investigate the status of chromatin within the repetitive sequences, nuclei from male and female mealybugs were treated with micrococcal nuclease or DNase I. The DNA derived was fractionated on agarose gels and Southern hybridised with DNA from clones nrc50 and nrc51. The results are shown in Figure 5. As shown previously (16) ~10% of the genome is resistant to nucleases, both MNase and DNase specifically in the males. But nrc50 shows 1:1 (lane 4) and nrc51 shows 5:1 (lane 6) distribution of intensity between the NRC fraction and the nuclease-susceptible fraction. In the females nrc50 is organised exclusively into nuclease-susceptible chromatin but nrc51 shows hybridisation at the NRC fraction though there is no ethidium bromide stainable DNA in this part of the gel (lane 5).

When nuclei are treated with DNase (16 U/10 OD of nuclei) there is a time-dependent decrease in the intensity of nrc51 hybridisation from undigested nuclei to those incubated with DNase I. Up to 25–30% hybridisation intensity remains resistant to DNase I in nuclei from males, while there is no detectable hybridisation under similar conditions in the nuclei from females (data not shown).

Distribution of nrc50 and nrc51 sequences within total NRC complement

It has been shown previously that NRC comprises heterogeneous DNA sequences ranging in size from 2 to 100 kb (16). If the repetitive sequences represented by nrc50 and nrc51

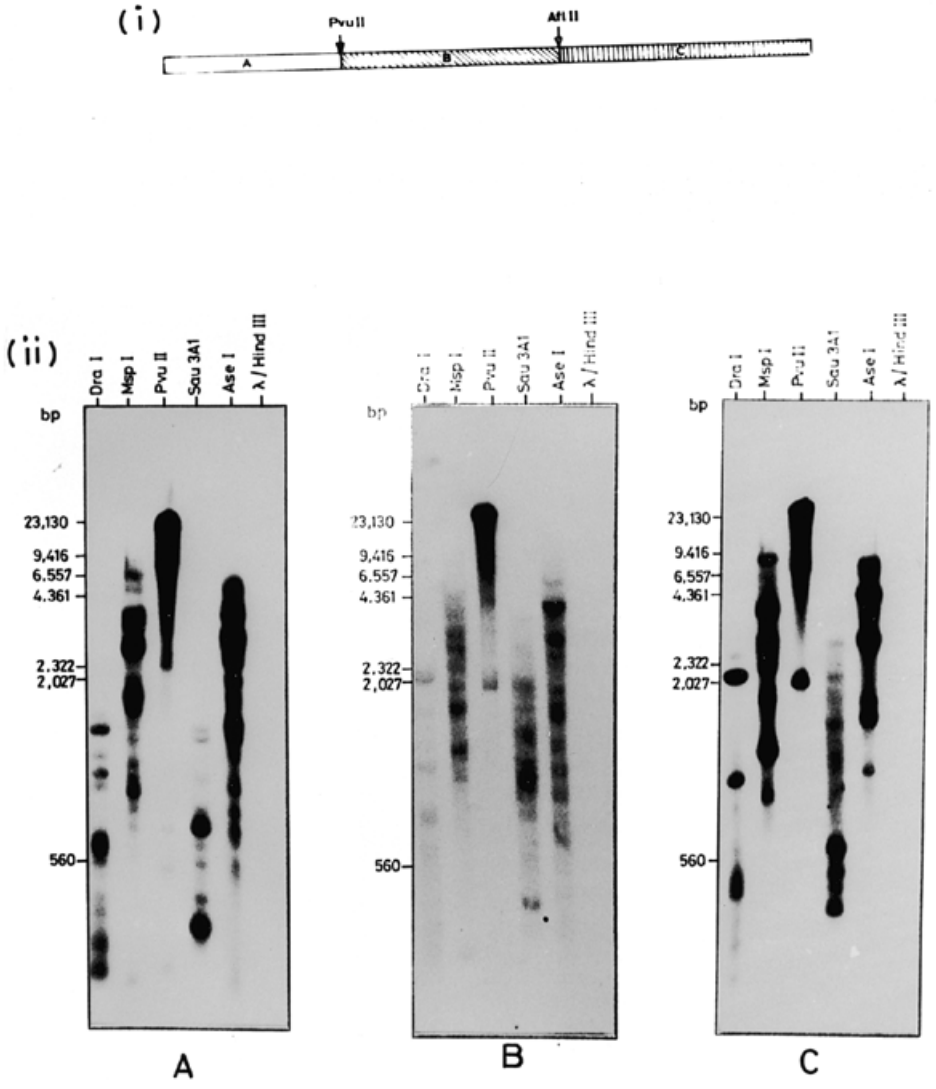

Figure 4. Identification of the repeat unit in nrc51. Southern hybridisations of mealybug genomic DNA with fragments from different regions of a clone nrc51 as probes. (**i**) Linear representation of nrc51 sequence showing the appropriate restriction sites and the nomenclature used for different regions. (**ii**) Autoradiograms of Southern hybridisations with different regions of nrc51 as indicated (A–C).

have any functional significance in organising DNA sequences into a nuclease-resistant conformation, they are expected to be part of several DNA fragments that are recovered in the NRC fraction. The distribution of nrc50 and nrc51 sequences within total NRC was therefore analyzed. Southern hybridisation of NRC DNA separated on low percentage agarose gel (0.4%) with clones nrc50 and nrc51 as probes was carried out. Figure 6 shows that nrc50 and nrc51 are not confined to one particular fragment in NRC but are distributed over several fragments ranging in size from 2 to >50 kb.

DISCUSSION

As mentioned earlier, in male mealybugs 50% of the genome, that is the entire genomic contribution from the male parent, appears heterochromatic cytologically and is transcriptionally silenced. We have shown previously that it is only ~10% of the male genome that has an unusual chromatin organisation which results in resistance to both MNase and DNase I (16). In an analysis of the nature of DNA sequences within NRC described here it is found that there are two classes of sequences that show unusual organisation: single-copy sequences like nrc7 and middle repetitive sequences like nrc50 and nrc51. The sequences represented in nrc50 and nrc51 are distributed all over the genome as shown by Southern hybridisation as well as FISH. The repeat unit within nrc50 is identified as $(ACGTCC)_6$. The other fragments from the insert in nrc50 are not repetitive. This suggests that the hexanucleotide $(ACGTCC)_6$ occurs in the context of different unique sequences. A similar analysis with nrc51 leads us to conclude that either nrc51 represents another repeat unit by itself or is a part of a larger repeat unit.

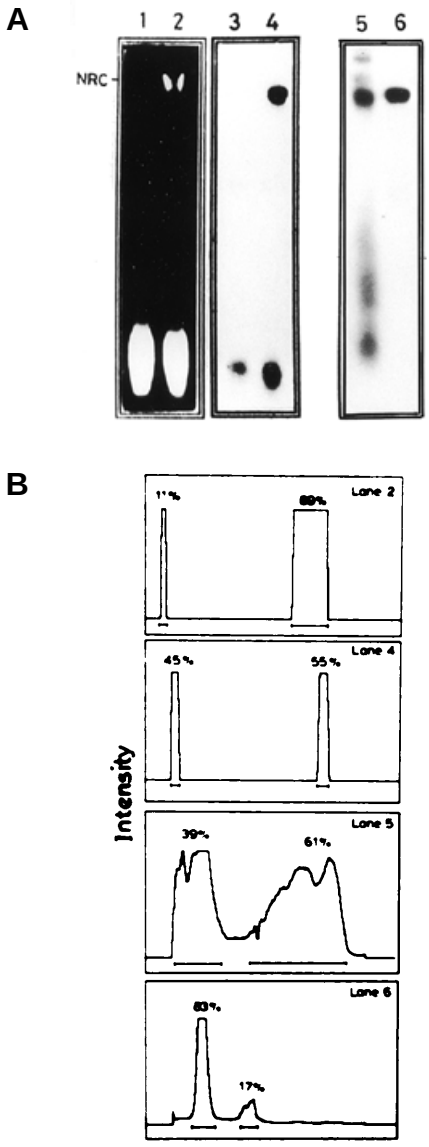

Figure 5. Hybridisation of nrc50 and nrc51 DNA to MNase digests of male and female chromatin. DNA extracted from chromatin digested with MNase for 60 min was electrophoresed on a 2% agarose gel, Southern blotted and probed with DNA from the clones nrc50 and nrc51. (**A**) Lanes 1, 3 and 5, MNase-digested female chromatin; lanes 2, 4 and 6, MNase digests of male chromatin. Lanes 1 and 2, ethidium bromide staining pattern; lanes 3 and 4, hybridisation pattern with nrc50; lanes 5 and 6, hybridisation pattern with nrc51. (**B**) Densitometric scan of lanes 2, 4, 5 and 6. The ratios of the peaks are represented as a percentage of the total intensity in a given lane, calculated in terms of net intensity which is the sum of the background subtracted pixel values in the band rectangle.

The two clones nrc50 and nrc51 containing middle repetitive sequences show a higher propensity to become organised into an NRC fraction. The enrichment of nrc50 and nrc51 sequences within the NRC is clear from the distribution of the nearly 50% of nrc50 sequences and 83% of nrc51 sequences in the unusually organised NRC fraction compared to the mono-nucleosomal fraction in MNase digests.

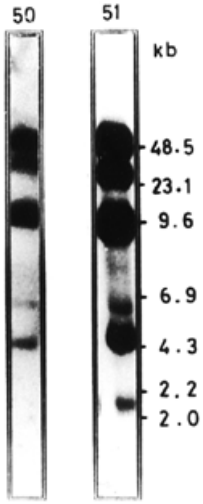

Figure 6. Hybridisation of nrc50 and nrc51 within the total NRC complement. The autoradiograms show the hybridisation of NRC DNA fractionated on 0.4% agarose gel, with ^{32}P-labelled nrc50 and nrc51.

The differential organisation of nrc51 is also reflected in the nearly complete and partial susceptibility to DNase I of nrc51 chromatin in females and males respectively. There is 25–30% remaining resistant to DNase I in males while there is no detectable hybridisation in females under the same conditions. However, there is intense hybridisation of DNA derived from nuclei of both females and males in samples not treated with DNase I. This suggests that the middle repetitive sequences represented by nrc50 and nrc51 are organised differently not only between the two sexes but within the male nuclei. In the male nuclei nrc50 and nrc51, like sequences on the maternally inherited euchromatic genome, are likely to be susceptible, while those on the paternally inherited heterochromatised chromosomes are organised into a nuclease-resistant conformation.

It is observed that nrc51 hybridises to the high molecular weight DNA region in female chromatin digests even though ethidium bromide staining in this region of the gel is not obvious (Fig. 5A). One interpretation of the results shown in Figure 5 could be that nrc51 sequences are not accessible to MNase even in euchromatic chromosomes, and may represent sequences involved in centromeric activity which is dispersed along the length of the chromosomes (14). It is also possible that there are contaminating male nuclei either because of insemination or due to mix-up of some male insects; morphological distinction between the two sexes is not marked at this stage in development (II instar). This contention is supported by the fact that in nuclei derived from females (Fig. 5B, lane 5) there is a much lower percentage of hybridisation to the NRC region compared to that from males and it is variable between experiments, and is absent in DNase I digests. The ability of DNase to cleave within nucleosomes may be one of the reasons for the difference in the percentage of resistant fraction seen in nuclei derived from males in DNase and MNase digestions. It has been shown that NRC is associated with nuclear matrix

(16). On isolation of matrix from male- and female-derived nuclei it is observed that nrc51 sequences are detected in the matrix fraction only in males and not in females (unpublished data).

Probable functional significance

The differential organisation of homologous chromosomes seen in mealybugs is similar to the inactivation of one of the X chromosomes in female mammals (22,23). In contrast to the single inactivation centre (Xic) demonstrated for the mammalian X chromosome (24,25), cytology of males derived from irradiated fathers suggested the presence of multiple centres of inactivation in the mealybug genome (13,14). Given this background, the distribution of nrc50 and nrc51 in mealybug genome, their presence in several of the heterogeneous NRC DNA fragments (Fig. 6) and their enrichment within the unusually organised chromatin of the male would raise the possibility of examining them as putative candidate sequences for multiple inactivation centres in the mealybug genome. There probably are two classes of sequences present as NRC: (i) those that mediate unusual chromatin organisation and (ii) those that are organised as NRC because of their proximity to the former class of sequences. This would explain why sequences as large as 100 kb exhibit nuclease resistance and matrix association. Efforts to screen nrc50- and nrc51-like sequences for their ability to influence chromatin organisation *in vitro* are under way.

It is argued that repetitive sequences associate with heterochromatin due to their 'hitch-hiking' tendency but this appears an unlikely explanation in the present case as the heterochromatic chromosomes are lost during meiosis and therefore do not contribute to sperm nuclei (12).

Earlier results from our laboratory (16) suggest that unusual chromatin organisation is perpetuated through spermatogenesis as NRC is detected in the sperm nuclei that are exclusively formed by the somatically euchromatic, maternally inherited genome of males (Fig. 7). Thus, it is possible that structural organisation of NRC is perpetuated through not only mitosis but also meiosis (8,9). Thus, the differential organisation of homologous chromosomes within the same nucleus and between the two sexes in mealybugs is a system where protein-mediated chromatin organisation as an epigenetic marking appears to be a distinct possibility.

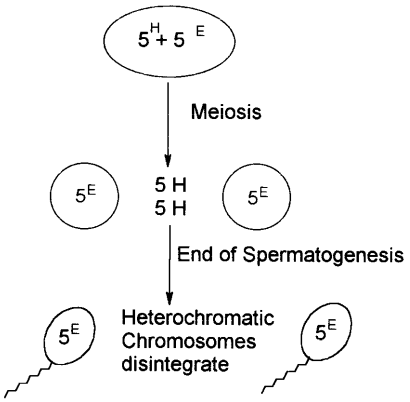

Figure 7. Schematic representation of spermatogenesis in mealybugs. H, heterochromatic haploid set; E, euchromatic haploid set.

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