HETEROCHROMATIN IN DROSOPHILA NASUTA : RESTRICTION ENZYME DIGESTION OF CYTOLOGICAL PREPARATIONS AND GENOMIC DNA.

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SUMMARY. In situ digestion of metaphase and polytene chromosomes and of interphase nuclei in different cell types of D.nasuta with restriction enzymes revealed that enzymes like AluI, EcoRI, HaeIII, Sau3a and SinI did not affect Giemsa-stainability of heterochromatin while that of euchromatin was significantly reduced; TaqI and SalI digested both heterochromatin and euchromatin in mitotic chromosomes. Digestion of genomic DNA with AluI, EcoRI, HaeIII, Sau3a and KpnI left a >21kb DNA band undigested in agarose gels while with TaqI, no such undigested band was seen. The AluI-resistant >21kb DNA hybridized in situ specifically with the heterochromatic chromocentre. Purified AT-rich satellite DNA of D.nasuta also hybridized in situ with heterochromatin regions while in southern hybridization, it hybridized specifically with the AluI-resistant >21kb DNA band. It is concluded that the satellite and other highly repetitive sequences present in the different heterochromatin blocks in karyotype of D.nasuta are remarkably homogeneous in their base sequence composition.

INTRODUCTION

A substantial amount of the genome of Drosophila nasuta is present as large pericentromeric blocks of heterochromatin on all the three pairs of larger chromosomes (1). Earlier cytological studies revealed these different blocks of heterochromatin of D.nasuta to be remarkably similar in their various attributes : i) C- and fluorescence banding patterns of all heterochromatin blocks were identical (1); ii) all the different blocks of heterochromatin coalesced together to form a single compact chromocentre in interphase and polytene nuclei (1,2); iii) on the basis of 5-bromodeoxyuridine-Giemsa staining patterns, it was inferred that all the heterochromatic regions contain asymmetric A-T rich DNA sequences (3); iv) DNA-ligands like Hoechst 33258, Distamycin A and Netropsin affected condensation of the different blocks of heterochromatin identically (4,5) and finally, v) all heterochromatin regions replicated synchronously in the late S (3,6). On the basis of these features, it appeared that all the heterochromatin regions in the genome of D.nasuta shared similar asymmetric A-T rich DNA sequences. An analysis of the satellite DNA of D.nasuta by Ranganath et al (7) revealed that there is only one A-T rich satellite in this species, which is present on all the heterochromatin blocks and which

accounts for only about 7-8% of total nuclear DNA. Since cytologically, the heterochromatin accounts for nearly 40% of the length of mitotic chromosomes of D.nasuta (1), it is obvious that other non-satellite DNA sequences comprise bulk of the heterochromatin of D.nasuta. The nature of these sequences is not known.

In recent years, in situ digestion of aceto-methanol fixed chromosomes with restriction endonucleases has been found to result in diverse banding patterns which allowed analysis of molecular organization of DNA sequences present in different regions (8-13). Differential staining of different chromosome regions following in situ digestion of fixed chromosomes with specific restriction enzymes has been suggested to be due to the differential loss of DNA. The differential loss could be related either to differential accessibility of the target sequences to cleavage by the restriction enzyme due to particular structural organization of chromatin domains or more likely to a differential distribution of the target sequences in different chromatin fractions (9,12-14). Thus restriction enzyme digestion of fixed cytological preparations is particularly useful in molecular differentiation of heterochromatin regions of different chromosomes or chromosome regions, which may appear similar in other cytological features (9,11-13).

With a view to know if the different heterochromatic regions in D.nasuta differ in some of their molecular properties, we examined effects of different restriction enzymes on cytological preparations of several cell types of D.nasuta. In addition, we also looked at the relationship between the AT-rich satellite DNA of D.nasuta and the DNA fraction that remained undigested with restriction enzymes like AluI. Our results showed that, in keeping with the earlier noted cytological uniformity, no difference was found between the different blocks of heterochromatin in chromosomes of D.nasuta with respect to sensitivity to restriction enzyme digestion in situ also. Satellite as well as other non-satellite (presumably highly repetitive) sequences present in the different heterochromatin blocks appear to be deficient in recognition sites for enzymes like AluI.

MATERIALS AND METHODS

A wild type strain of Drosophila nasuta, maintained in laboratory on standard food at 20°±1°C, was used.

Restriction enzyme digestion of cytological preparations

Metaphase chromosome preparations of brain ganglia of late third instar larvae were made by the air-dry method as described by Lakhotia and Kumar (1). Polytene chromosome squashes were obtained from salivary glands of late third instar larvae in the usual manner except that the aceto-orcein/carmine staining step prior to squashing was omitted. In addition, squash preparations of aceto-methanol (1:3) fixed interphase cells from early embryos (~4h post-oviposition), brain ganglia of late third instar larvae, pupae and adults and the ovarian follicle and nurse cells of adult females were also made in 50% acetic acid. Coverslips of squash preparations were flipped off with a razor blade after the preparations were stored at -70°C for 5 to 16h. The slides were rinsed in absolute ethanol and air-dried.

The chromosome preparations of larval brain ganglia were digested with the following restriction endonucleases : AluI, EcoRI, HaeIII, Sau3a, SalI, SinI and TaqI. All other cytological preparations were digested only with AluI. For digestion of the cytological preparations with restriction endonucleases, 20-25ul of appropriate reaction buffer containing 10-30 units of the enzyme was put on the slide, covered with a coverslip and incubated at 37°C (65°C in case of TaqI) for 16-20h. After completion of digestion, the slides were washed in 5mM EDTA, dehydrated through ethanol grades and air-dried. Parallel control slides were incubated only in the respective buffer without the enzyme. Finally, the preparations were stained with 5% Giemsa, mounted with D.P.X. mountant and examined by bright-field microscopy.

Restriction digestion of genomic DNA

Genomic DNA from adult male flies was purified by the usual procedure involving SDS-Proteinase K lysis, Phenol-chloroform extraction, ethanol precipitation and RNAse treatment. Only unsheared DNA preparations were used for restriction digestion. Purified DNA samples were digested with AluI, EcoRI, HaeIII, Sau3a, KpnI or TaqI using appropriate reaction buffers and other conditions. To ensure complete digestion, excess enzyme (5-10 units/ug DNA) was used over a period of 16h. The digested DNA samples were fractionated on standard 0.8% Agarose gels containing ethidium bromide (15). HindIII digested lambda-DNA was used as the size marker.

Electroelution of AluI-resistant high mol wt DNA

A >21kb genomic DNA band left undigested by AluI (see Results) was electroeluted from preparatory 0.8% agarose gels. After completion of the gel run, the bright band at >21kb position was cut with a sharp razor blade and its DNA eluted following Maniatis et al (15).

Isolation of satellite DNA from D.nasuta

The A-T rich satellite (7) in the genomic DNA of D.nasuta was isolated using $CsCl_2$ gradient containing Hoechst 33258 as described by Manuelidis (16). The samples were centrifuged at 38000 rpm for 12h in a Beckman L870M

ultracentrifuge using a vertical rotor (Ti65a). The satellite DNA band was isolated, dialysed overnight against .5x TBE, extracted twice with phenol-chloroform and chloroform-isoamyl alcohol and precipitated with ethanol and dissolved in TE (10mM Tris, 1mM EDTA, pH 8).

In situ and Southern hybridization

The electroeluted AluI DNA and the satellite DNA were nick-translated using either ^{3}H-dNTPs (all four labelled dNTPs from Amersham) or ^{35}S-dATP (Amersham) and used for in situ hybridization with preparations of larval brain ganglia and salivary glands of D.nasuta following Pardue (17).

AluI digested genomic DNA of D.nasuta was separated on 0.8% Agarose gel and blotted on Genescreen nylon membrane using 6XSSC as transfer medium. UV-crosslinked DNA on the membrane was hybridized with isolated satellite DNA labelled by nick-translation using ^{32}P-dCTP (Amersham) as per Maniatis et al (15).

RESULTS

Restriction digestion of metaphase chromosomes

Examples of stained metaphase plates digested with the different restriction endonucleases are shown in figure 1. It was seen that except for SalI and TaqI, all other enzymes produced a typical C-band staining of metaphase chromosomes: digestion with AluI, EcoRI, HaeIII, Sau3a and SinI caused very reduced Giemsa staining of all euchromatic regions while the heterochromatin blocks on all chromosomes appeared very dark stained as seen after typical C-banding (1). With these restriction enzymes, the Giemsa staining of Y chromosome (see inset in fig. 1b) also closely

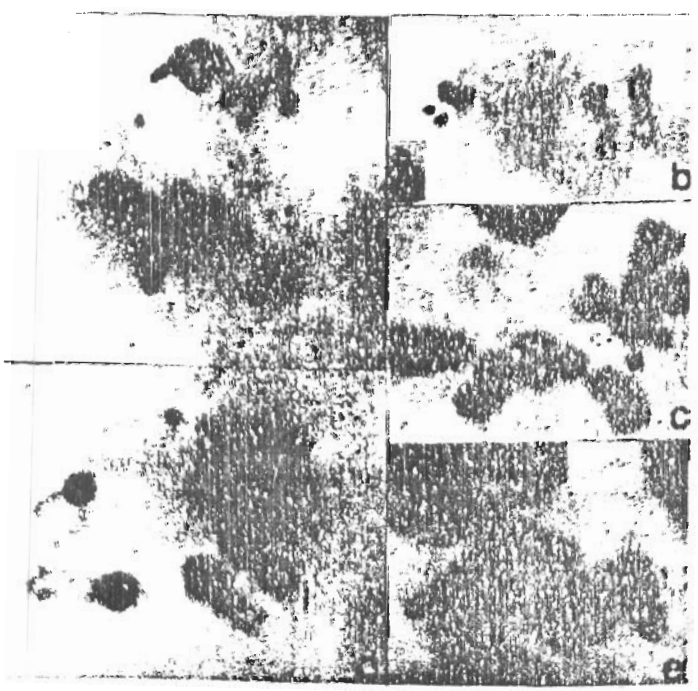


Fig.1 Giemsa stained metaphase plates from brain ganglia of D.nasuta larvae : (a) control (no enzyme) or after different enzyme treatments (b - AluI, c - EcoRI, d - Sau3a and e - HaeIII). The inset in b shows Y-chromosome from a male metaphase after AluI digestion.

resembled the pattern seen after C-banding (1). No notable difference was found between the Giemsa staining pattern of metaphases digested with the above 5 restriction enzymes (Fig.1). However, digestion with SalI or TaqI resulted in a significant reduction of Giemsa stainability of both eu- as well as heterochromatin regions. Due to the poor stainability, metaphases digested with TaqI or SalI are not illustrated. None of the enzymes produced any banding in the euchromatin regions (Fig.1)

Giemsa staining of other cell types after AluI digestion

AluI-digested polytene chromosomes in squash preparations of salivary glands of D.nasuta stained poorly with Giemsa except for the whole of alpha-heterochromatin in the chromocentre (2), a band at the base and one band in middle of chromosome 4 (fig. 2a). The intranucleolar DNA mass (18) also appeared to be less affected by AluI digestion. AluI digested interphase nuclei from embryos or brain ganglia of larvae, pupae or adult showed intense staining of only the single chromocentre with rest of the nuclear chromatin appearing very light stained. Examples of control and AluI digested cells of larval brain ganglia are shown in fig. 2b-c.

Ovarian follicle and nurse cells in adult females endoreplicate, with the latter being highly polyploid. In both cell types, AluI digestion reduced Giemsa staining of all regions except the single chromocentre (fig. 2d) which remained as darkly stained as in control nuclei. It is significant that in spite of their different degrees of endoreplication, size of the chromocentre was the same as in diploid embryonic cells.

Thus in every cell type examined, the heterochromatic chromocentre was found to be completely resistant to AluI digestion.

Restriction endonuclease digestion of genomic DNA

Ethidium bromide staining of genomic DNA from adult males of D.nasuta, digested with the different enzymes mentioned in Materials and Methods and separated on 0.8% Agarose gels, revealed that all enzymes, except TaqI, left a high mol wt DNA band (>21kb) undigested (fig. 3). As a result, the >21kb AluI-resistant band appeared very distinct. After TaqI digestion, the >21kb band was not seen (fig. 3).

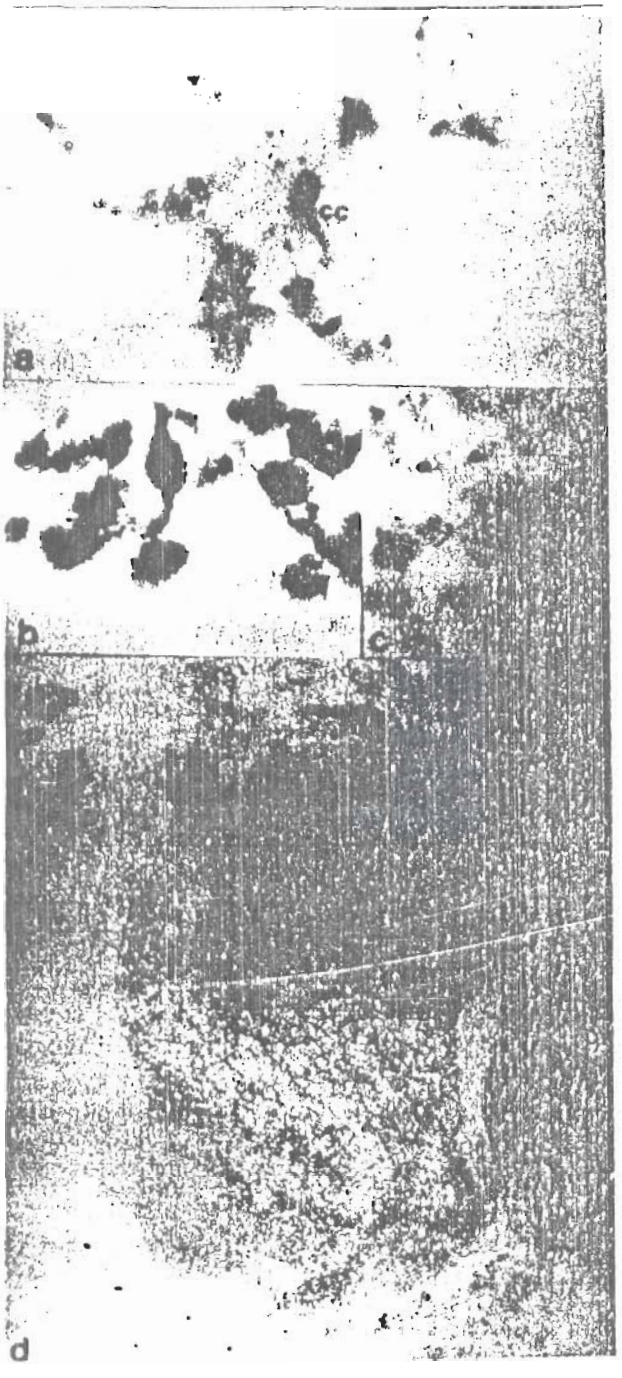


Fig. 2 Effect of AluI digestion on different cell types of D.nasuta : **a** - chromocentre (cc) and chromosome 4 (4) of AluI digested polytene nucleus showing intense staining of the alpha-heterochromatin and of two bands on chromosome 4; **b** and **c** - control (b) and AluI (c) treated cells from larval brain ganglia; **d** - a large nurse cell (NC) and a group of follicle cells (FC); arrow marks the small chromocentre in the highly endoreplicated nurse cell.

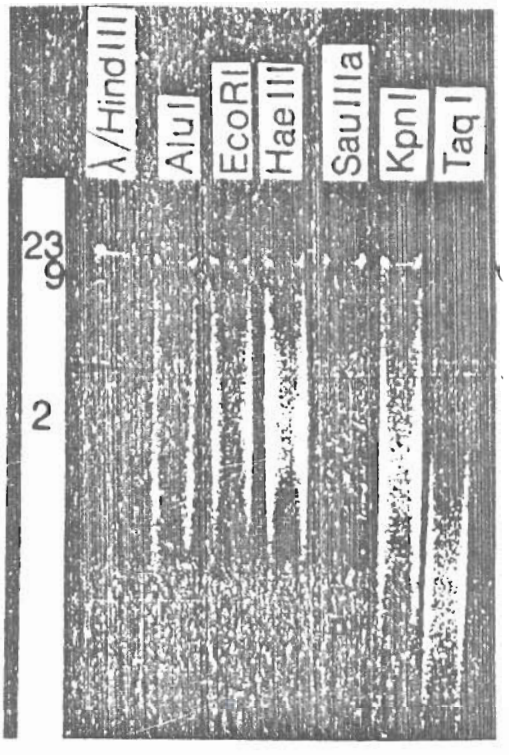
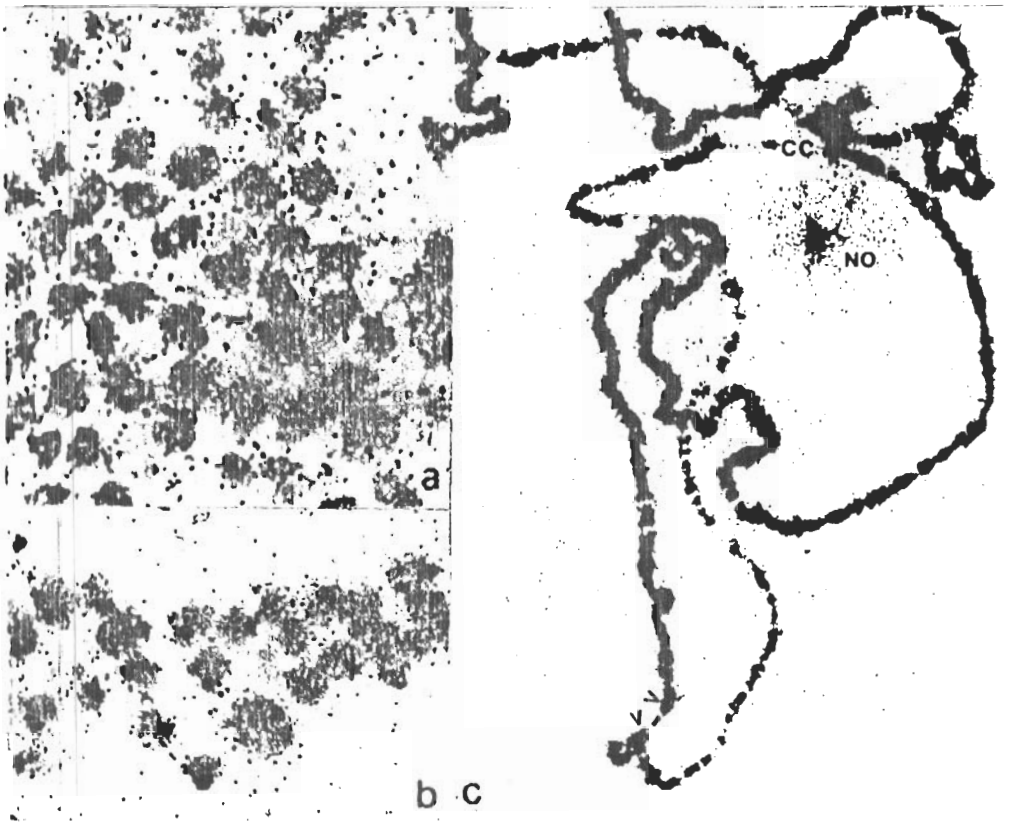


Fig. 3 Ethidium bromide staining of genomic DNA of D.nasuta digested with the various enzymes indicated. Molecular weights (in kb) of some of the marker bands in HindIII digested lambda DNA lane are indicated. Note the bright band at top in all genomic DNA lanes except TaqI.

In situ hybridization of AluI-resistant and satellite DNA

After isolation of the >21kb AluI resistant genomic DNA of D.nasuta, it was labelled by nick-translation and hybridized in situ with brain cell nuclei

of D.nasuta. As seen in the example in fig. 4a, the hybridization was more or less restricted to the heterochromatic chromocentre region only. Likewise, the purified AT-rich satellite DNA of D.nasuta also hybridized in situ to the chromocentre only in brain cells (fig. 4b). When the satellite DNA was hybridized in situ to polytene chromosome squashes, most prominent labelling was noted on the alpha-heterochromatin and intra-nucleolar DNA. In addition, distinct labelling was also seen on telomeres of some chromosomes (fig. 4c).



< Fig.4

Fig.5

Fig. 4 In situ hybridization of the >21kb AluI-resistant DNA with larval brain nuclei (a), of the satellite DNA with brain nuclei (b) and with polytene chromosomes (c) of D.nasuta. In c, the nucleolus (NO), chromocentre (CC) and tips of chromosomes X and 3 (arrowheads) are indicated.

Fig. 5 Southern hybridization of isolated satellite DNA of D.nasuta with AluI digested genomic DNA of D.nasuta - the left lane shows EtBr staining of the AluI digested DNA while the right lane shows the autoradiogram after southern hybridization.

Southern hybridization of satellite DNA with AluI digested genomic DNA

AluI digested genomic DNA was transferred to nylon membrane and hybridized with ^{32}P-labelled satellite DNA of D.nasuta. As seen in fig. 5, only the >21kb AluI-resistant band hybridized with the satellite DNA.

DISCUSSION

Earlier cytological studies in our laboratory showed that the different pericentromeric heterochromatin blocks in chromosomes of D.nasuta were remarkably similar in their C- and fluorescence-banding, 5-bromodeoxyuridine Giemsa staining and sensitivity to DNA-ligands like Hoechst 33258, Distamycin A etc (1,3-5). The present results showed that the cytological similarity of the different heterochromatin blocks in chromosomes of D.nasuta is due to similarity at the molecular level. Except SalI and TaqI, none of the other restriction enzymes tested in this study affected Giemsa stainability of any of the heterochromatin blocks in cytological preparations of D.nasuta, although all euchromatin regions were severely affected. Refractoriness of heterochromatin to the action of these enzymes could be due either to particular properties of chromatin structure and organization of heterochromatin which did not allow action of these enzymes or to absence of recognition sites for these enzymes in the DNA sequences comprising heterochromatin in D.nasuta. Although the first alternative cannot be ruled out, the latter possibility appears more likely in view of the earlier reports in literature (9,10,13,14) and the following observations in the present study: i) while AluI did not affect heterochromatin in any of the cell types (interphase cells in embryo or brain ganglia; mitotic cells in larval brain; polytene nuclei in larval salivary glands and polyploid nuclei in ovarian follicle and nurse cells), SalI and TaqI appeared to readily affect heterochromatin regions of mitotic cells; thus the condensed heterochromatin regions were not totally refractory to loss of chromicity following restriction endonuclease digestion in situ; ii) a high mol wt DNA band was left undigested in purified genomic DNA of D.nasuta by all those enzymes that also did not affect heterochromatin staining in situ while enzymes like TaqI which digested heterochromatin, also did not leave a high mol wt DNA band in gels and iii) the specific in situ hybridization of the gel purified high mol

wt AluI resistant DNA with chromocentre heterochromatin showed that the heterochromatin of D.nasuta contains DNA sequences that do not have or have only infrequent sites for AluI. Therefore in all likelihood the C-band effect of AluI and the other restriction enzymes seen in this study is due to the DNA sequences in heterochromatin of D.nasuta being poor in recognition sites for the enzymes.

D.nasuta genomic DNA has only one AT rich satellite fraction (7). Our results showed that this satellite DNA hybridized specifically to the AluI-resistant high mol wt DNA in southern blots, while in cytological preparations, it hybridized mainly with the chromocentre heterochromatin. This observation suggests that the satellite DNA of D.nasuta is devoid of or has infrequent AluI sites. Since enzymes like EcoRI, HaeIII, Sau3a, SinI etc also do not affect heterochromatin of D.nasuta, sites for these enzymes may also be infrequent in the satellite DNA fraction.

Cytologically, the heterochromatin content in D.nasuta chromosomes is about 40% of chromosome length (1) while the single satellite sequence was reported (7) to be only about 7-8% of D.nasuta genome. If this is indeed so, much of the heterochromatin in D.nasuta should be comprised of other non-satellite DNA sequences. In the light of present results it would therefore appear that sites for enzymes like AluI are infrequent in these non-satellite DNA sequences too and that these sequences are more or less uniformly distributed in different blocks of heterochromatin.

Satellite and highly repetitive sequences comprising heterochromatin are known to be underreplicated in endoreplicating cells of Drosophila (see 19,20 for recent reviews). Accordingly the size of the AluI-resistant heterochromatic chromocentre in highly polytenized salivary gland nuclei as well as in the endoreplicating follicle and nurse cells was found to be small. Hammond and Laird (21) compared the extent of underreplication and the spatial organization of satellite and certain other repetitive sequences in these three cell types of D.melanogaster : they concluded that in the follicle cells which undergo only 2-3 endoreplication cycles, the satellite DNA sequences remain at 2C level while in the highly endoreplicated nurse cells, the satellite sequences replicate in later endoreplication cycles. Moreover, these authors also concluded that in the

nurse cells, the satellite sequences associated with different heterochromatin blocks are not as tightly held together as in salivary gland polytene nuclei but are more loosely associated and in rare cases may even be widely separated so that a compact chromocentre perhaps does not exist in nurse cells of D.melanogaster. Our present results revealed a different organization of heterochromatin in follicle and nurse cells of D.nasuta. The AluI-resistant dark-stained chromocentre in the very highly endoreplicated large nurse cells was as small as in the follicle or early embryonic cells. Moreover, like in embryonic, brain or follicle cells, the AluI-resistant chromocentre was always seen as a single compact block in the ovarian nurse cells of D.nasuta. This means that unlike the situation reported in nurse cells of D.melanogaster (21), the pericentromeric heterochromatin blocks of different chromosomes of D.nasuta are as tightly associated with each other as in typical polytene or mitotic cell types. The differences in the spatial organization of heterochromatin in ovarian nurse cells of D.melanogaster (21) and in D.nasuta (present results) may be related to the fact that while the heterochromatin in D.melanogaster is comprised of more than one type of satellite sequences (22), the DNA sequences in heterochromatin of D.nasuta are, as noted above, much more homogeneous and thus may condense together. However, another point may also be noted in this context : Hammond and Laird (21) used in situ hybridization of satellite sequences with nurse cells to monitor the quantity (extent of endoreplication) and spatial distribution of heterochromatin. This approach would detect the satellite sequences present anywhere in the nucleus, not necessarily only those that are located within the heterochromatin only and thus the information so obtained cannot be directly correlated to chromocentre. In our case, the cytological identity of chromocentre is very distinct leaving no scope for such ambiguity. Indeed, using Hoechst 33258 fluorescence to locate heterochromatin, we found the chromocentre in ovarian nurse cells of D.melanogaster to be organized as compactly as in the other cell-types (unpublished observations).

None of the restriction enzymes used in our study produced any banding patterns in the euchromatin regions of mitotic chromosomes although a

majority of these enzymes are known to produce G- or R-bands in mammalian metaphase chromosomes (13). Mitotic chromosomes of Drosophila do not show G-bands or replication bands also (20,23). The absence of restriction enzyme-induced banding of mitotic chromosomes of Drosophila further supports the view that the functional and higher order organization of mitotic chromosomes is different in Drosophila and mammals (20).

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