

Restriction enzyme digestion of heterochromatin in *Drosophila nasuta*

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Abstract. *In situ* digestion of metaphase and polytene chromosomes and of interphase nuclei in different cell types of *Drosophila 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 *Sall* digested both heterochromatin and euchromatin in mitotic chromosomes. Digestion of genomic DNA with *AluI*, *EcoRI*, *HaeIII*, *Sau3a* and *KpnI* left a 23 kb DNA band undigested in agarose gels while with *TaqI*, no such undigested band was seen. The *AluI* resistant 23 kb DNA hybridized *in situ* specifically with the heterochromatic chromocentre. It appears that the digestibility of heterochromatin region in genome of *Drosophila nasuta* with the tested restriction enzymes is dependent on the availability of their recognition sites.

Keywords. *Drosophila*; heterochromatin: restriction enzyme digestion.

1. 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, occupying nearly 40% of the length of the mitotic chromosomes (Lakhotia and Kumar 1978). Earlier cytological studies revealed these different blocks of heterochromatin of *D. nasuta* to be remarkably similar in their various attributes such as C- and fluorescence banding patterns (Lakhotia and Kumar 1978), coalescing together to form a single compact chromocentre in interphase and polytene nuclei (Lakhotia and Kumar 1978; Kumar and Lakhotia 1977), containing asymmetric A-T rich DNA sequence (Lakhotia *et al* 1979) and effects of DNA ligands like Hoechst 33258, Distamycin A and Netropsin (Lakhotia and Roy 1981, 1983). These features suggested that the different heterochromatin regions in the genome of *D. nasuta* shared similar asymmetric A-T rich DNA sequences. A single A-T rich satellite DNA, present on all the heterochromatin blocks, is reported to account for only 7–8% of total nuclear DNA of *D. nasuta* (Ranganath *et al* 1982). The nature of other sequences constituting rest of the heterochromatin is not known.

In recent years, *in situ* digestion of aceto-methanol fixed or unfixed chromosomes with restriction endonucleases has been found to result in diverse banding patterns which allows analysis of molecular organization of DNA sequences present in different regions (Lima-de Faria *et al* 1980; Miller *et al* 1983; Bianchi *et al* 1985; Mezzanotte 1986; Mezzanotte *et al* 1986; Babu 1988; Burkholder 1989; Lopez-Fernandez *et al* 1989; Miller and Miller 1990). Restriction enzyme digestion of fixed

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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 (Miller *et al* 1983; Mezzanotte 1986; Mezzanotte *et al* 1986; Babu 1988).

With a view to know if the different heterochromatic regions in *D. nasuta* differ in their cytological organization, we examined effects of different restriction enzymes on cytological preparations of several cell types of *D. nasuta*. 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*. Satellite as well as other non-satellite (presumably highly repetitive) sequences present in the different heterochromatin blocks thus appear to be deficient in recognition sites for enzymes like AluI.

2. Materials and methods

A wild type strain of *D. nasuta*, maintained in laboratory on standard food at $20^{\circ} \pm 1^{\circ}\text{C}$, was used.

2.1 Restriction enzyme digestion of cytological preparations

Metaphase chromosome preparations from brain ganglia of late third instar larvae were made by the air-dry method as described by Lakhotia and Kumar (1978). 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 (~4 h 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 16 h. The slides were rinsed in absolute ethanol and air-dried.

Chromosome preparations of larval brain ganglia were digested with the following restriction endonucleases. AluI, EcoRI, HaeIII, Sau3a, Sall, SmaI and TaqI (Amersham, UK). All other cytological preparations were digested only with AluI. For digestion of the cytological preparations with restriction endonucleases, 20-25 μl 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 16-20 h. After completion of digestion, the slides were washed in 5 mM 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 DPX mountant and examined by bright-field microscopy.

2.2 Hoechst 33258 staining of ovarian nurse and embryonic cells

To localize the chromocentric heterochromatin, cytological preparations of ovarian nurse and follicle cells and blastoderm cells from 4 h old embryos (after egg laying)

were stained with Hoechst 33258 (5 $\mu\text{g/ml}$) for 10 min in light proof boxes. Stained preparations were mounted in McIlvaine buffer (pH 5.5) for observation in a leitz MPV-3 cytophotometer (using a 100 W ultra high pressure mercury burner, a 50X NPL-Fluotar oil immersion objective and the B filter block-UV-violet excitation).

2.3 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. Each DNA preparation was checked on agarose gels for possible shearing and only unsheared DNA preparations were used for restriction digestion. DNA samples were digested with excess (5–10 units/ μg DNA) AluI, EcoRI, HaeIII, Sau3a, KprI or TaqI restriction enzymes for about 16 h using appropriate reaction buffers and other conditions. The digested DNA samples were fractionated on standard 0.8% agarose gels containing ethidium bromide (Maniatis *et al* 1983). HindIII digested λ -DNA was used as the size marker.

2.4 Electroelution of AluI-resistant high molecular weight DNA

The 23 kb genomic DNA band left undigested by AluI (see §3) was electroeluted from preparatory 0.8% agarose gels. After completion of the gel run, the bright band at 23 kb position was cut with a sharp razor blade and the DNA electroeluted following Maniatis *et al* (1983).

2.5 *In situ* hybridization

The electroeluted AluI DNA was nick-translated using ^3H -dNTPs (all four labelled dNTPs from Amersham) and used for *in situ* hybridization with preparations of larval brain ganglia of *D. nasuta* following Pardue (1986).

3. Results

3.1 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 Sall 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 (Lakhotia and Kumar 1978). With these restriction enzymes, the Giemsa staining of Y chromosome (see inset in figure 1b) also closely resembled the pattern seen after C-banding (Lakhotia and Kumar 1978). No notable difference was found between the Giemsa staining pattern of metaphases digested with the above 5 restriction enzymes (figure 1). However, digestion with Sall or TaqI resulted in a significant reduction of Giemsa stainability of both eu- as well as heterochromatin regions

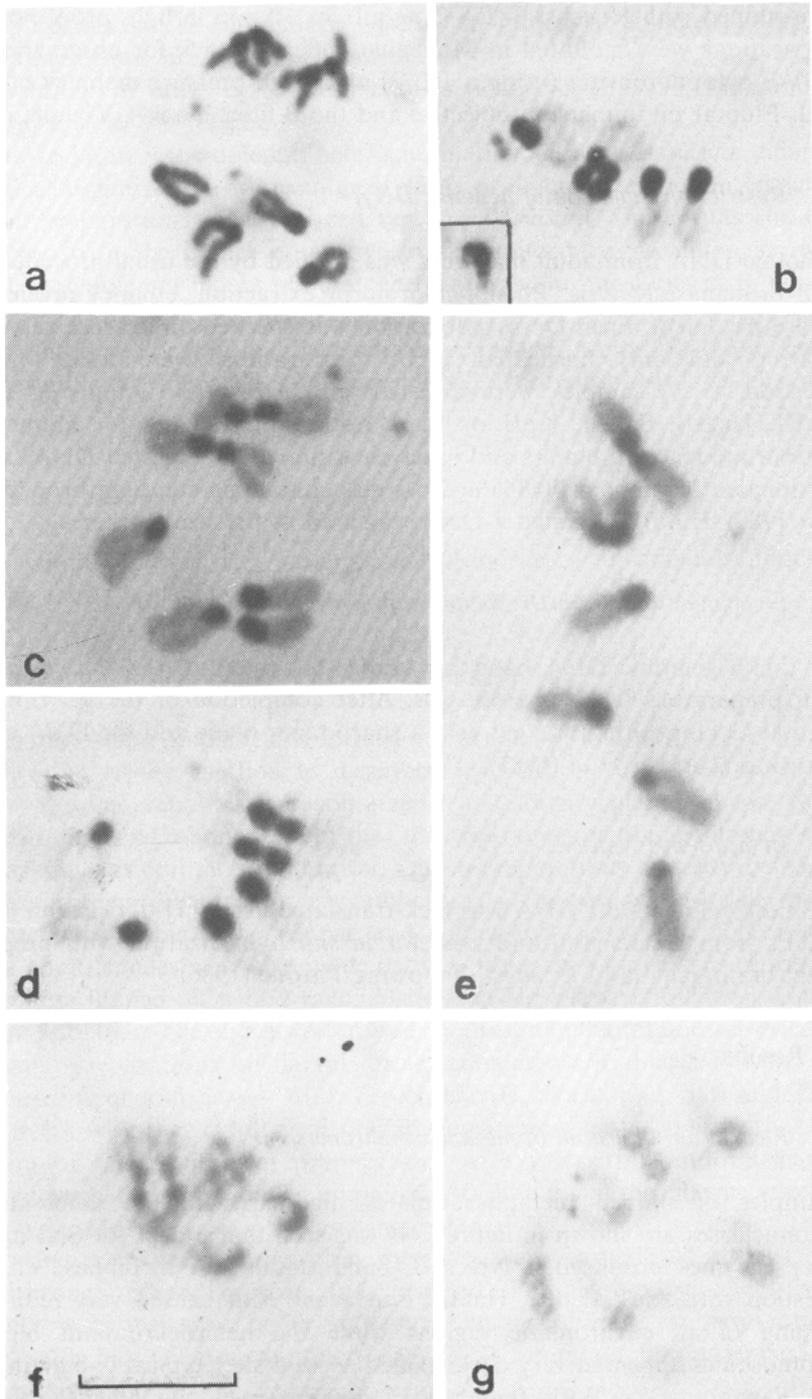


Figure 1. Giemsa stained metaphase plates from brain ganglia of *D. nasuta* larvae. (a) Control (no enzyme) or after different enzyme treatments, (b) AluI, (c) EcoRI, (d) Sau3a, (e) HaeIII, (f) TaqI, (g) SalI. The inset in (b) shows Y-chromosome from a male metaphase after AluI digestion. The scale bar in this and figures 2, 3 and 5 indicates 10 μ m.

(figure 1f, g). None of the enzymes produced any banding in the euchromatin regions (figure 1).

3.2 *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 α -heterochromatin in the chromocentre (Kumar and Lakhota 1977), a band at the base and one band in middle of chromosome 4 (figure 2a). The intranucleolar DNA mass (Lakhota and Roy 1979) also appeared to be less affected by AluI digestion. AluI digested interphase nuclei from embryos of brain ganglia or larvae, pupae or adult showed intense staining of only the single chromocentre with rest of the nuclear chromatin appearing very light stained.

The follicle and nurse cells in ovaries of adult females endoreplicate, with the latter being highly polyploid (up to 1500C). However, the homologous chromatids in these cell types are not as organized as in larval salivary gland cells and thus no polytene chromosomes are seen in nurse cells. In both cell types, AluI digestion reduced Giemsa staining of all regions except the single chromocentre (figure 2b) which remained as darkly stained as in control nuclei. It is significant that in spite of their very different degrees of endoreplication, the size of the chromocentre was same in these two cell types and was comparable to that in diploid embryonic cells.

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

3.3 *Hoechst 33258 fluorescence pattern of ovarian nurse, follicles and embryonic blastoderm cells*

The Hoechst 33258 stained nuclei both from the large nurse and smaller follicle cells show a single, similar sized brightly fluorescing chromocentre (figure 3b) as seen in larval salivary gland polytene nuclei (Lakhota 1984). This Hoechst-bright region corresponds with the region that stains dark with Giemsa after AluI digestion (see figure 2b). The early embryonic nuclei do not have compact chromocentres as may be seen in figure 3a. However, the size of the Hoechst-bright regions in these diploid embryonic cells compares with the size in endo-replicated nurse and follicle cells.

3.4 *Restriction endonuclease digestion of genomic DNA and in situ hybridization of AluI resistant DNA*

Ethidium bromide staining of genomic DNA from adult males of *D. nasuta*, digested with the different enzymes mentioned in §2 and separated on 0.8% agarose gels, revealed that after digestion with all enzymes, except TaqI, a high molecular weight DNA band (23 kb) was left undigested (figure 4). As a result, the 23 kb AluI-resistant band appeared very distinct. After TaqI digestion, the 23 kb band was not seen (figure 4).

When the nick translated 23 kb AluI-resistant DNA was hybridized *in situ* with

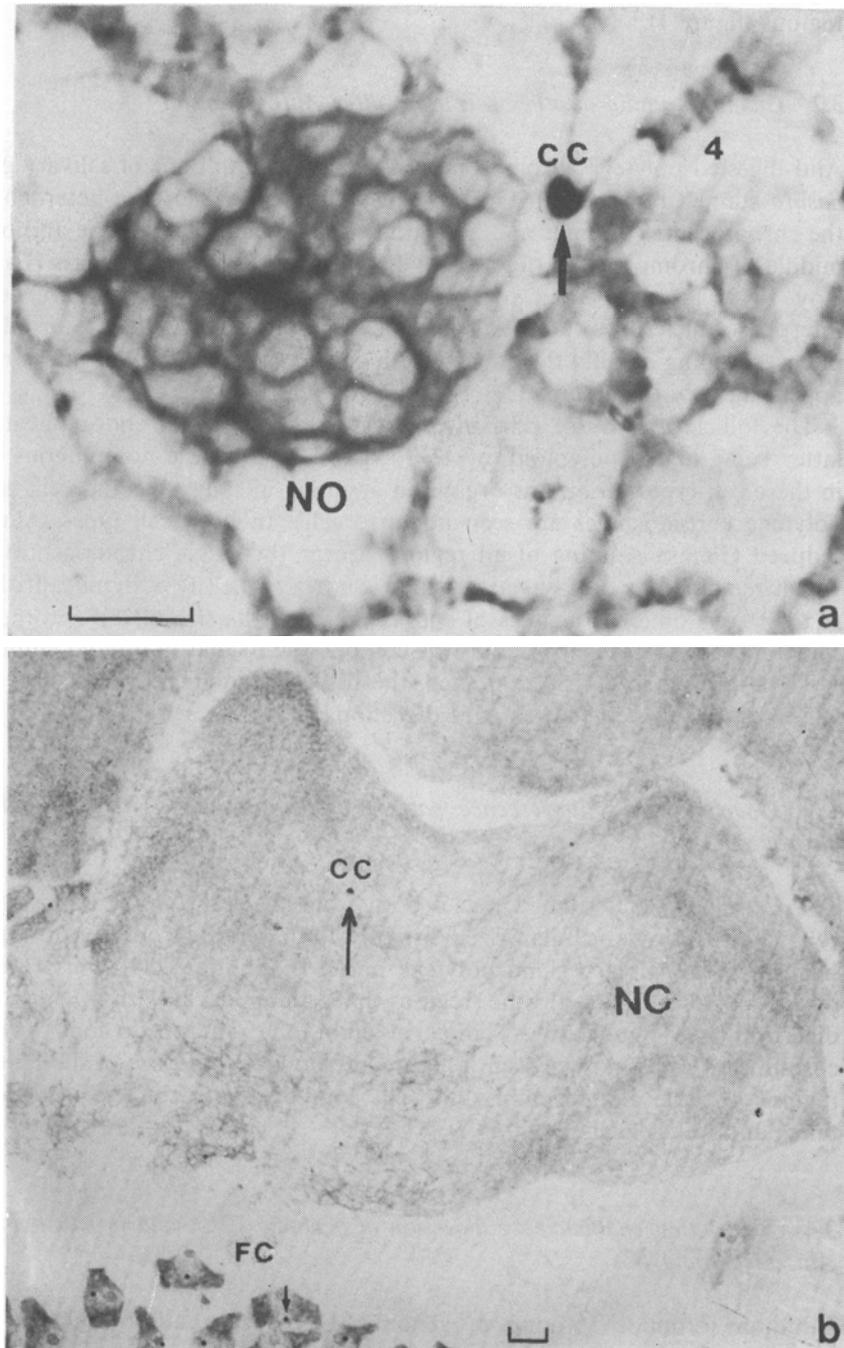


Figure 2. (a) Part of an AluI digested polytene nucleus showing intense staining of the α -heterochromatin (arrow) and of two bands on chromosome 4 (NO = nucleolus), (b) AluI treated large nurse cell (NC) and a group of follicle cells (FC) from adult ovary. Arrow marks the small chromocentre in the highly endoreplicated nurse cell.

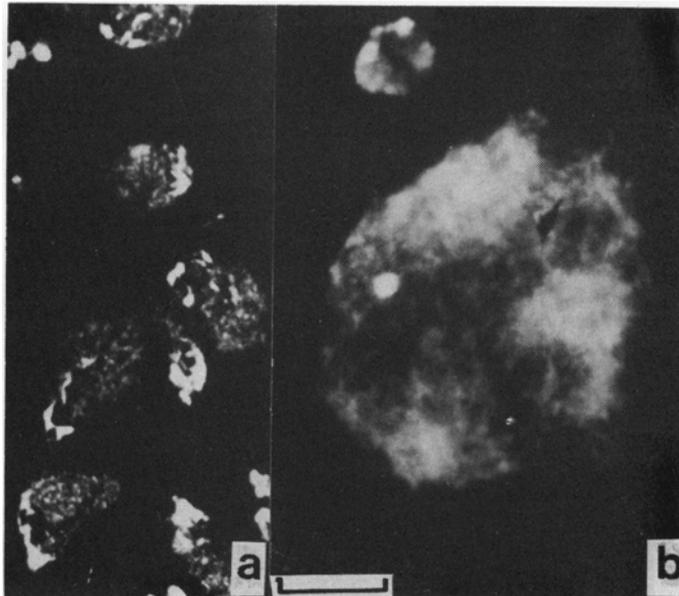


Figure 3. Hoechst 33258 fluorescence stained nuclei from (a) early embryos and (b) adult ovarian nurse and follicle cells.

brain cell nuclei of *D. nasuta*, the hybridization was more or less restricted to the heterochromatic chromocentre region only (figure 5).

4. Discussion

The effect of restriction enzymes on the fixed chromosome preparations have been variously ascribed to be primarily due to chromatin conformation or to the distribution of the recognition sites for those enzymes in the genome or to both (reviewed by Miller and Miller 1990). However, the view that the availability of recognition sequences play a more important role in the production of restriction bandings, has received significant support from various studies. In the present study, except Sall and TaqI, none of the other restriction enzymes tested affected Giemsa stainability of any of the heterochromatin blocks in cytological preparations of *D. nasuta*, although all euchromatin regions were severely affected. This 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 the 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 (Miller *et al* 1983; Bianchi *et al* 1985; Babu 1988; Lopez-Fernandez *et al* 1989) and our following observations: (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

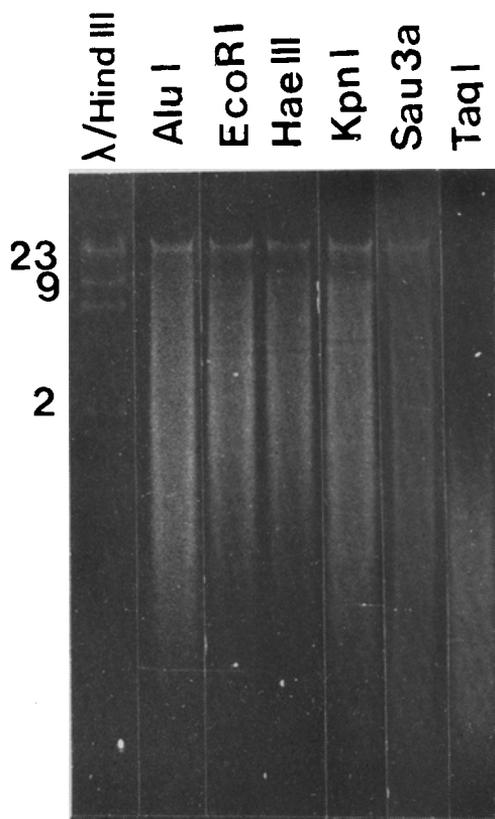


Figure 4. Ethidium bromide staining of genomic DNA of *D. nasuta* digested with different enzymes indicated.

Molecular weights (in kb) of some of the marker bands in HindIII digested λ -DNA lane are indicated. Note the bright band at the top in all genomic DNA lanes except TaqI.

nurse cells), Sall 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 molecular weight 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 molecular weight DNA band in gels, (iii) The specific *in situ* hybridization of the gel purified high molecular weight 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. In a recent detailed study on the mechanism of action of restriction enzymes on fixed and unfixed mammalian metaphase chromosomes, Burkholder (1989) found that while digestion with certain restriction enzymes was influenced to some extent by local chromatin organization, the effects produced by enzymes like AluI, HaeIII, etc., reflected the distribution of restriction sites along the chromosomal DNA. Therefore, in all likelihood the C-band effect of AluI and the other restriction

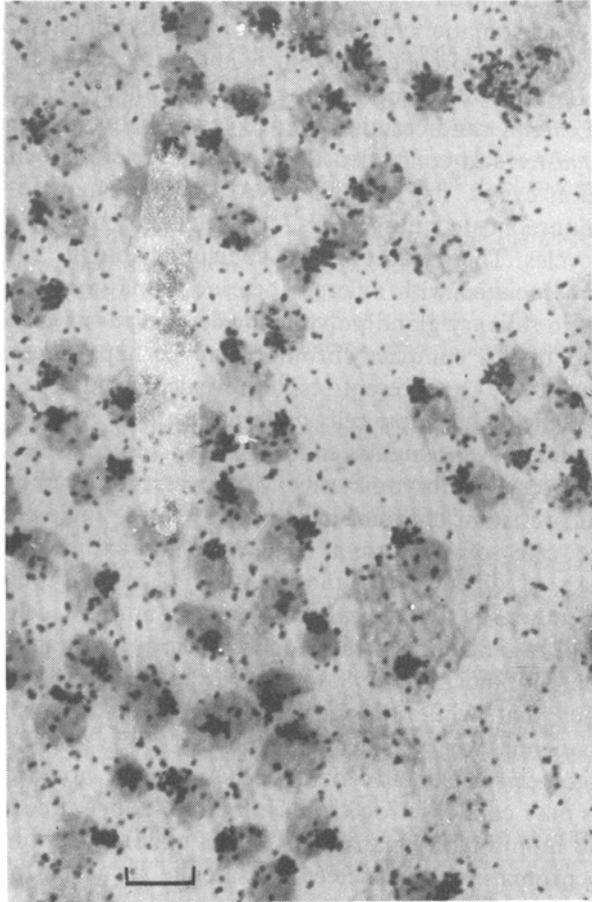


Figure 5. *In situ* hybridization of the 23 kb AluI-resistant DNA with larval brain nuclei.

enzymes seen in this study is due to the DNA sequences in heterochromatin of *D. nasuta* being poor in recognition sites for the enzymes.

Cytologically, the heterochromatin content in *D. nasuta* chromosomes is about 40% of chromosome length (Lakhotia and Kumar 1978) while the single satellite sequence was reported (Ranganath *et al* 1982) 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 in the population of *D. nasuta* studied by us. The content and distribution of heterochromatin is known to vary intra- as well as inter-specifically in different members of the *D. nasuta* subgroup of species (Ranganath *et al* 1982; Hatsumi *et al* 1988). Application of *in situ* restriction digestion in these instances is expected to help in understanding the basis of polymorphism in heterochromatin content in this group of species.

Satellite and highly repetitive sequences comprising heterochromatin are known to be underreplicated in endoreplicating cells of *Drosophila* (see Spradling and Orr-

Weaver 1988; Raman and Lakhotia 1990 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 (1985) compared the extent of underreplication and the spatial organization of satellite and certain other repetitive sequences in these three cell types of *D. melanogaster* and 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. 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 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 a single compact block in the ovarian nurse cells of *D. nasuta*, suggesting that the pericentromeric heterochromatin blocks of different chromosomes of *D. nasuta* were 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* (Hammond and Laird 1985) 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 (Lohe and Roberts 1988), the DNA sequences in heterochromatin of *D. nasuta* are, as noted above, much more similar and thus may condense together. Hammond and Laird's (1985) use of *in situ* hybridization to monitor the quantity (extent of endoreplication) and spatial distribution of heterochromatin would detect the satellite sequences present in the euchromatin domains also. Thus the information 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. nasuta* to be organized more or less as compactly as in the other cell types.

None of the restriction enzymes used in our study produced any banding pattern 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 (Babu 1988). Mitotic chromosomes of *Drosophila* do not show G-bands or replication bands also (Holmquist 1989; Raman and Lakhotia 1990). 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 (Raman and Lakhotia 1990).

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