Heterochromatin in mitotic chromosomes of *Drosophila nasuta*

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Abstract

The heterochromatin in mitotic cells of larval neural ganglia of *Drosophila nasuta* has been analysed by C-banding and by fluorescence studies. All chromosomes, except the 'dot'-like 4th chromosome pair, carry large blocks of heterochromatin which are darkly stained by C-banding, and which fluoresce uniformly brightly with Hoechst 33258, quinacrine mustard, and acridine orange. These heterochromatic segments make up about 40% of the total metaphase chromosome length. The heterochromatic segments also fluoresce brightly with all the dyes at the anaphase stage. In interphase nuclei, all the heterochromatic segments form a common, compact and homogeneous mass which fluoresces brightly with the three fluorochromes used. The size of the bright chromocentre is similar with all the dyes. It is suggested that the heterochromatic segments of different chromosomes are relatively homogeneous in their DNA base sequences, which are likely to be A–T rich in view of their bright fluorescence with Hoechst 33258 as well as quinacrine mustard.

Introduction

Heterochromatin in *Drosophila* cells has received considerable attention in recent years in view of its disproportionate replication in different cell types, particularly the polytene cells (Rudkin, 1965; Gall *et al.*, 1971; Lakhotia, 1974), and a possible involvement of heterochromatin in cellular transcription and other functions (Lakhotia and Jacob, 1974; Lindquist *et al.*, 1975; Spradling *et al.*, 1975; Sandler, 1972). The differential replication of heterochromatin in different cell types may be related to the process of cellular differentiation (Rudkin, 1972; Lakhotia, 1974). We are studying this aspect in *Drosophila nasuta*, a member of the *immigrans* species group. In this paper, we present our observations on the localization and the fluorescence patterns of heterochromatin in the mitotic cells of *Drosophila nasuta.*

Materials and methods

A wild strain of *Drosophila nasuta*, collected from the University campus, was used for these studies. The larvae were grown at 20° ± 0.5°C in standard corn meal-agar food. The neural ganglia from the late third instar larvae were dissected out in *Drosophila* Ringer solution (pH 7.2), and air-dried chromosome preparations were made following the technique of Stock *et al.* (1972) with some modifications. The ganglia were treated with colchicine in Ringer (1 μg/ml) for 1 h at 20°C following which they were transferred to a hypotonic solution (0.67% trisodium citrate) for 30 min. The ganglia were then fixed in freshly prepared acetomethanol (1:3) for 2 h with three changes.

In some specific instances, the colchicine and/or hypotonic pretreatments were omitted and the ganglia were fixed directly in acetomethanol. A small drop of warm 60% acetic acid was taken on a warm (55°C) slide, and the fixed ganglia from one larva were placed onto this drop. Within a very short time the cells dissociate from

he tissue mass and at this stage a few drops of acetomethanol were poured on the slide and air-dried. These air-dried preparations were either stained with Giemsa stain or processed for C-banding or fluorescence analysis.

C-banding

After several trials, the following procedure was adapted for C-banding of *D. nasuta* brain ganglia chromosomes. Air-dried preparations were treated with 0.0014 N NaOH in 2 x SSC at 20°C for 150 sec and washed with three changes (2–3 min each) of 2 x SSC. After passing through three changes of 70%, and two changes of 90% alcohols (2 min each), the slides were dried and stained with 5% Giemsa stain (pH 7.0) for 7.5 min.

Fluorescence banding

Hoechst 33258 (H), quinacrine mustard dihydrochloride (QM) and acridine orange (AO) have been used for fluorescence banding. Prior to staining with the fluorochromes, the air-dried slides were hydrated by passing through 90% (10 min) and 70% (10 min) alcohols, and then two changes of distilled water. In the case of QM staining, a buffer (pH 5.5) was used in place of distilled water. The dye concentrations (in distilled water) and staining time for the different fluorochromes were as follows: H – 5 µg/ml for 10 min, QM – 0.5 mg/ml for 30 min, and AO – 0.1 mg/ml for 10 min.

Following the staining, the preparations were washed in distilled water except for QM, where buffer (pH 5.5) was used instead. The slides were mounted in McIlvaine buffer (pH 4.0 or 5.5) with or without glycerol. The mounting medium did not significantly modify the fluorescence patterns. The fluorescence patterns were examined by incident illumination in a Carl Zeiss (Jena) 'Fluoval' fluorescence microscope with HBO 200 burner, and BG 12/4 excitation and OG4 barrier filters. Micrographs were taken at an initial magnification of x 400 on OrWo DK5 or OrWo NP15 films.

Observations

Mitotic chromosomes of *Drosophila nasuta*

In neural ganglia preparations, metaphase plates showed eight chromosomes including a pair of sex chromosomes (XX in female and XY in male), a pair of small 'dot' chromosomes and two pairs of large autosomes (Figure 1). The X chromosome was a large acrocentric with the proximal half being heterochromatic. The Y-chromosome, of similar size as the X, was sub-metacentric and in normal Giemsa stained preparations, the tips of both arms of the Y-chromosome appeared lightly stained compared to the middle region which was typically heterochromatic. Occasionally in very extended chromosomes, this region gave a banded appearance (Figure 1a); however, this differentiation was obscured in condensed plates (Figure 1c). One of the large autosomal pair was metacentric (chromosome 2), and the other pair was acrocentric (chromosome 3). The metacentric chromosome pair carried very large blocks of darkly stained heterochromatin on either side of the centromere. The acrocentric pair had a small heterochromatic segment near the centromere.

On all the chromosomes, the junction of heterochromatin and euchromatin was marked by a constriction; this junctional region always remained synapsed even when the sister chromatids in the rest of heterochromatic regions were desynapsed (Figure 1b). It also appeared that the heterochromatin, and euchromatin junction on the

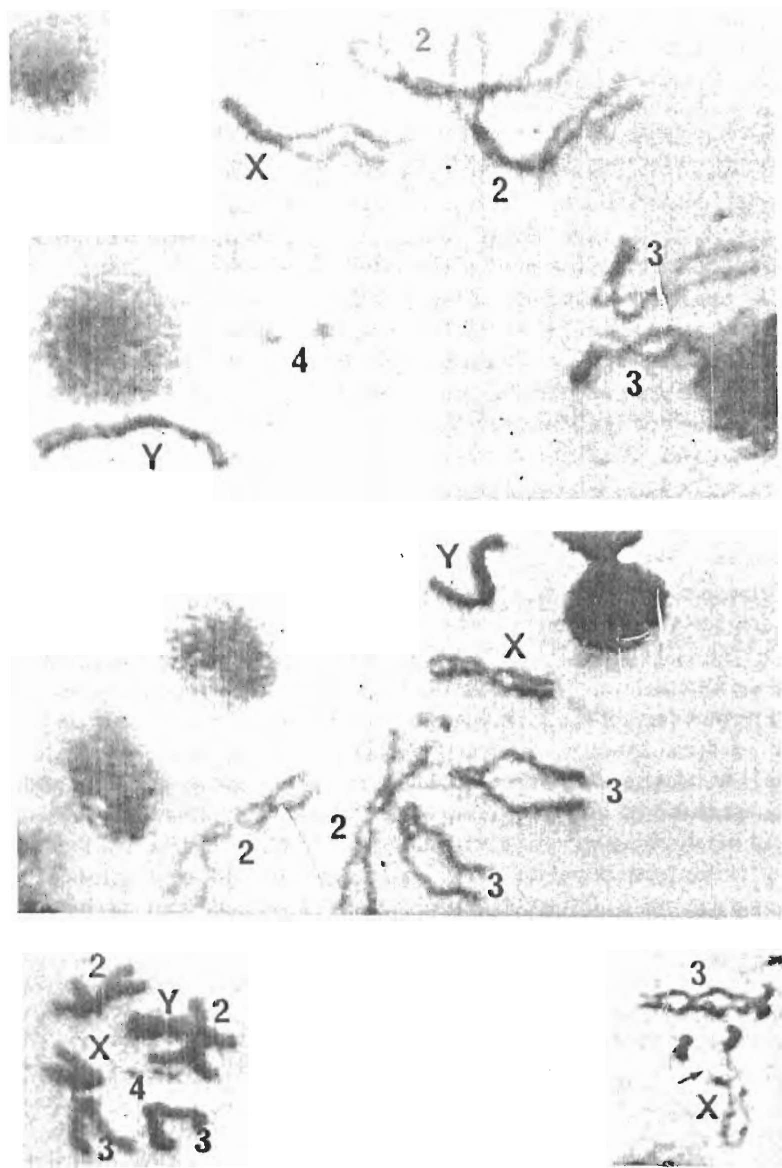

Figure 1 Giemsa stained metaphase chromosomes of larval neuroblast cells of *Drosophila nasuta*. (a) Highly extended chromosomes with a banded appearance of heterochromatin segments, particularly the Y chromosome; (b) extended chromosomes with a prominent constriction at the junction of heterochromatic and euchromatic segments on all chromosomes; (c) highly condensed chromosomes: the heterochromatic segments, particularly of the Y chromosome, do not show any internal differentiation as in (a); (d) part of a metaphase plate in which the heterochromatic segment has broken away from the euchromatic segment in one X chromosome at the 'weak point' (arrow). Figure 1a (top); Figure 1b (centre); Figure 1c (bottom, left); Figure 1d (bottom, right). x 2,000.

X-chromosome had a 'weak point' since in some metaphase plates (Figure 1d) and in many anaphase stages (Figures 4b and 4c), this region broke or appeared 'achromatic' and considerably stretched.

C-banding

In general, the C-band positive regions coincided with the darkly stained regions seen with normal Giemsa staining. The proximal half of the X-chromosome took a uniformly dark stain with C-banding (Figure 2). The sub-metacentric Y-chromosome had two blocks of C-positive heterochromatin, one on either arm close to the centromere; the tips of both arms were relatively lightly stained. Among the autosomes, the metacentric pair had two large pericentromeric blocks of C-positive segments. The C-positive region on one arm was smaller than that on the other arm. The acrocentric pair had a small segment near the centromere. Occasionally, this C-positive segment on the acrocentric chromosome pair showed heteromorphism: one chromosome having a smaller block of C-positive heterochromatin than its homologue (Figure 2). The small 4th chromosomes were almost unstained with C-banding.

Fluorescence patterns

Mitotic chromosomes

All the three fluorochromes, namely, Hoechst 33258 (H), quinacrine mustard (QM) and acridine orange (AO), used in the present study gave a differential fluorescence of heterochromatin and euchromatin. The brightly fluorescing regions corresponded, in general, to the heterochromatic regions localized by C-banding (Figure 3), i.e. all the C-banded regions (except the Y chromosome, see below) were uniformly bright with all the three dyes. However, the overall brightness of fluorescence varied with the dye used: H-staining gave maximum bright fluorescence and the differentiation of the heterochromatin was also very pronounced; AO gave minimum differential fluorescence of heterochromatin and euchromatin; with QM the differential fluorescence of heterochromatic segments was well marked, but the total intensity of fluorescence was less. With all the three dyes, the euchromatic segments showed uniform dull fluorescence.

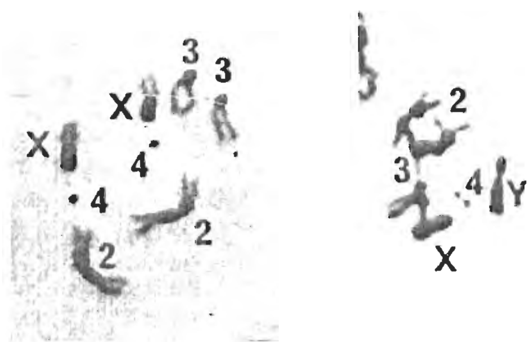

Figure 2 C-banded metaphase chromosomes of female (a) and male (b) larval neuroblast cells of *D. nasuta*. Note the lack of C-banding on the tips of both arms of the Y chromosome in (b). Figure 2a (left); Figure 2b (right). x 2,000.

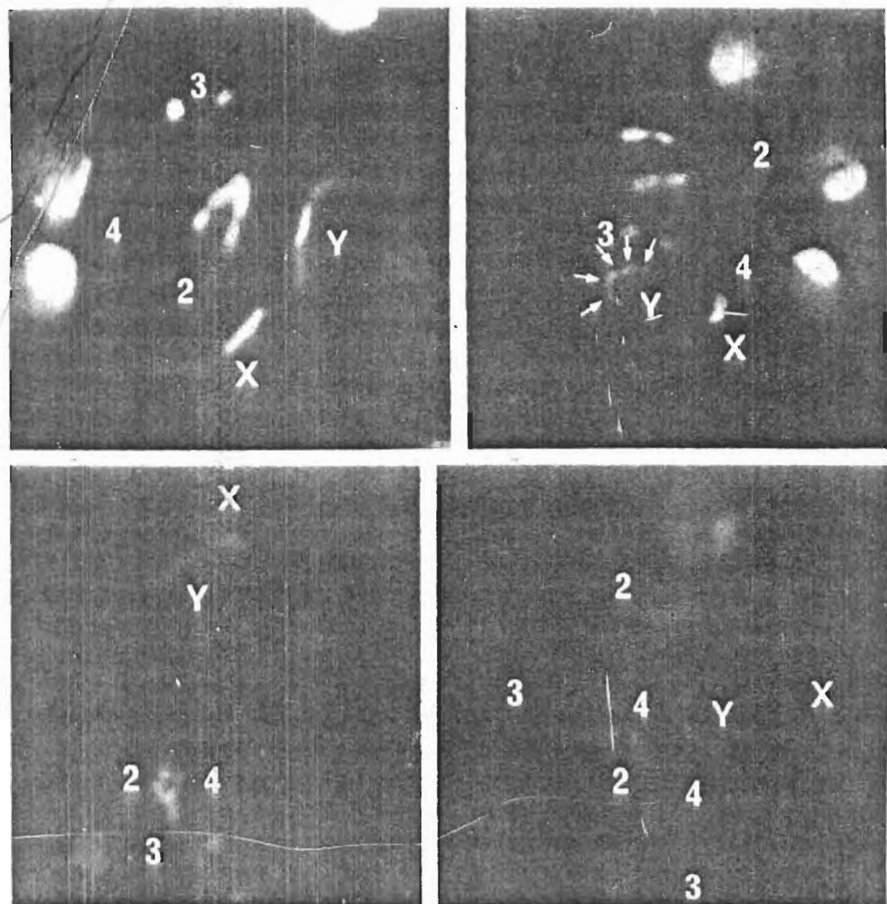

Figure 3 Metaphase chromosomes of neuroblast cells of *D. nasuta* stained with H (a,b), QM (c) and AO (d). In (b) the different bright segments on the Y chromosome seen after H-staining have been arrowed; this sub-divison of Y chromosome fluorescence is not always clear as in (a). Figure 3a (top, left); Figure 3b (top, right); Figure 3c (bottom, left); Figure 3d (bottom, right). x 2,000.

The size difference in the heterochromatic segment on chromosome pair 3, noted with C-banding, was also seen in many plates after fluorescence staining (Figures 3a and 3b). The 4th chromosome pair, which was C-negative, manifested dull fluorescence with all three dyes, and being very small in size was occasionally almost invisible after fluorescence staining.

The Y chromosome gave characteristic fluorescence patterns, particularly in extended plates (Figures 3a and 3b). The C-positive block in the short arm had a bright fluorescence with H as well as QM, while the distal region of the short arm was dull. The C-banded region on the long arm was uniformly bright with H and QM. In some plates an indication of two H-dull bands in this segment of the Y chromosome was seen

(Figure 3b). The C-negative region in the distal segment of long arm had one bright band with H and the rest of this segment was dull. With QM, the entire distal C-negative region gave dull fluorescence (Figure 3c). The overall fluorescence of the Y chromosome with QM was not as bright as of the other heterochromatic segments of the nucleus. With AO, the differential fluorescence of different segments of the Y chromosome was not well demarcated (Figure 3d) as with the other two dyes. Nevertheless, the C-positive segments had a slightly brighter fluorescence than the C-negative regions.

A few anaphase plates were also examined after fluorescence staining (Figure 4). It was seen that at anaphase stage also the heterochromatic regions of all chromosomes were brightly fluorescing with H, QM as well as with AO. An estimate of the heterochromatin content of metaphase chromosomes was obtained by measuring the relative area occupied by C-positive or H-bright regions. C-band treated metaphase plates showed about 40% of total metaphase chromosome length to be heterochromatic; with H-fluorescence, the proportion of the bright regions was about 38% of the total length of all the chromosomes. The values for male and female nuclei were similar.

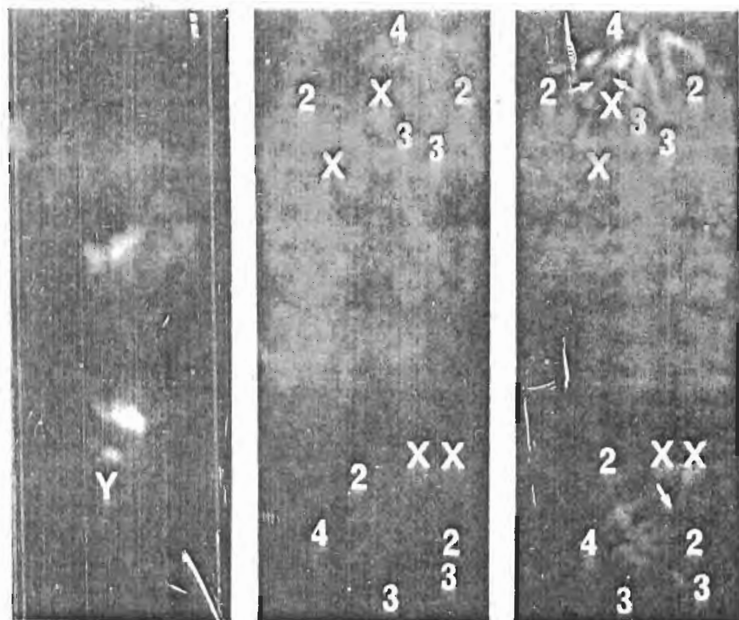

Figure 4 Anaphase chromosomes stained with H (a), QM (b) and AO (c). The acrocentric nature of the X chromosome and chromosome pair 3 is very clear in these anaphase figures (b and c, see Discussion). Also note the fluorescence negative 'weak point' on the X chromosome (arrows in c). Figures 4b and c represent the same anaphase figure which had been first stained with QM, destained in running water for 2 h and restained with AO. The anaphase figure in 4a seems to be aberrant since chromosomes have apparently segregated without chromatid disjunction. Figure 4a (left); Figure 4b (centre); Figure 4c (right). x 2,000.

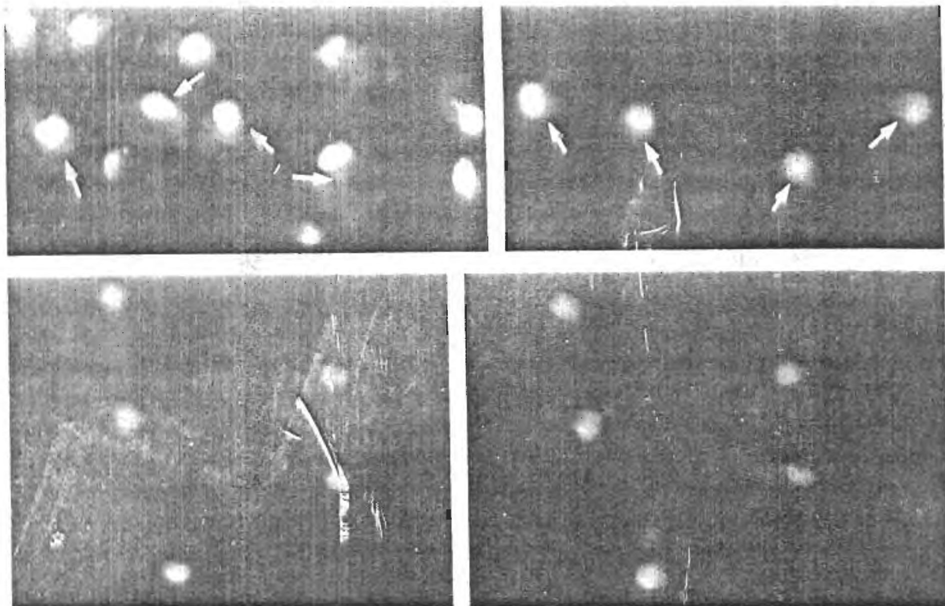

Figure 5 Smaller sized interphase nuclei of larval neuroblast cells showing the brightly fluorescing chromocentre. In male nuclei, stained with H (a) and QM (b), a less brightly fluorescing structure is seen closely associated with the very bright chromocentre (arrows). Figures 5c and d show the same female nuclei stained first with QM and subsequently (after destaining in running water) with AO. The size of the bright chromocentre in these nuclei is similar with the different fluorescent dyes. Figure 5a (top, left); Figure 5b (top, right); Figure 5c (bottom, left); Figure 5d (bottom, right). x 2,000.

Interphase nuclei

Interphase nuclei from brain ganglia cells of third instar larvae had a prominent, darkly stained (with normal Giemsa as well as with C-banding) region, in both the sexes. This region was always associated with the nuclear envelope on one side, and presumably represents the common chromocentre formed by the heterochromatic segments of different chromosomes. It is to be noted that in brain cells of third instar larvae of *D. nasuta*, interphase nuclei of different sizes, including some typical polytene nuclei, were seen, and in these nuclei the size of the chromocentre showed considerable variation. The nuclear size variation and its possible significance will be discussed separately.

In the present analysis, we have selected only the nuclei of small dimensions. The fluorescence patterns of these smaller interphase nuclei have been studied from larval brain ganglia fixed without prior hypotonic and colchicine treatment. In these preparations, the chromocentre was nearly always brightly fluorescing with all the three dyes in both the sexes (Figure 5). The fluorescence of the euchromatic regions was very dull with QM as compared to that with H and AO. The bright chromocentre in most nuclei was a compact mass associated with the nuclear envelope; no subdivisions within this mass could be identified with any of the three dyes. However, in nuclei from male larvae, stained with H or QM, a small region with relatively less bright fluorescence (compared to the rest of the chromocentre) was closely associated

with the chromocentre (Figures 5a and 5b). A similar less bright region was not seen in female nuclei or in nuclei of either sex stained with AO.

In order to ascertain if the size of the H and QM bright chromocentre was similar, the area of the brightly fluorescing region with H and QM, respectively, was measured in smaller interphase nuclei from non-hypotonically pretreated brain ganglia of female larvae. For this purpose, the brain complex from each larva was separated into two lateral halves, and each half used to make separate slides. These slides of sister brain ganglia were stained with H and QM, respectively, and the area of brightly fluorescing chromocentres in smaller sized nuclei with either of the dyes was measured by photographing the nuclei and then enlarging the negatives to a final magnification of x 3,200. A total of 191 nuclei were measured for QM and 146 for H fluorescence. The mean area (± SE) of the bright chromocentre with QM was $1.77 \pm 0.03\ \mu m^2$ and with H it was $1.84 \pm 0.03\ \mu m^2$. A Student's t test analysis showed that these two means were not significantly different.

Discussion

The karyotype of *Drosophila nasuta* has been described by Ray Chaudhuri and Jha (1969), Sajjan and Krishnamurthy (1971), Wakahama and Kitagawa (1972, quoted in Sajjan and Krishnamurthy, 1972), Kumar *et al.* (1975) and Kumar and Lakhotia (1977). Ray Chaudhuri and Jha (1969) described a karyotype with 2n = 12 chromosomes; however, the identity of species described by these authors is doubtful (Sajjan and Krishnamurthy, 1971). The karyotype of *D. nasuta* described by Sajjan and Krishnamurthy (1971), and Wakahama and Kitagawa (1972), is essentially similar to that observed by us (Kumar *et al.*, 1975; and present observations) in *D. nasuta* collected from Varanasi in Northern India. Sajjan and Krishnamurthy (1971) have identified the Y chromosome as metacentric ('V'-shaped) while our observations, and those of Wakahama and Kitagawa (1972), show the Y to be submetacentric. It may be noted here that in very extended prometaphase and metaphase plates, the X chromosome and chromosome 3 often gives an impression that they are metacentric and sub-telocentric, respectively (see Figure 1b). Indeed in one of our earlier short reports (Kumar and Lakhotia, 1977), we erroneously noted the X to be metacentric, and chromosome pair 3 to be sub-telocentric. Now, however, we have also examined several anaphase stages to confirm the centromeric locations on different chromosomes, and these observations confirm that the X as well as chromosome 3 are acrocentrics.

Our main interest in *D. nasuta* karyotype is its heterochromatin content. The proportion of heterochromatin (about 40%) is substantial. The uniformity of all the heterochromatic segments on different chromosomes with respect to their fluorescence patterns etc is remarkable. A number of *Drosophila* species have been examined for the fluorescence patterns of their chromosomes (Vosa, 1970; Ellison and Barr, 1971; Barr and Ellison, 1971; Hennig *et al.*, 1972; Holmquist, 1975a), but in none of these species all the C-band heterochromatic segments give a uniform fluorescence with H as well as QM.

In *D. virilis*, which also has a very large amount of heterochromatin, the different heterochromatic segments vary with respect to their fluorescence with H and QM (Holmquist, 1975a). On the other hand, in *D. nasuta*, except for the Y chromosome, all heterochromatic segments fluoresce identically with H and QM. In contrast to *D. virilis* (Holmquist, 1975a), in *D. nasuta*, there is no segment which is H-bright but QM-dull or vice-versa.

The fluorescence patterns of interphase nuclei of larval brain cells also suggest a homogeneity of different heterochromatic segments of *D. nasuta*. The area of the bright chromocentre in nuclei of similar size, is comparable after H or QM staining; this is in agreement with similar fluorescence patterns of the mitotic chromosomes with these two dyes. It is also important to note that in *D. nasuta* interphase nuclei, the bright chromocentre appears homogeneous with no indication of any subdivisions as have been noted in nuclei of *D. virilis* and related species (Holmquist, 1975b).

In *D. virilis*, which has three different satellite sequences associated with heterochromatic segments, the interphase nuclei have a tripartite chromocentre (Holmquist, 1975b). Several other studies have also suggested that similar DNA sequences of heterochromatin tend to condense as one mass in interphase nuclei (Barr and Ellison, 1972; Mayfield and Ellison, 1975; Schmid *et al.*, 1975). In view of these, we think that the single chromocentre formed by the different heterochromatic segments of *D. nasuta* chromosomes, reflects a relative homogeneity of DNA sequences associated with the heterochromatin on different chromosomes.

On the basis of H and QM fluorescence patterns, some inferences can be drawn about the nature of DNA sequences associated with the heterochromatic regions (Holmquist, 1975a). Although a variety of factors like chromosome condensation, DNA-protein interaction etc, may modify the H or QM fluorescence, it is generally believed that the base sequences play a significant role in causing certain chromosome regions to be very brightly fluorescing. A very bright fluorescence with H and QM has been thought to be due to DNA sequences being enriched in A–T base pairs (Ellison and Barr, 1972; Jalal *et al.*, 1974; Comings, 1975; Comings *et al.*, 1975; Holmquist, 1975a). In *D. nasuta* all the C-band positive segments (except the Y chromosome) are very brightly fluorescing at the metaphase stage with H as well as QM, and also to some extent with AO. Thus, it seems to us that all these heterochromatic segments on the autosomes and the X chromosome are enriched in A–T base pairs.

The fluorescence of anaphase chromosomes of *D. nasuta* is also interesting. Usually it is observed that as a result of greater condensation of chromatin at anaphase, the differential fluorescence of heterochromatin, particularly with QM, disappears (Holmquist, 1975a). In *D. nasuta*, however, H and QM as well as AO, give a differentially bright fluorescence of all the heterochromatic segments even at the anaphase stage. This further suggests that in *D. nasuta*, the bright fluorescence of heterochromatin is particularly due to A–T rich base sequences rather than other secondary factors. An analysis of DNA sequences of *D. nasuta* would be useful in this respect.

The Y chromosome of *D. nasuta*, like the Y chromosome of other species of *Drosophila* (Holmquist, 1975a), appears banded with H. However, with QM, the Y chromosome fluorescence is neither as bright nor does it appear so distinctly banded. In addition, some H-bright segments are QM dull. This difference in the fluorescence of the Y chromosome with H and QM, as compared to other chromo-

somal heterochromatin, may reflect a difference in the type of repetitious DNA and/or chromosomal proteins associated with the Y chromosome. It seems that the relatively less bright region (with H and QM) seen to be associated with the chromocentre in male interphase nuclei, is related to the Y chromosome since a similar structure is not present in female larvae. Probably this less bright region represents the distal segments of both the arms of the Y chromosome which are dull when fluorescing with H and QM at metaphase. It seems that the tips of both arms of the Y chromosome have an organisation different from the typical heterochromatic segments since these are C- as well as fluorescence-negative. In addition, it has also been observed (unpublished data) that these tip segments in brain ganglia cells, complete DNA synthesis earlier than the rest of the Y chromosome.

In conclusion it is suggested that the large amount, and relative homogeneity, of heterochromatin in *Drosophila nasuta* may be very useful in further studies on the organisation, replication and role of heterochromatin in different cell types. These studies are in progress in our laboratory.

Note added in proof

After submitting the manuscript for publication we have seen an earlier report by Travaglini *et al*. (1972) on the satellite DNA sequences of *D. nasuta*. According to this study, unlike other *Drosophila* species examined, *D. nasuta* has two types of poly dAT satellites and these account for 30% of DNA. No other types of satellite sequences were found by these authors in *D. nasuta*. These data support our inferences drawn on the basis of fluorescence patterns that in *D. nasuta*, the heterochromatin has A-T rich sequences and that different heterochromatic segments appear homogenous with respect to their base composition.

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