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Replication in *Drosophila* chromosomes

XII. Reconfirmation of underreplication of heterochromatin in polytene nuclei by cytofluorometry*

S.C. Lakhotia
Cytogenetics Laboratory, Department of Zoology, Banaras Hindu University, Varanasi 221 005, India

Abstract. It is widely known that the bulk of the pericentromeric heterochromatin (α-heterochromatin) does not replicate during polytenization in *Drosophila.* However, a recent DNA-Feulgen cytophotometric study (Dennhöfer 1982a) has claimed equal polytenization of all heterochromatin regions. To re-examine this issue, the amount of Hoechst 33258-bright heterochromatin in non-polytene and polytene nuclei in salivary glands and Malpighian tubules of late third instar larvae of *D. nasuta* has been compared by cytofluorometry. Since the amount of Hoechst 33258-bright heterochromatin is similar in non-polytene and polytene nuclei in spite of the latter having an enormously high euchromatin DNA content, it is concluded that the α-heterochromatin does not replicate during polytenization. The present results further indicate that in the polytene nuclei of Malpighian tubules the α-heterochromatin remains at the 2C level whereas in salivary gland polytene nuclei it varies between the 2C and 4C levels.

Introduction

Fifty years ago a comparison of the relative amounts of heterochromatin in diploid and polytene nuclei led Heitz (1934) to distinguish between the α- and β-heterochromatin and to propose that the α-heterochromatin does not participate in polytenization. This classical observation on underreplication of heterochromatin in polytene nuclei of *Drosophila* has subsequently been confirmed by a variety of techniques like (1) DNA-Feulgen cytophotometry (Rudkin and Schultz 1961; Rudkin 1964), (2) analysis of satellite and repetitive DNAs (Jones and Robertson 1970; Gall et al. 1971), and (3) electron and fluorescence microscopy (Lakhotia 1974; Lakhotia and Mishra 1980; Kumar and Lakhotia 1977). However, in spite of the fairly conclusive evidence for underreplication of heterochromatin and certain other sequences in polytene nuclei of *Drosophila* (see reviews by Spradling and Rubin 1981; Lakhotia 1982; Endow 1982), a controversy has been raised by the recent DNA-Feulgen cytophotometric data of Dennhöfer (1982a, b). By combining ^{3}H-thymidine autoradiography with DNA-Feulgen cytophotometry, Dennhöfer has claimed that in salivary gland polytene nuclei of *D. melanogaster,* all portions of the genome, including the *X* and *Y* heterochromatin, replicate equally. Since this claim raises a whole series of questions relating to all the previous results on underreplication of heterochromatin during polytenization in *Drosophila,* I have re-examined this issue by cytofluorometry in *D. nasuta.* All the larger chromosomes of *D. nasuta* carry big blocks of pericentromeric heterochromatin, which in interphase nuclei come together to form a well-defined single chromocentre (Lakhotia and Kumar 1978). All the heterochromatin regions in *D. nasuta* share similar A-T rich DNA sequences and fluoresce very brightly with quinacrine mustard (QM) or Hoechst 33258 (Lakhotia and Kumar 1978; Lakhotia et al. 1979; Lakhotia and Roy 1981; Ranganath et al. 1982). These properties of heterochromatin in *D. nasuta* permit a precise estimation of the relative amounts of hetero- and euchromatin regions in different cell types by cytofluorometry of Hoechst 33258 or quinacrine mustard-stained preparations. In the present study, therefore, the amount of Hoechst 33258-bright heterochromatin in different sized polytene nuclei of larval salivary glands and of Malpighian tubules has been compared with that in the non-polytene salivary gland imaginal disc nuclei. The rationale was that if the heterochromatin regions were replicating in step with polytenization of euchromatin regions, the absolute amount of Hoechst 33258-bright heterochromatin should increase in proportion to the polyteny level so that the amount of Hoechst-33258-bright material relative to euchromatin remains constant irrespective of polyteny levels. On the other hand, if heterochromatin regions were not polytenizing, the absolute amount of Hoechst 33258-bright regions would remain similar in non-polytene and polytene nuclei resulting in a progressive decline in the relative amount of heterochromatin in higher polyteny nuclei. The present results show that the α-heterochromatin regions do not participate in polytenization in *Drosophila.*

Material and methods

A wild strain (Varanasi) of *D. nasuta,* reared at 20±1° C under standard laboratory conditions was used. Eggs were collected at hourly intervals, and the larvae were grown in uncrowded dishes on yeast-supplemented food at 20°±1° C. Very late third instar male larvae (about 1–2 h before prepupal stage) were taken. Salivary glands (with their ducts) and Malpighian tubules of each larva were dissected out in Poels' (1972) salt solution and transferred

* I would like to dedicate this paper to the memory of E. Heitz to commemorate 50 years of α- and β-heterochromatin

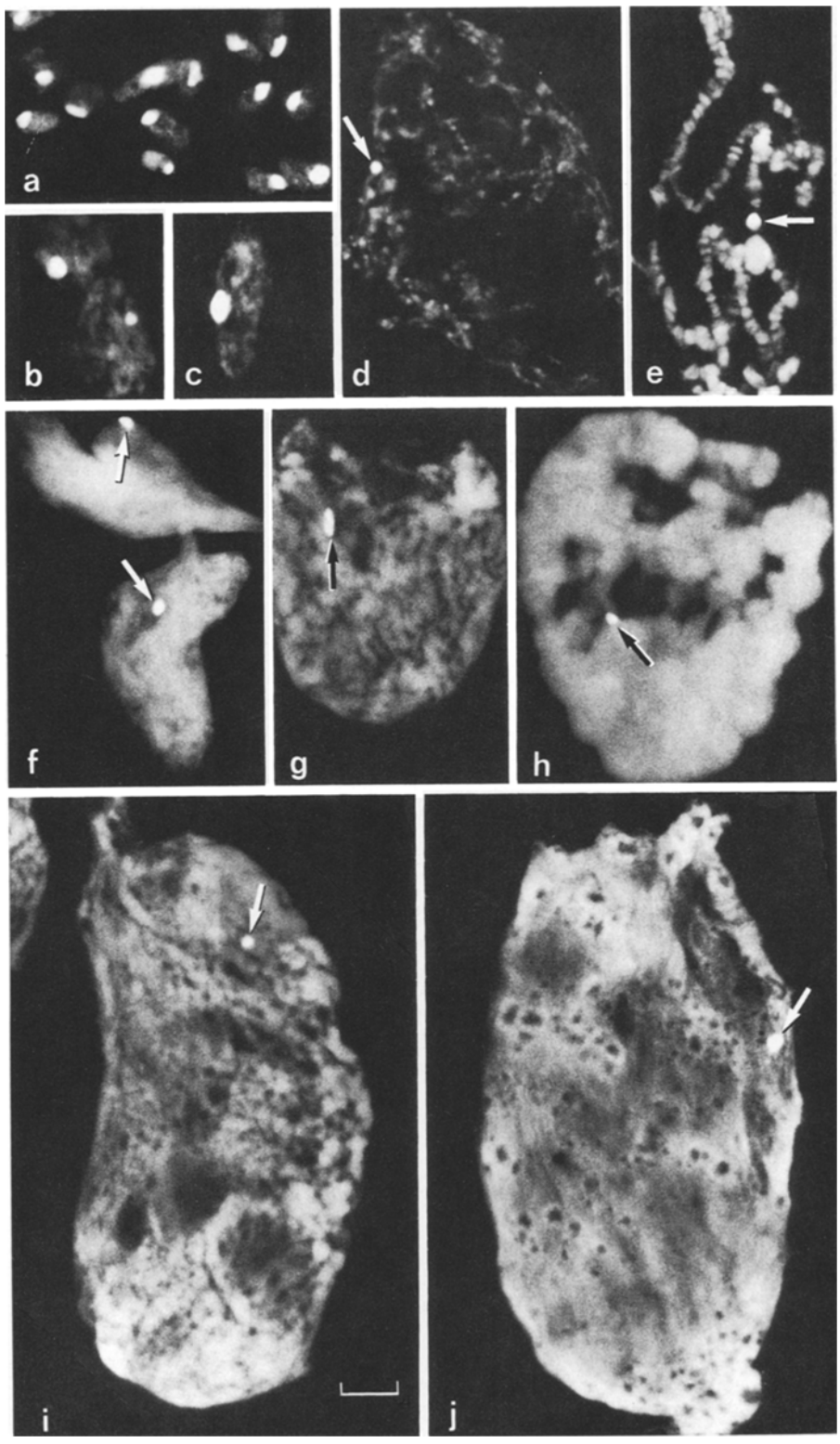

Fig. 1. Hoechst 33258-bright heterochromatic chromocentre in non-polytene salivary gland imaginal disc nuclei (a–c) and in polytene nuclei from fixed Malpighian tubules (d), fixed salivary glands (e) and unfixed salivary glands (f–j). Note the generally similar size of the H-bright region (*arrows* in d–j) in different non-polytene and in polytene nuclei of various polyteny levels in fixed and unfixed preparations. In some non-polytene nuclei (b–c) the nuclear or H-bright areas are unusually large. Bar represents 10 μm

to a fresh slide. The salivary glands (SG) and Malpighian tubules (MT) were kept in separate areas of the same slide so that their nuclei could be processed identically but could also be distinguished under the microscope. The SG and MT were either squashed in 50% acetic acid after a brief fixation with 3:1 methanol-acetic acid (fixed SG and MT preparations) or were lightly squashed in the salt solution without any prefixation (unfixed SG and MT preparations). The former preparations yield typical polytene chromosome spreads whereas in the latter, the polytene chromsomes lose their morphology and each nucleus appears as a more or less homogeneous mass (see Dennhöfer 1982a). All squash preparations were immediately frozen on −70° C ice and the coverslips pried off with a blade after 30 min. The slides were then quickly immersed in a jar containing fresh 3:1 methanol-acetic acid. After 10–15 min, the slides were

rinsed in absolute ethanol and air dried. All slides were digested with 0.2% RNase (Sigma) for 1 h at 37° C. After the RNase treatment, the slides were stained with 5 µg/ml Hoechst 33528 (H) for 10 min, washed with distilled water and mounted with a pH 5.5 McIlvaine buffer. The coverslips were sealed with DPx mutant (BDH). After storage for 24–48 h in the dark at 4° C, the slides were examined in a Leitz MPV-3 cytophotometer using a 100 W ultrahigh pressure mercury burner, a 50× NPL-Fluotar oil immersion objective and the B filter block (UV-violet excitation). The fluorescence emission of nuclei or the heterochromatin region (see Results) in different preparations was measured using continuously variable measuring and field diaphragms. Photomicrographs were taken with a Leitz Vario-Orthomat camera using Ilford HP5 (400ASA) film, developed with "Promicrol" (May and Baker) ultrafine grain developer for hard contrast.

Results

In H-stained polytene nuclei from fixed as well as unfixed tissues, the single heterochromatic chromocentre could be very distinctly identified by its fluorescence which was much brighter than in the rest of the nuclear material (Fig. 1). The non-polytene imaginal cells at the junction of duct and gland (Berendes and Ashburner 1978) also displayed a single distinct H-bright chromocentre (Fig. 1a–c) in preparations of fixed as well as unfixed salivary glands.

The values for total nuclear fluorescence and for H-bright chromocentre fluorescence (in arbitrary fluorescence units, AFU) were measured for each non-polytene (salivary gland imaginal disc) and polytene nucleus in unfixed salivary gland preparations. The values for respective regions of a given cell type in different preparations were similar. The data pooled from salivary glands of six male larvae are presented in Figure 2. The total nuclear fluorescence values for the different non-polytene imaginal cells showed a restricted distribution, with a majority between 8–20 AFU (Fig. 2). Likewise, the heterochromatin fluorescence values in the majority of non-polytene nuclei ranged between 3–10 AFU. Presumably, these nuclear and chromocentre fluorescence values reflect the G_1, S, and G_2 range of AFU. In the same preparations, the nuclear fluorescence values of salivary gland polytene nuclei ranged from 200 to about 4,500 AFU. Since there was a positive correlation between the nuclear area and the total nuclear fluorescence values (data not presented but see Fig. 1), the higher AFU reflected higher levels of polyteny. In the present study, no attempt was made to assign the measured nuclei to different polyteny classes on the basis of their nuclear fluorescence values. Nevertheless, it is obvious from a general comparison of the nuclear fluorescence values of the samples of non-polytene and polytene nuclei that the polytene nuclei in the higher range (>4,000 AFU) correspond to nuclei having completed at least eight or nine cycles of polytene replication. It is very noteworthy, therefore, that in spite of the enormous increase in the nuclear fluorescence values, the area as well as the fluorescence values of the H-bright heterochromatin region in these polytene nuclei remained in the same range (3–11 AFU) as in the non-polytene nuclei (Figs. 1 and 2). In fact, some non-polytene nuclei displayed a much larger H-bright heterochromatin and higher fluorescence value than seen in any polytene nucleus (see Fig. 1c). In preparations of unfixed MT also, the fluorescence values

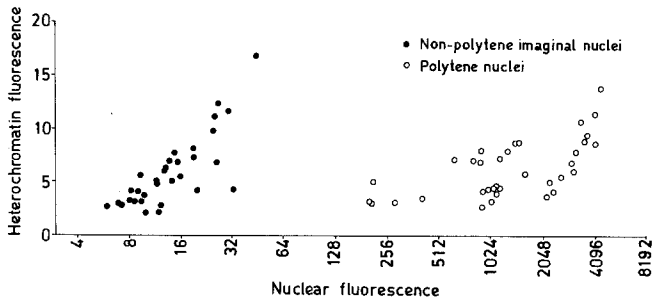

Fig. 2. Relative amounts (expressed in arbitrary fluorescence units, AFU) of total nuclear chromatin (abscissa, log scale) and of the H-bright heterochromatin (ordinate, linear scale) in different non-polytene (●) and polytene (○) nuclei in Hoechst 33258-stained preparations of unfixed salivary glands from male larvae

Table 1. Hoechst 33258 fluorescence values (in AFU) of the H-bright heterochromatin in non-polytene and polytene cells of *D. nasuta*

Fluorescence value class (AFU)	Frequency (%) of nuclei				MT Polytene
	SG Non-polytene		SG Polytene		
	Fixed	Unfixed	Fixed	Unfixed	Fixed
1.5 to 4.5	45.4	36.5	36.1	25.6	81.0
4.6 to 7.5	36.3	38.5	30.6	34.9	18.9
7.6 to 10.5	6.1	25.0	25.0	23.2	0.0
10.6 to 13.5	12.1	0.0	8.3	16.3	0.0
Mean value	5.5	5.8	6.2	6.7	3.9
±S.G.	±0.5	±0.3	±0.4	±0.1	±0.1
Total no. cells	32	52	37	43	58

of the H-bright regions were found not to increase with nuclear fluorescence values (data not presented) but remained in the lower range of non-polytene salivary gland imaginal disc nuclei (see below).

Since squashing the glands or Malpighian tubules without prefixation destroyed polytene chromosome morphology, the H-bright regions could have also suffered distortion and thus their fluorescence values could be in error. To check this, the fluorescence values of the H-bright regions in preparations of fixed and unfixed glands were compared. The data (Table 1) show that the fluorescence values of the H-bright region in fixed and unfixed gland imaginal and polytene nuclei were similar (also see Fig. 1). Thus the fluorescence values of the H-bright regions as measured in unfixed preparations were not artifactual. The data in Table 1 further reveal that the means as well as the distributions of fluorescence values of the H-bright regions in salivary gland imaginal and polytene nuclei were very similar.

The H-bright region in MT polytene nuclei generally appeared smaller than that in SG polytene nuclei (see Fig. 1d, e). Correspondingly, the fluorescence values of the H-bright region in MT polytene nuclei were significantly smaller than in SG polytene nuclei and corresponded to the lower range of values obtained in SG imaginal cells (see Table 1).

Discussion

The quantum of H-fluorescence of chromatin is primarily dependent upon its base sequence (Comings 1975; Hauser-Urfer et al. 1982). Since all the heterochromatin regions

in the genome of *D. nasuta* share similar A-T rich sequences, they specifically fluoresce very brightly when stained with H or QM (Lakhotia and Kumar 1978; Ranganath et al. 1982). In the present study, the H-fluorescence values were compared between the different cell types of the same individual and therefore, the measured values would not be modified by variations in base sequence but would basically depend upon the amount of H-fluorescing chromatin. Thus the fluorescence values of the H-bright chromocentre region in *D. nasuta* cells provide direct information on the total amount of heterochromatin in nuclei of different polytene and non-polytene cell types.

It is well known that compared to the imaginal non-polytene nuclei, most of the polytene nuclei in late third instar larval salivary glands have endoreplicated for 8–10 cycles (Berendes and Ashburner 1978). This is reflected in the present study in the large differences in nuclear fluorescence values of H-stained SG imaginal and polytene nuclei, respectively. The equal fluorescence of the H-bright region in the presumptive 2C/4C population of imaginal cells and in the highly polytenized nuclei, therefore, implies that the heterochromatin regions have not polytenized in step with euchromatin regions. A similar conclusion was reached earlier (Kumar and Lakhotia 1977) due to non-incorporation of 5-bromodeoxyuridine in the α-heterochromatin region in polytene nuclei of *D. nasuta* and due to its morphologically similar size in nuclei of different polyteny levels. The present cytofluorometric measurements quantitatively confirm the earlier qualitative observations. When stained with QM also (data not presented here), the fluorescence values of the QM-bright chromocentre in polytene and non-polytene nuclei are similar as in the H-stained preparations.

The H- or QM-staining in *D. nasuta* permits a precise identification of the heterochromatin regions. Thus, cytofluorometry has a distinct advantage over DNA-Feulgen cytophotometry. In the latter preparations, the heterochromatic chromocentre, particularly the α- and β-heterochromatin regions cannot be precisely delimited (Dennhöfer 1982a, b) and thus the estimates of heterochromatin DNA content are subject to error. The base sequence-specific fluorescence exposes the heterochromatin regions for quantification in any kind of preparation. The fortuitous homogeneity (with respect to its base sequence and other cytological properties) of the different heterochromatin blocks in *D. nasuta* chromosomes is a special advantage in this type of study. However, even if all heterochromatin regions are not H- or QM-bright, cytofluorometry will still provide information about polytenization of *those sequences in heterochromatin that are H- or QM-bright*. In an earlier study in *Drosophila* species in which only some heterochromatin regions are H- or QM-bright (Lakhotia and Mishra 1980), the H- or QM-bright material was found to be generally similar in area in polytene and non-polytene cell types of a given species. The present quantitative information in *D. nasuta* confirms the conclusion in the previous study that the similar area of the H- or QM-bright region in polytene and non-polytene cell types reflects underreplication of heterochromatin in polytene cells.

The present results are thus contrary to Dennhöfer's (1982a, b) conclusion but reconfirm the previous evidence derived from a variety of approaches (see Introduction) that the α-heterochromatin fails to replicate during polytenization in *Drosophila*. It is to be noted that while concluding against underreplication, Dennhöfer could not satisfactorily explain how all the earlier diverse evidence in favour of underreplication could be reconciled with equal polytenization of heterochromatin. It appears that methodological limitations have influenced Dennhöfers (1982a, b) conclusion. Dennhöfer measured the total DNA-Feulgen content of polytene nuclei and compared the observed values with those expected on complete doublings. In such measurements of total nuclear DNA content, the relative contributions made by hetero- and euchromatin DNA cannot be distinguished. If the different euchromatin and intercalary heterochromatin sequences polytenize unequally, as has been proposed in several recent studies (Laird 1980; Lakhotia and Sinha 1983; Zhimulev et al. 1982), the total nuclear DNA-Feulgen values would only reflect the net balance of over- and underreplication of different sequences and the net value may lie within the confidence intervals of the values expected on uniform and complete doublings. Besides, when measuring the total nuclear DNA content, the absolute difference between non-polytene and the highly polytenized nuclei becomes enormous; it remains possible that the DNA-Feulgen values do not maintain an absolute linearity over such a vast range of DNA content. The cytofluorometric approach in the present study, on the other hand, provides a direct comparison of specific DNA sequences and thus provides a more reliable estimate of heterochromatin content in non-polytene and polytene cells.

Comparison of the H-fluorescence values in MT and SG polytene nuclei reveals that the extent of underreplication of heterochromatin differs in the two tissues and also within the different SG polytene nuclei, the heterochromatin content differs by one doubling interval. Since the H-fluorescence values of the H-bright region in MT polytene nuclei are restricted to the lower range encountered in SG imaginal nuclei, it may be suggested that the α-heterochromatin in MT polytene nuclei remains at the 2C level. By comparison, the α-heterochromatin in different SG polytene nuclei seems to vary between 2C and 4C levels. Earlier observations on fluorescence patterns of heterochromatin in polytene nuclei of different species of *Drosophila* (Lakhotia and Mishra 1980) also indicated that the extent of underreplication of α-heterochromatin varies within a narrow range in different SG polytene nuclei. Whether the extra doubling of the α-heterochromatin in some SG nuclei is related to these nuclei in general having higher levels of polyteny remains to be ascertained.

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