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EM Autoradiographic Studies on Polytene Nuclei of *Drosophila melanogaster*

III. Localisation of Non-replicating Chromatin in the Chromocentre Heterochromatin

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Abstract. Replication in the chromocentre heterochromatin of salivary gland polytene nuclei of *Drosophila melanogaster* has been examined by ^{3}H-thymidine EM autoradiography. *In vitro* pulse labelling of salivary glands from late third instar larvae showed that the chromocentre heterochromatin replicates in synchrony with the euchromatin in the nucleus. Within the chromocentre region, the central compact mass, identified earlier as the alpha heterochromatin, did not incorporate ^{3}H-thymidine at any stage of the S, while the surrounding beta heterochromatin was always labelled in nuclei with labelled euchromatin. In a second set of experiments, growing larvae from just after hatching till late third instar stages, were fed on food containing ^{3}H-thymidine, and at the end of larval life, the incorporation in salivary gland nuclei was examined by EM autoradiography. A grain density analysis of the EM autoradiographs revealed that the alpha heterochromatin does not replicate at all from after hatching till late third instar while the beta heterochromatin replicates as much as the euchromatin. Non-replication of the alpha heterochromatin provides the explanation for the lowered amount of heterochromatin in the polytene nuclei compared to their diploid counterparts. Implications of these observations on the organization of chromocentre heterochromatin in polytene nuclei and its homology to the heterochromatic regions in mitotic chromosomes are discussed.

Introduction

In a previous paper (Lakhotia and Jacob, 1974b) we have described the ultrastructural organization of the chromocentre heterochromatin in larval salivary gland polytene nuclei of *D. melanogaster*. It was shown that in this part of the polytene nuclei, two distinct chromatin organizations are recognizable, which recall the classical "alpha" and "beta" heterochromatin described by Heitz (1934). It was also shown that the region identified as the beta heterochromatin, actively incorporates ^{3}H-uridine, while the compact and centrally located mass, identified as the alpha heterochromatin, is totally inactive in this respect. In the present paper, the replicative organization of the chromocentre heterochromatin has been examined by EM autoradiography of unsquashed larval salivary glands of *D. melanogaster* and it is shown that the alpha heterochromatin does not undergo any replication while the beta hetero-

chromatin replicates to the same extent as the euchromatin in polytene nuclei. A preliminary report on this aspect was presented earlier (Lakhotia and Jacob, 1973b).

Material and Methods

Two sets of experiments were done to study the replication in the chromocentre region of salivary gland nuclei of *D. melanogaster*. In the first, excised salivary glands from late third instar larvae were pulse labelled with ^{3}H-thymidine (1 mC/ml; sp.act., 23.7 C/mM; Radiochemical Centre, Amersham) for 10 minutes, fixed and processed for EM autoradiography (see below). In the second set, larvae from just after hatching till late third instar stages were raised on a food supplemented with ^{3}H-thymidine (5 μC/g of food) at 24° C; at the end of feeding, salivary glands from these larvae were dissected out and without any further treatment fixed and processed for EM autoradiography.

EM Autoradiography. The labelled salivary glands from both the experiments were fixed, washed, dehydrated and embedded in an Epon-Araldite mixture as described earlier (Lakhotia and Jacob, 1974a). Pale-gold sections were obtained and mounted on Formvar-Carbon coated 200 mesh Nickel grids. A monolayer of Ilford L4 emulsion was applied on the grids using the "loop" method of Caro and van Tubergen (1962). The autoradiographic exposure for the pulse labelling experiments was 10 days and for the feeding experiments up to 60 days. The photographic development of the preparations in the pulse labelling experiments was by the usual procedure using Kodak D 19b and acid fixer; in the case of the feeding experiments, a gold-latensification step preceded development with D19b to increase the autoradiographic efficiency (Rogers, 1967). All the preparations were double stained with aqueous uranyl acetate and lead citrate through the emulsion after photographic development. Preparations were examined under an AEI EM6 electron microscope at 60 kV.

Observations

As has been described earlier (Lakhotia and Jacob, 1974b), the chromocentre heterochromatin in late third instar salivary gland nuclei of *D. melanogaster* is organized into a central compact mass of alpha heterochromatin and the surrounding network of small dense blocks which form the more extensive beta heterochromatin. The beta heterochromatin finally gives way to the euchromatin at the base of each chromosome arm. It may be mentioned that since sections of unsquashed glands have been examined, different nuclei are sectioned along different planes and, therefore, the orientation of the alpha and beta heterochromatin seen is not always the typical. However, several characteristics of the chromocentre organization described earlier (Lakhotia and Jacob, 1974b) permit unmistakable identification of the alpha and beta heterochromatin in the EM autoradiographs.

I. Replication in Chromocentre Region in Late Third Instar Larvae

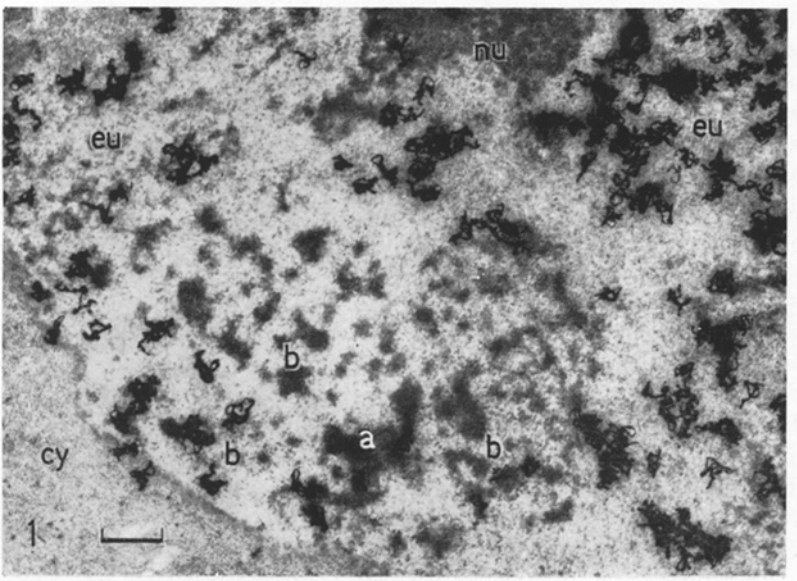
The results of the pulse labelling experiments provide information on the replicative organization of the chromocentre region during the last

few replication cycles in the salivary gland polytene nuclei. The chromocentre region is always unlabelled in nuclei with no labelling on euchromatic regions but nuclei with some labelling on euchromatin have some labelling on the chromocentre region also. Figs. 1–5 show some representative types of labelling of the chromocentre region observed after a 10 min pulse of ^{3}H-thymidine. The labelling in the euchromatic regions of nuclei in these examples is comparable to the different types of labelling patterns seen in the light (Rodman, 1968) and electron microscope autoradiographs of these chromosomes after ^{3}H-thymidine pulse labelling (Lakhotia and Jacob, 1973a, 1974a). In nuclei with a heavy continuous type of labelling over euchromatin, the alpha heterochromatin is completely unlabelled; the beta heterochromatin shows some incorporation of ^{3}H-thymidine, but this is considerably less when compared to the euchromatin (Fig. 1). Nuclei with medium continuous labelling of the euchromatin have nearly similar degree of labelling in the beta heterochromatin, but the alpha is completely unlabelled (Fig. 2). The chromocentre region is heavily labelled in nuclei with heavy discontinous labelling of euchromatic chromosome arms; in these types also the alpha heterochromatin is unlabelled. In the example shown in Fig. 3, the alpha heterochromatin, though itself free of any grains, is encircled by beta heterochromatin with clusters of grains. Examination of serial sections of this particular chromocentre region revealed that in every section examined, the alpha mass was free of any developed silver grain and was likewise, ringed by grain clusters on the beta heterochromatin. It, therefore, seems that the closely surrounding grain clusters are not due to the labelling of the alpha heterochromatin, but due to a very heavy incorporation in the adjoining beta heterochromatin. Nuclei with medium discontinuous labelling patterns also have their alpha heterochromatin completely unlabelled while the beta is rather well labelled (Fig. 4). Nuclei with low discontinuous labelling of euchromatin usually show a relatively greater incorporation in the chromocentre region, but in this case too, the labelling is confined to the beta heterochromatin only (Fig. 5).

In summary, the results of EM autoradiography after ^{3}H-thymidine pulse labelling of late third instar salivary glands, indicate that the alpha heterochromatin is completely unlabelled in all stages of the replication cycle, while the beta heterochromatin incorporates ^{3}H-thymidine in synchrony with the euchromatic regions in the nucleus.

II. Replication in Chromocentre Region during Larval Development

The results of the pulse labelling experiments have shown non-replication of the alpha heterochromatin mass during the late third



Figs. 1—5. EM autoradiographs of polytene nuclei of salivary glands pulse labelled *in vitro* with ^{3}H-thymidine for 10 min. The incorporation of ^{3}H-thymidine in the chromocentre region in nuclei with different types of labelling patterns in the euchromatic regions is shown. The bars represent 1 μm. *a* the alpha heterochromatin; *b* the beta heterochromatin; *cy* cytoplasm; *eu* euchromatin; *nu* nucleolus
Fig. 1. A nucleus with nearly heavy continuous labelling in euchromatic regions; the chromocentre region, in contrast, is rather poorly labelled. The alpha heterochromatin is entirely unlabelled while some parts of the beta heterochromatin are labelled. ×8000

instar stages. To see if this part replicates in the earlier stages of polytenic growth of salivary glands, the second set of experiments was done in which the larvae were fed with ^{3}H-thymidine throughout their growth. This would make ^{3}H-thymidine available during all the polytenic replication cycles occurring in salivary glands after hatching of larvae till pupation.

The incorporation of ^{3}H-thymidine administered through food was restricted to nuclear areas (Figs. 6 and 7). Nucleolar DNA was also seen to be labelled, the details of which will be presented elsewhere. The labelling in the chromocentre region was analyzed in detail and it was seen that in all the EM autoradiographs examined, the alpha heterochromatin appears almost completely unlabelled while the beta heterochromatin and euchromatin are heavily labelled. Figs. 6 and 7 show two typical examples of labelling seen in the chromocentre region; the un-

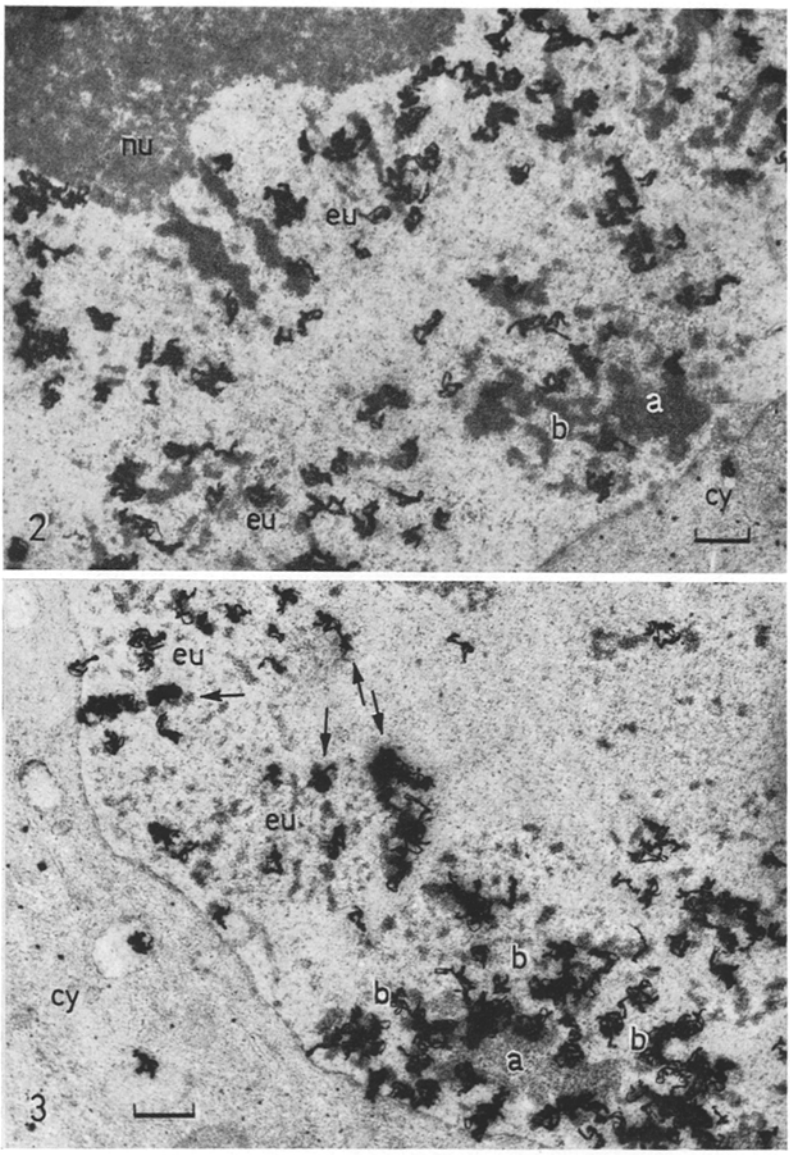


Fig. 2. An example of medium continuous labelling of euchromatin; chromocentre region is also well labelled. The alpha heterochromatin, however, is completely unlabelled. ×7000

Fig. 3. The euchromatic chromosome regions in this example show a heavy discontinuous labelling with the bands heavily labelled (arrow). The alpha heterochromatin mass is clearly unlabelled but the dense blocks of beta heterochromatin are showing heavy incorporation. ×8000

labelled alpha heterochromatin mass is obvious. It may also be noted that the labelling in the beta heterochromatin as well as euchromatin is pronounced on the more electron dense regions, in accordance with their higher DNA density.

A more precise information on the relative incorporation of ^{3}H-thymidine into the alpha and beta heterochromatin was obtained by analyzing the number of silver grains per unit area of the different regions by an empirical method, described previously (Lakhotia and Jacob, 1974b). Briefly, in this procedure the areas of the alpha and beta heterochromatin and the euchromatin in each autoradiograph (all exposed for 54 days and developed after gold-latensification, see Material and Methods) were measured and the number of silver grains in these respective areas counted. Since the alpha and beta heterochromatin are continuous with each other, a "peripheral" zone was delineated at the junction of these two regions to take into account the limits of autoradiographic resolution. The extent of the peripheral zone was defined by drawing lines on the enlarged micrographs at a distance equivalent to 1600 Å on both sides of the presumed junction of the alpha and beta heterochromatin. The distance of 1600 Å represents the "half-distance" or the HD value for our preparations calculated on the basis of data provided by Salpeter *et al.* (1969). Grains overlying the alpha heterochromatin, internal to the peripheral zone, were taken to be due to a true labelling of this region while grains located within the peripheral zone as defined above, could be due to radioactivity of the transition zone of the alpha and beta heterochromatin and, therefore, not ascribable unequivocally to one of the two. The silver grains seen over the beta heterochromatin outside the limits of the peripheral zone were likewise taken to be due to the incorporation in the beta region itself. The euchromatin areas were selected randomly in each autoradiograph and included band, interband and puffed regions. In these preparations, the background was significant, probably due to the longer exposure and the gold-latensification step. The number of silver grains in randomly selected section-free areas of the grids were counted and used for the background correction. A total of 30 EM autoradiographs were analyzed and the results are presented in Table 1. It is seen that the labelling on the alpha heterochromatin is not above the background level while the other regions are well labelled. The relative labelling intensity is similar in the peripheral and beta heterochromatin areas; it is slightly lower in the euchromatin regions. The alpha heterochromatin was seen to be labelled only in 6 of the 30 EM autoradiographs analyzed, with one silver grain in each of the 6 instances; the peripheral zone was labelled in 20 of the 30 EM autoradiographs. The beta heterochromatin and euchromatin regions, of course, were labelled in every case.

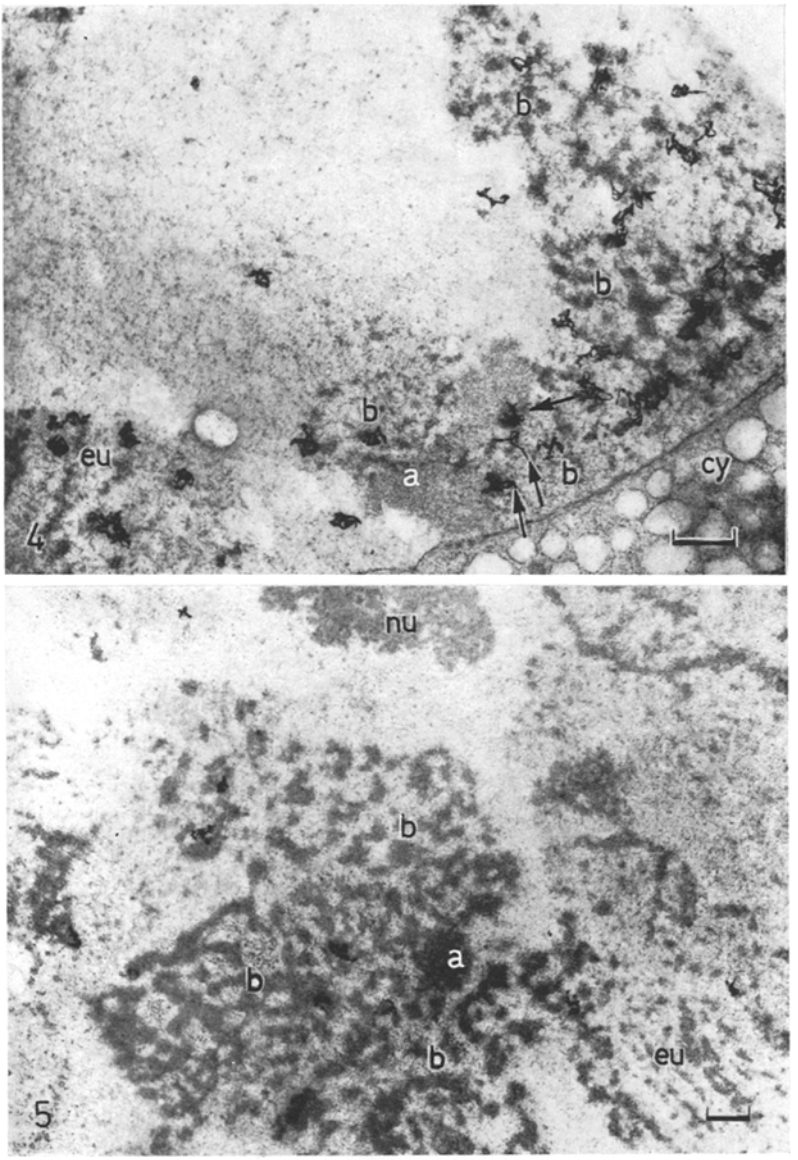
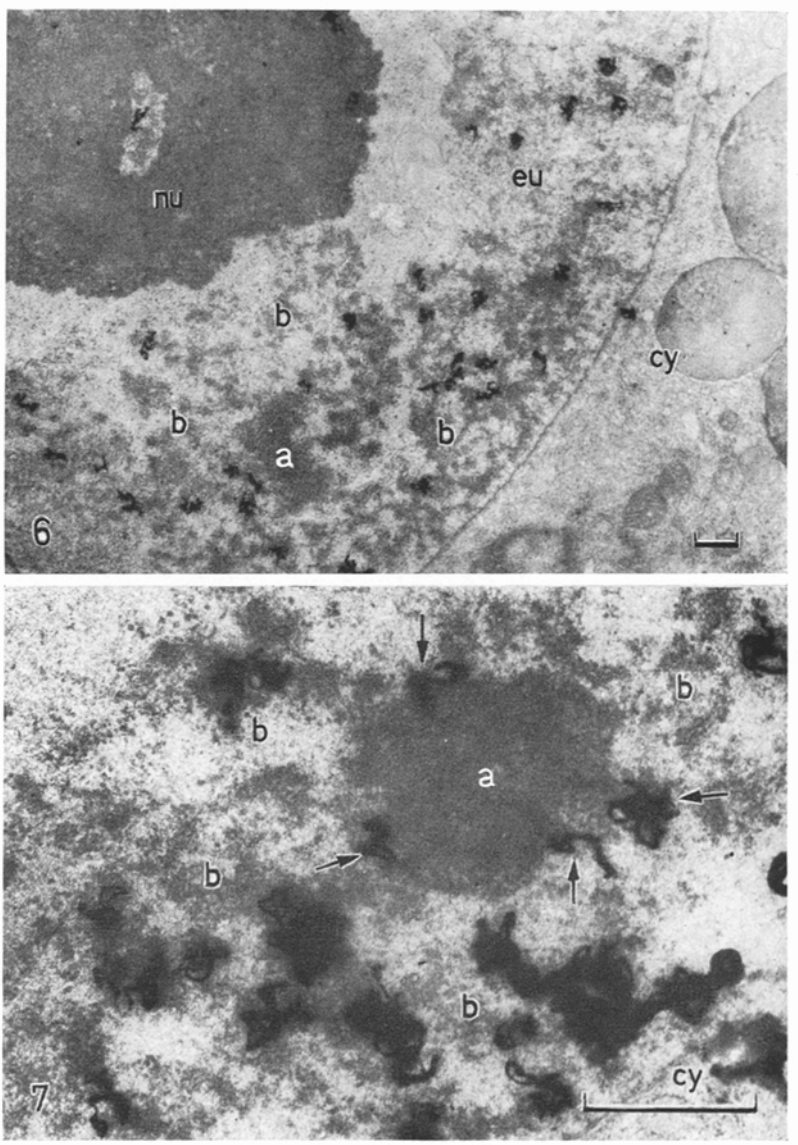


Fig. 4. The chromocentre region of a nucleus showing medium discontinuous labelling of the euchromatin. Only a small part of euchromatin is seen here on the lower left corner. The alpha heterochromatin mass is completely unlabelled except a few grains (arrows) at the junction of alpha and beta heterochromatin. The beta heterochromatin is well labelled. $\times 8000$

Fig. 5. The chromocentre region of a nucleus with very low discontinuous labelling of euchromatin. The beta heterochromatin shows a relatively greater incorporation of ^{3}H-thymidine. The alpha heterochromatin, however, is again completely unlabelled. $\times 5500$



Figs. 6—7. EM autoradiographs of polytene nuclei of salivary glands from larvae fed throughout with ^{3}H-thymidine as described in the text. Symbols are same as in Figs. 1—5

Fig. 6. Part of a nucleus showing the completely unlabelled alpha heterochromatin mass, surrounded by the labelled beta heterochromatin. The euchromatin chromosome arm leading from the beta heterochromatin on the right upper corner is also labelled. It may be noted that the labelling is pronounced on the more electron

Table 1. Analysis of silver grains in different areas of the chromocentre and euchromatin after continuous feeding of larvae with ^{3}H-thymidine

Region	Total area μ^2	Total no. of grains	Average no. of grains/100 μ^2	
			Observed	Corrected for background
Alpha heterochromatin	61.66	6	9.74	0.24
Peripheral zone	81.02	32	43.97	34.47
Beta heterochromatin	1542.06	602	45.65	36.15
Euchromatin	1392.64	430	31.90	22.40
Background	1640.00	156	9.50	—

Discussion

Heitz (1934) in his classical studies on heterochromatin in *Drosophila* suggested that the portions of genome composed of "alpha" heterochromatin, like the whole of the Y-chromosome and some proximal segments of the X and autosomes, do not "grow" during polytenization; this results in the polytene nuclei having a very small amount of heterochromatin compared to the diploid nuclei (also see Fujii, 1942). Although the question of disproportionately lower amount of heterochromatin in *Drosophila* polytene nuclei compared to their diploid counterparts has been examined repeatedly by different techniques, no clearcut answer has been available. Some cytophotometric data on the DNA contents in larval salivary gland nuclei of *D. melanogaster* have indicated a geometric progression of DNA values with increasing polyteny (Rodman, 1967; Rasch, 1970), implying that there is no underreplication of some DNA during polytenization. However, more extensive Feulgen cytophotometric data of Rudkin (1965, 1969, 1972) have indicated that in the larval salivary gland nuclei of *D. melanogaster* there is a gradual reduction in the proportion of DNA in the centric heterochromatin with increasing polyteny of the nuclei. Rudkin (1965) has considered three possible ways by which this underrepresentation of centric hetero-

dense regions. Some label may also be seen on the nucleolus. Cytoplasm is free of incorporation. ×5500

Fig. 7. The alpha heterochromatin mass and a part of beta heterochromatin shown at a higher magnification. The alpha mass is clearly unlabelled; there are a few grains (arrows) at the junction of the alpha and beta heterochromatin, considered here as the "peripheral" zone. The dense blocks of beta heterochromatin are very heavily labelled. Abundance of small RNP particles in the beta heterochromatin, described earlier (Lakhotia and Jacob, 1974b) may also be noted. ×22500

chromatin in the polytene nuclei is brought about. He favoured a "partial" replication model which assumes that some part of the chromocentral DNA (about 19–20% of diploid DNA) never replicates in salivary gland polytene nuclei while the rest of it replicates along with the euchromatic regions. But neither his cytophotometric data nor the several autoradiographic studies carried out in the squashed preparations of ^{3}H-thymidine labelled salivary glands (reviewed by Rudkin, 1972) could precisely locate or identify this non-replicating chromatin within the chromocentre of *Drosophila*. This, obviously, has caused some ambiguity about the extent of heterochromatin involved in non- or under-replication and the organization of the chromocentre region as a whole. A combined autoradiographic and cytophotometric study on polytene nuclei in the brain ganglion cells of *D. hydei* by Berendes and Keyl (1967) led them to suggest that in polytene nuclei of brain too the relative amount of heterochromatin is less compared to the diploid nuclei and that this lowered amount of heterochromatin is the result of a "slow" replication of heterochromatin compared to the euchromatic regions.

In the present study, replication in the chromocentre region has been examined in detail by EM autoradiography. It is clear from the observations on glands pulse labelled with ^{3}H-thymidine in late third instar stages that replication in chromocentre as a whole is not grossly asynchronous with the euchromatin. This suggests that, unlike in the brain ganglion cells of *D. hydei* (Berendes and Keyl, 1967), the underreplication of chromocentre heterochromatin in the salivary gland nuclei of *D. melanogaster* is not achieved by a "slow" replication.

The present results support the "partial" replication model suggested by Rudkin (1965). A complete absence of labelling of the central mass of alpha heterochromatin in the chromocentre region after the pulse labelling with ^{3}H-thymidine in late third instar stage suggests that this part of the genome may correspond to the non-replicating chromatin suggested by Rudkin on the basis of his cytophotometric studies. The EM autoradiographs obtained after the pulse labelling could not be subjected to any grain count analysis because of the limited sample size and the fact that nuclei showing different types of labelling would have different kinetics of ^{3}H-thymidine incorporation in eu- and heterochromatin and, therefore, not suitable for a common analysis. The sample size as a whole is greatly limited because of the small dimensions of the chromocentre region (Lakhotia and Jacob, 1974b) and the low frequency of replicating nuclei in late third instar larvae (Rodman, 1968). However, even in absence of such analysis, the results clearly show that the alpha heterochromatin is not replicating during the late third instar though the beta heterochromatin is replicating with euchromatin.

The results of ^{3}H-thymidine feeding experiments show that the alpha heterochromatin is not replicating even in the early stages of polytenization. The EM autoradiographs obtained in this set of experiments have been subjected to an empirical analysis to estimate the relative incorporation of ^{3}H-thymidine in different regions of nuclei. In the present case, the labelling intensity depends at least on two factors, viz., the relative DNA density and the number of replication cycles undergone by the region under consideration. On the basis of chromatin organization and electron density it may be presumed that the alpha and beta heterochromatin have higher DNA density than euchromatin (Lakhotia and Jacob, 1974b); this presumption has been substantiated in general by observations on ^{3}H-actinomycin D binding in different regions of these nuclei (Lakhotia, unpublished). If all the regions had undergone equal number of replications, the grain densities in different regions of EM autoradiographs would be expected to be in proportion to the DNA densities of respective regions. In beta heterochromatin, the grain density has been seen to be higher than in average euchromatin areas. In view of the apparent higher DNA density of the former region, this may indicate that these two regions undergo equal number of replication cycles after hatching of larvae. It is certainly unlikely that the beta heterochromatin is replicating more than euchromatin. The synchronous labelling of the beta heterochromatin and euchromatin in late third instar after the *in vitro* pulse of ^{3}H-thymidine also seems to rule out a differential replication in the two regions. If the alpha heterochromatin were also replicating as much as the beta heterochromatin, one would expect to observe a high labelling density on this region also: with the mean value of 36.15 grains/100 μ^2 of beta heterochromatin, about 22 grains would have been observed on the 61.66 μ^2 of the alpha heterochromatin examined. Actually, however, no grains (after correcting for background, see Table 1) were seen on the alpha heterochromatin. The lack of labelling of the alpha heterochromatin after ^{3}H-thymidine feeding throughout the larval development together with the similar results after the pulse labelling in the late third instar stages, indicates that this region in the salivary gland polytene nuclei is not replicating from hatching till pupation stages. However, it is not possible to say from these results if the alpha heterochromatin replicates during the very first or second endomitotic duplication cycles, which in some salivary gland nuclei may occur before hatching of the larvae (Rudkin, 1972). Nevertheless, the observations show that in the salivary gland nuclei of *D. melanogaster*, the alpha heterochromatin mass located in the centre of the chromocentre region does not replicate at all (or may replicate only during the very early polytenic duplication cycles), while the beta heterochromatin at the base of each chromosome arm replicates all along with the euchro-

matin in the nucleus. It may be mentioned that the failure to detect this non-labelling part of the chromocentre region in light microscope autoradiographs was obviously due to the fact that in the squashed preparations the alpha heterochromatin is surrounded on all sides by the beta heterochromatin and, thus, an unlabelled alpha heterochromatin is not detectable in such autoradiographs.

A correlation of the present results with other studies on the distribution of repetitive and satellite DNA sequences in diploid and polytene nuclei of *D. melanogaster* and the cytophotometric data on DNA content may provide information on the involvement of different mitotic chromosome regions in the formation of the chromocentre in salivary gland nuclei. In *D. melanogaster* it is now known that in mitotic chromosomes, the centric regions and the Y-chromosome and in polytene nuclei, the chromocentre region are enriched in several families of repetitive and/or satellite sequences (Jones and Robertson, 1969; Rae, 1970; Blumenfeld and Forrest, 1971, 1972; Botchan *et al.*, 1971; Gall *et al.*, 1971; Perreault *et al.*, 1973). Interestingly, the total content of some of these repetitive sequences in polytene and diploid nuclei is the same inspite of the differences in ploidy (Gall *et al.*, 1971; Blumenfeld and Forrest, 1972). In mitotic chromosomes, these particular sequences are located in the X- and autosomal centric and pericentric heterochromatin and the Y-chromosome (Gall *et al.*, 1971; Perreault *et al.*, 1973); in polytene chromosomes it has been suggested that these same sequences are located in what may correspond to the alpha heterochromatin (Rae, 1970; Botchan *et al.*, 1971; Gall *et al.*, 1971). These and the present observations taken together, suggest that the alpha heterochromatin block in salivary gland nuclei of *D. melanogaster* is formed by the fusion of the centric and pericentric heterochromatin of the X and autosomes (and the bulk of Y chromosome in male). On the basis of Rudkin's (1965) cytophotometric data, it would then appear that about 19–20% of each chromosome (the X and autosomes) in the diploid female cell contributes to the non-replicating alpha heterochromatin while the remaining small fraction of heterochromatin in the diploid cell (about 3% of diploid DNA) forms the replicating beta heterochromatin at the base of each chromosome arm in the polytene nuclei. Thus although the alpha heterochromatin may represent as much as about 20% of diploid DNA, it comes to represent a very minor fraction in the fully formed polytene nuclei; the beta heterochromatin on the other hand, remains nearly in the same proportion in the two types of nuclei. On the basis of *in situ* hybridization and other studies, Gall *et al.* (1971) suggested that (i) the alpha heterochromatin consists of highly repetitive (satellite) sequences, probably not replicating during polytenization, and that (ii) the beta heterochromatin represents a region in the mitotic chromosome con-

taining repetitive sequences of which at least some replicate during polytenization. Evidence obtained here confirms the non-replicating nature of the alpha heterochromatin, but with respect to the beta heterochromatin, the present observations show that this represents mitotic chromosome regions all of which replicate in step with euchromatin during polytenization. *In situ* hybridization studies at the EM level would be very informative for a complete understanding of the organization of heterochromatin in diploid and polytene nuclei of *Drosophila*.

The location of ribosomal cistrons in the alpha heterochromatin of the X- and Y-chromosomes of *D. melanogaster* (Cooper, 1959; Ritossa and Spiegelmann, 1965) raises the interesting aspect of control of replication of the alpha heterochromatin and rDNA in polytene nuclei (Rudkin, 1972). While the alpha heterochromatin remains unreplicated in polytene nuclei, the rDNA cistrons located within this region replicate (Rodman, 1969; Pardue *et al.*, 1970; Lakhotia, unpublished), although not to the same polytenic levels as the euchromatin (Hennig and Meer, 1971; Spear and Gall, 1973). Obviously, the alpha heterochromatin and the rDNA cistrons are under independent control for their replication in polytene nuclei. The localization of almost all the rDNA cistrons within the nucleolus in polytene nuclei (Pardue *et al.*, 1970) and the difficulty of assigning the nucleolar organizer region to specific band/s of salivary gland chromosomes (Viinikka *et al.*, 1971) may be significant in this context.

The significance of non-replication of such a large amount of DNA in polytene nuclei may be sought in the functional needs and the fate of these cells. The non-replicating regions are the kinetochore and pericentric heterochromatic regions. These polytene nuclei never re-enter mitotic phase and thus, presumably, do not require the kinetochore and associated activity. As also suggested by Rudkin (1972), it would be economical for the cell not to replicate the "non-essential" DNA (in these cells) to the high polytenic levels since this will not only require a large amount of metabolic precursors, but will also create unnecessary structural and organizational problems. We have shown previously (Lakhotia and Jacob, 1974b) that the beta heterochromatin, which is replicating, is also actively transcribing in these nuclei, while the alpha heterochromatin is completely inactive in transcription and apparently, "non-essential" to the polytene nuclei in larval salivary glands of *Drosophila* (also see Shah *et al.*, 1973).

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