Experimental Cell Research 86 (1974) 253–263

EM AUTORADIOGRAPHIC STUDIES ON POLYTENE NUCLEI OF *DROSOPHILA MELANOGASTER*

II. *Organization and Transcriptive Activity of the Chromocentre*

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SUMMARY

The organization and the transcriptive activity of the chromocentre region of salivary gland nuclei of *Drosophila melanogaster* have been examined by electron microscopy and EM autoradiography. The heterochromatic chromocentre region is organized into a central compact block of chromatin, surrounded by a large number of smaller, interconnected blocks. The latter are continued into the euchromatic chromosome arms. The two types of chromatin organization in the chromocentre are reminiscent of the classical α- and β-heterochromatin. Two types of ribonucleoprotein-like particles regularly occur in the β-heterochromatin. EM autoradiography after short (5 min) in vitro pulse of ^{3}H-uridine shows that the β-heterochromatin is as active in RNA synthesis as the euchromatin in the nucleus; the α-heterochromatin is completely inactive. It is suggested that the two types of particles seen in the β-heterochromatin are products of RNA synthesis occurring in this region. These observations raise doubts on the general validity of the notion of total inactivity of constitutive heterochromatin.

During the process of polytenization in the larval salivary gland cells of *Drosophila melanogaster*, the centric heterochromatin regions of mitotic chromosomes fuse together to form the chromocentre [5, 11, 16] from which the different euchromatic chromosome arms radiate out. The ultrastructure of this region in salivary gland chromosomes was studied by Sorsa [19] in sections of squashed preparations. We have re-examined this aspect by electron microscopy of unsquashed glands for better ultrastructural and topographical preservation, and we have also studied RNA synthesis in the heterochromatic chromocentre region by EM autoradiography after in vitro pulse labelling of the glands with ^{3}H-uridine.

MATERIAL AND METHODS

Salivary glands from late third instar male and female larvae of a wild strain (Oregon K$^+$) of *D. melanogaster* were double-fixed in glutaraldehyde and osmium tetroxide and embedded in an Epon-Araldite mixture by our usual procedure [12]. Ultrathin sections showing silver to pale-gold interference colours (800–1 000 Å) were collected on Formvar-carbon-coated 200 mesh copper (or nickel for EM autoradiography) grids, double-stained with uranyl acetate and lead citrate and examined in an AEI EM6 electron microscope. For light microscope observations, semithin sections (0.5 μm thick) from the same Epon-Araldite blocks were obtained and stained with a 3% basic fuchsin solution in 70% ethyl alcohol. Staining was done at room temperature for 3–5 min and the slides with sections differentiated in 70% alcohol, dehydrated and examined under phase contrast optics.

For studying RNA synthesis, the excised salivary glands from late third instar larvae were incubated in *Drosophila* Ringer solution containing 1 mCi/ml of ^{3}H-uridine (spec. act., 31 Ci/mM; Radiochemical Centre, Amersham) for 5 min. Fixation, washing

and processing for EM autoradiography were as described earlier [12]. Exposure for EM autoradiography was up to 20 days.

Owing to the very small size of the chromocentre region in the large salivary gland nucleus, the frequency with which a section through the chromocentre can be examined under electron microscope is extremely low. For this reason, a very large number of preparations from different glands had to be examined for this study.

OBSERVATIONS

Ultrastructure of the chromocentre region

The entire chromocentre region is considerably more electron dense than the rest of the chromatin and as also noted earlier by Sorsa [19], is rather disorganized compared with the highly regular structure of the bands and interbands. It has also been observed in the present study that the chromocentre always lies near the nuclear membrane at one end of the rather spheroid polytene nucleus.

Examination of serial sections, both under light and electron microscope, reveals that the chromocentre in male as well as female glands, is composed of two distinct components (fig. 1) which recall the original classification into α- and β-heterochromatin by Heitz [7]. No appreciable difference in the chromocentre organization could be detected between male and female nuclei. As can be seen in fig. 1, there is a single, more or less homogeneous mass of compact chromatin in the centre of the chromocentre region, surrounded on all sides by a large reticulated area of many small interconnected dense blocks; this area gives a granular appearance under light microscope and is continued into the euchromatic regions of chromosome arms (fig. 1). The central block of dense chromatin is termed the α-heterochromatin and the granular area of small dense masses as the β-heterochromatin.

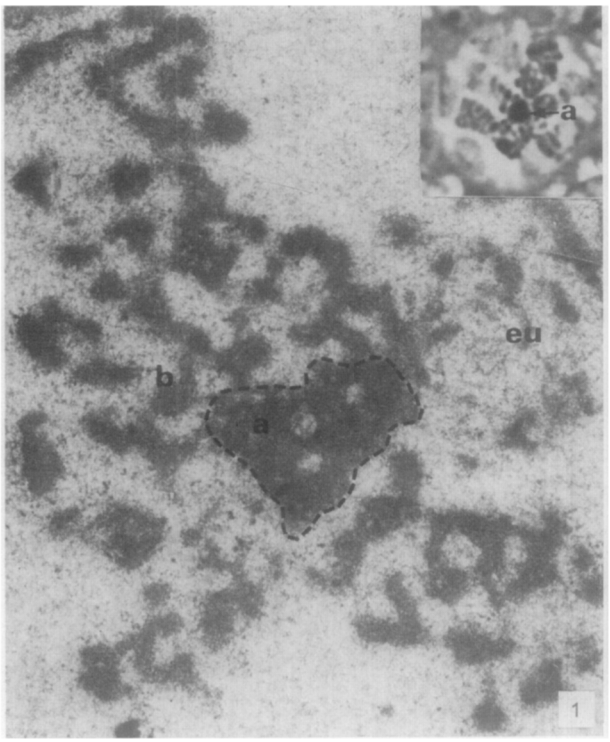
The single compact block or mass of α-heterochromatin consists of densely packed fibrils (fig. 2). This block has been seen to measure up to about 3–4 μm in its largest dimension in a section. There is no internal differentiation, although occasionally small less dense areas can be noted in the region (figs 1–3). The constituent fibrils are clearly seen to be about 30 Å thick in high resolution micrographs (fig. 2) and they often occur in pairs, giving the impression of units about 70–100 Å in thickness, as previously reported by Sorsa [19].

The α-chromatin is surrounded on all sides by a larger area of β-heterochromatin which occurs as many small and irregularly placed dense fibrillar masses, forming a kind of loosely interconnected network (figs 1, 3, 4). The central mass is connected by groups of fibrils with the surrounding dense blocks (figs 1–3); the small dense blocks are also interconnected in a similar fashion (figs 2–3). The fibrils in these blocks and the interconnections are about 30 Å thick, and as in the

Fig. 1. Montage of two electron micrographs of a section through the chromocentre region showing the central dense block of α-heterochromatin (*a*) surrounded by the network of smaller dense blocks forming the β-heterochromatin (*b*); the latter continues into the euchromatic chromosome arms (*eu*). × 12 000. The inset shows the chromocentre region seen in a semi-thin section (0.5 μm) under light microscope. The central compact α- and the surrounding granular-looking β-heterochromatin are clearly visible; the different euchromatic chromosome arms leading out from the chromocentre can also be seen. × 2 500.

Fig. 2. High resolution electron micrograph of a part of α-heterochromatin (*a*) and the immediately adjoining blocks of β-heterochromatin (*b*). 30 Å thick fibrils are seen in all regions of α- and β-heterochromatin and also in the interconnections (*arrows*). × 90 000.

Fig. 3. Electron micrograph of chromocentre region showing parts of α- (*a*) and β-heterochromatin (*b*). The α-chromatin is free of any particles; in the β-chromatin region a large number of the smaller type of particle (described in the text) are seen placed close to the dense chromatin masses. A small area of cytoplasm is also seen in the lower right corner and it may be noted that the cytoplasmic ribosomes (*er*) and these particles in the beta chromatin appear to be nearly of the same size. × 52 500.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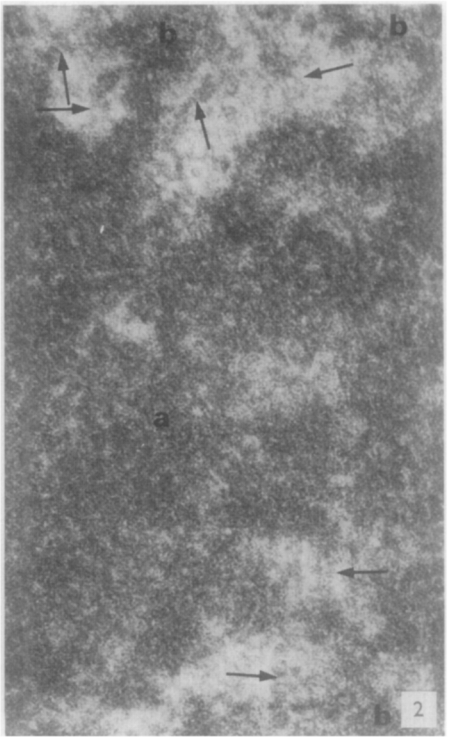
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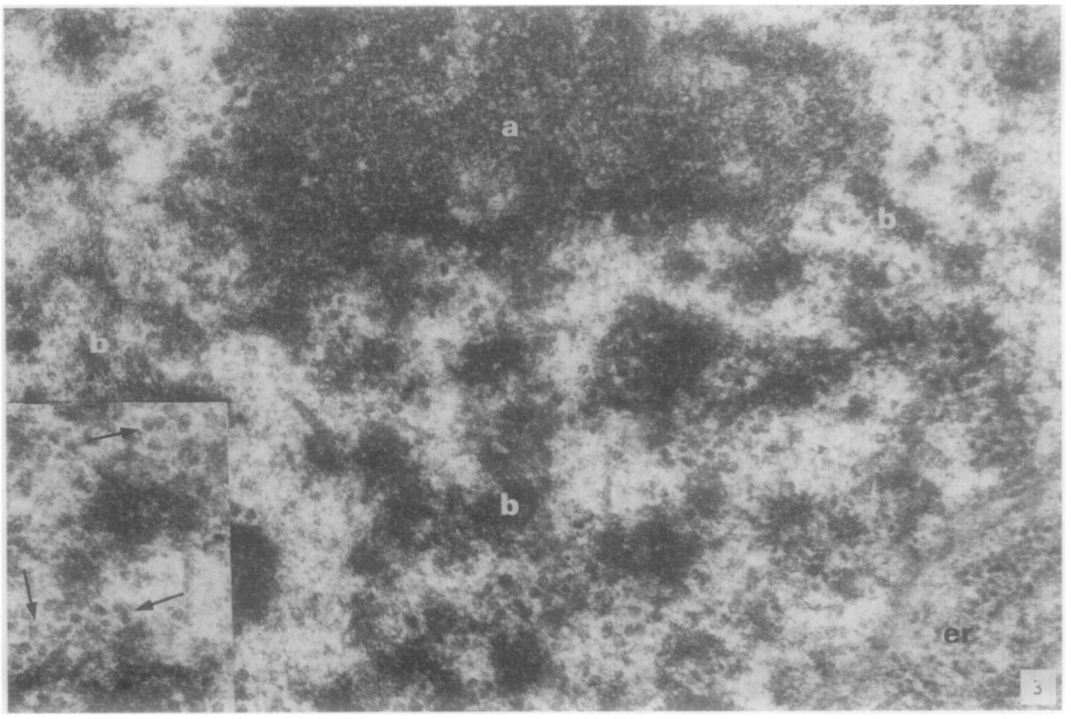
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α-chromatin, they often occur in pairs or higher groups (fig. 2).

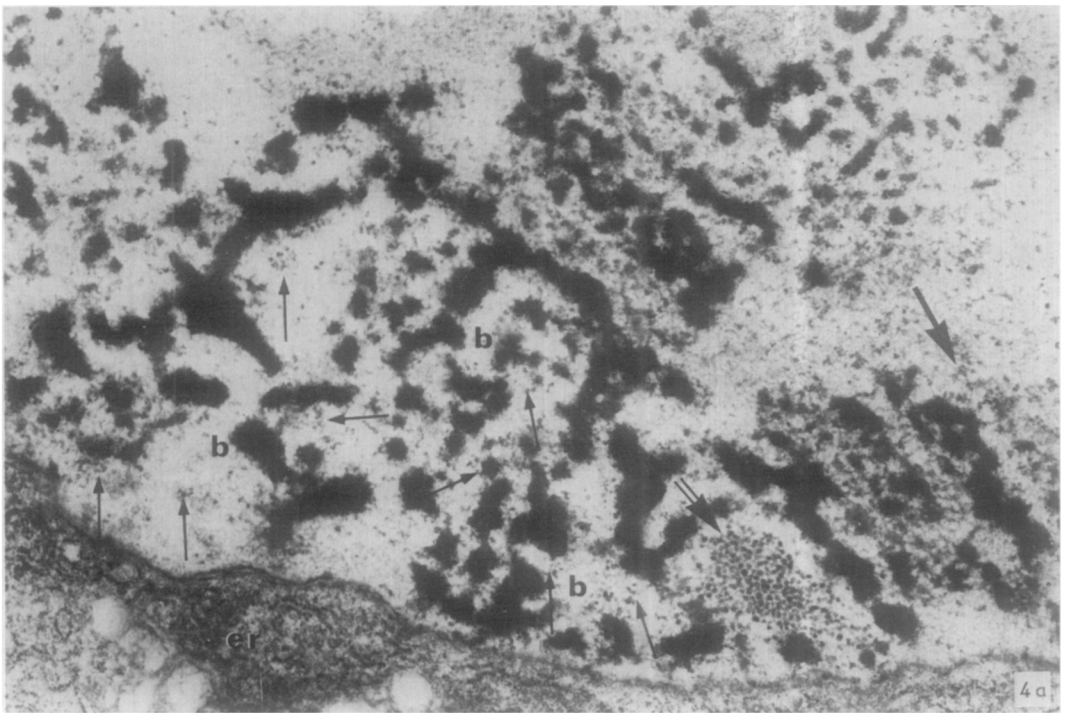
The outer area of the network of dense masses (β-heterochromatin) is finally continued into the different chromosome arms (figs 1, 4*a*). The junction between the heterochromatic chromocentre and the euchromatic chromosome arms is marked by the low electron density of the latter regions and by a transition of the apparently disorganized fibrils of the β-heterochromatin into the regularly arranged fibrils of bands and interbands (fig. 4*a*). The individual chromosomes leading out from the chromocentre are not easily identifiable in these preparations.

In majority of preparations examined, the β-chromatin region is characterized by the presence of large number of particles, reminiscent of typical ribonucleoprotein (RNP) particles of puffed regions in polytene chromosomes. Two types of particles have been observed with respect to their size and location. The first type (fig. 3) is nearly of the same size as the cytoplasmic ribosomes (about 250 Å) and these particles occur throughout the β-heterochromatin. A closer examination reveals that the particles tend to be closely associated with the chromatin masses. Particles of the second type (fig. 4*a*, *b*) are larger (about 450–500 Å) and appear to be organized in clusters restricted to an area of β-heterochromatin close to or within the zone of transition to one of the chromosome arms. Although the chromosome arm, at the base of which these particles are clustered, could not be identified with certainty, examination of some micrographs suggests that this could be the X-chromosome. For example, in fig. 4*a* the chromosome arm, at the base of which these particles are located, seems to resemble the most proximal euchromatic part of the X-chromosome on the basis of its banding pattern (compare the banding pattern of this chromosome segment with the electron micrograph of the base of X-chromosome of *D. melanogaster* presented in fig. 2 of Sorsa [19]). Particles of both the types have been seen in the β-heterochromatin region of male and female nuclei. No such particles, however, have been seen in the α-chromatin.

^{3}*H-uridine incorporation in the chromocentre*

In the EM autoradiographs, the incorporation of ^{3}H-uridine varies considerably from nucleus to nucleus even within the same gland. In every instance, however, the nucleolus shows much higher grain density than the chromosomal regions. In some preparations, there is also some labelling of the nucleoplasm and cytoplasm but this is considerably lower compared with the labelling of chromosomes. In general, the euchromatic regions of chromosomes and the chromocentre regions appear to be labelled more or less uniformly (fig. 5). Within the chromocentre region, the majority of grains lie over the small dense masses of β-hetero-

Fig. 4. (*a*) Electron micrograph of a section through the junctional region of the chromocentre and chromosome arms showing the β-heterochromatin (*b*) at the base of three euchromatic chromosome arms. The α-heterochromatin is not visible in this section. The β-heterochromatin of the chromosome on the lower right corner of the micrograph has a cluster of the larger type of particles (*double arrow*) described in the text. Banding pattern of the euchromatic part of this particular chromosome immediately following the cluster of particles suggests this to be the most proximal euchromatin part of the X-chromosome (see text). These particles are clearly larger than the cytoplasmic ribosomes (*er*) or the smaller type of particles seen in the rest of the chromocentre (*single arrows*) and RNP particles in some parts of chromosome arms (*heavy arrow*) and nucleoplasm. Particles of the larger type are not seen in any other chromosome regions. ×13 600. (*b*) A high magnification micrograph of a part of β-heterochromatin at the base of a chromosome arm (not shown here) with a large number of the larger type of particle clustered in several groups. ×52 500.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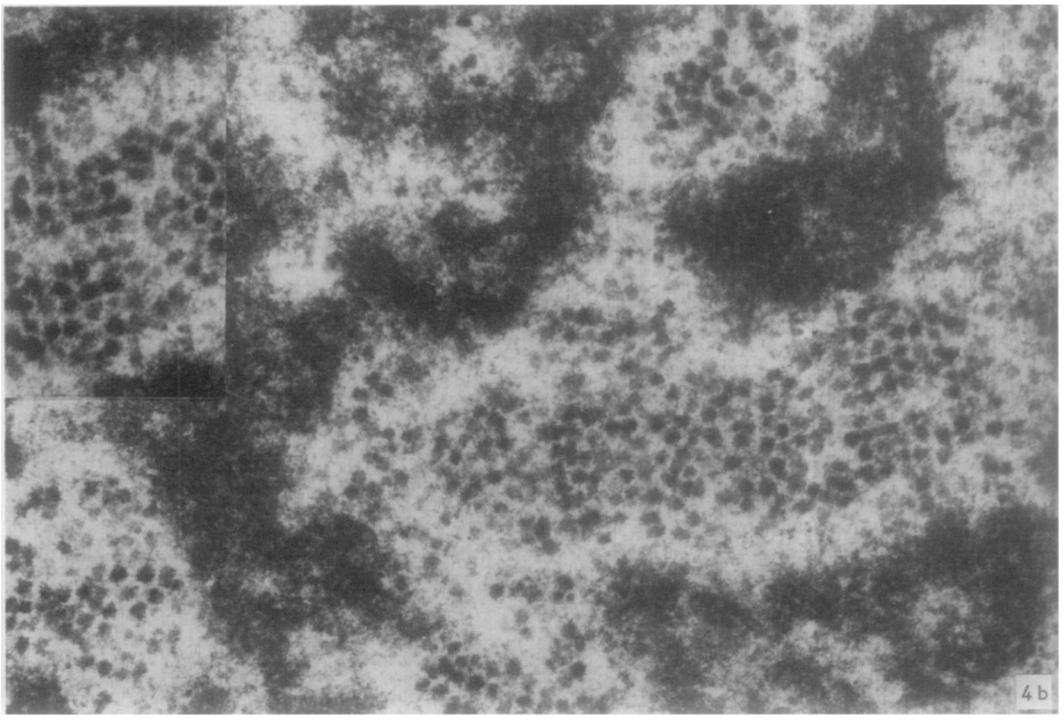
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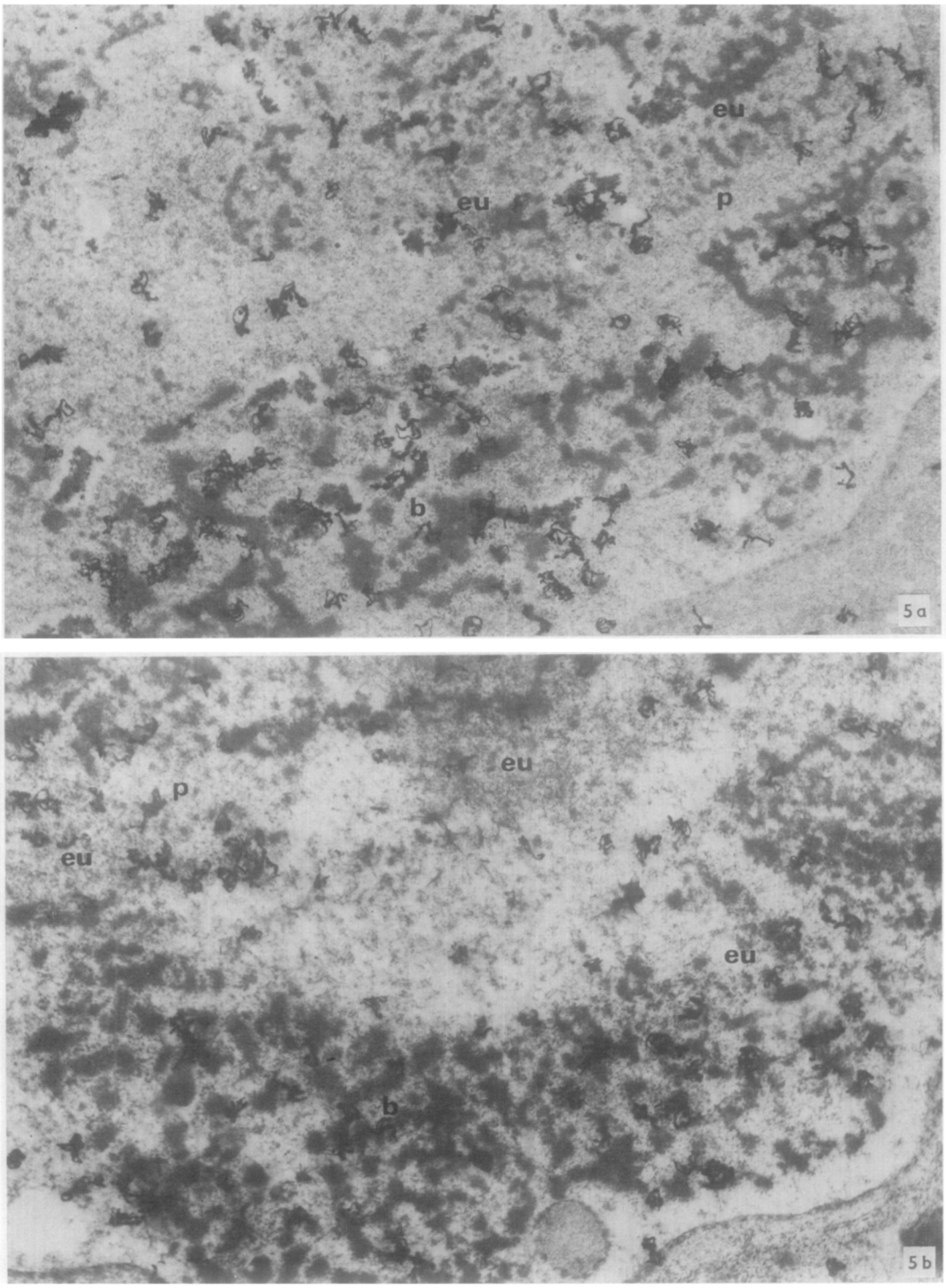

Fig. 5. (*a*, *b*) EM autoradiographs of salivary glands pulse labelled with ^{3}H-uridine for 5 min and exposed for 20 days. In both, the β-heterochromatin (*b*) and euchromatin (*eu*) areas with a high degree of labelling are seen. The α-heterochromatin is not visible in these sections. Some puffed regions (*p*) may also be seen. In both instances there is some labelling of the nucleoplasm and cytoplasm. $\times$ 7 000.

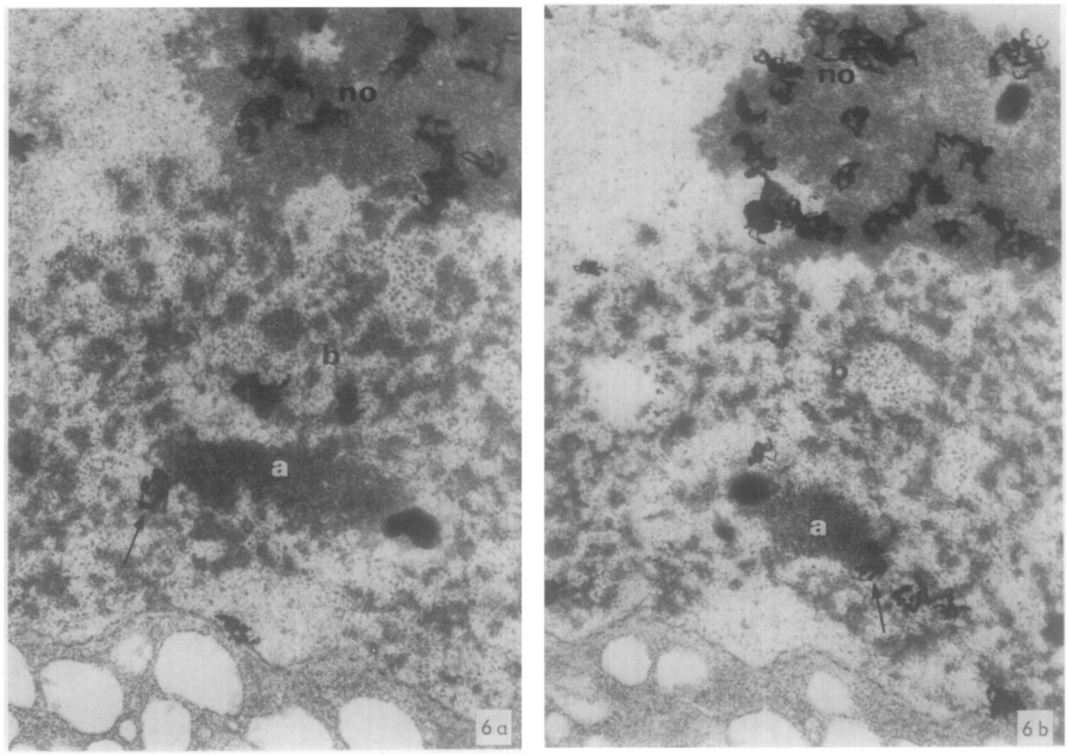

Fig. 6. (*a*, *b*) EM autoradiographs prepared as in fig. 5, showing two serial sections of the chromocentre region and nucleolus (*no*) of a low-labelled nucleus. The α-chromatin (*a*) in both is completely unlabelled while the β-chromatin (*b*) is clearly labelled. RNP particles may also be noted in β-heterochromatin. Grains seen near the junction of the α- and β-heterochromatin (*arrows*) have been considered to belong to the 'peripheral' zone described in the text. In these autoradiographs, there is no labelling of the nucleoplasm or the cytoplasm. Labelling density of nucleolus is much higher than the β-heterochromatin or euchromatin (not shown). (*a*) × 13 000; (*b*) × 11 000.

chromatin, but in a few cases, grains have also been noted over the particles described earlier. The α-chromatin region has not been seen to be labelled in any preparation examined (fig. 6*a*, *b*).

An estimate of the relative labelling over the α- and β-heterochromatin and the typical euchromatic areas has been obtained by comparing grains per unit area of these regions. For this purpose, from the enlarged autoradiographs, total areas of the α-, where present, the β-heterochromatin and euchromatin were measured separately. The areas of euchromatin selected were away from the chromocentre regions and included both puffed and non-puffed regions. Within the chromocentre region, the α- and β-chromatins are contiguous with each other and because of the limitations of autoradiographic resolution, the grains lying near the border of the two can not be unequivocally ascribed to one or the other. For this reason, an empirical method of analysis was adopted as was also done by Jurand & Jacob [9] for a comparable situation. In our preparations, the EM autoradiographic resolution can be estimated to be about 1 600 Å on the basis of data provided by Salpeter et al. [17]. Accordingly, lines at a distance equivalent to 1 600 Å were marked on the enlarged prints

Table 1. *Analysis of silver grains in different areas of the chromocentre and euchromatin after in vitro pulse labelling of salivary glands with ^3H-uridine*

Region[a]	Total area[b] μm^2	Total no. of grains	Grains/ 100 μm^2
α-Heterochromatin	26.48	0	—
β-Heterochromatin	1 639.84	774	47.20
Euchromatin	1 301.24	596	45.80
'Peripheral' zone	33.84	12	35.46

[a] For details, see text.
[b] Total area scored in 30 EM autoradiographs; α-heterochromatin area was absent in some of the EM autoradiographs analysed.

on either side of the border between the α- and β-chromatin; the area between these two lines is termed the 'peripheral' zone. Any grains located over the α-region internal to the peripheral zone, were taken to be due to radioactivity located in this region itself, while grains lying within the peripheral zone could be due to crossfires from either the α- or the β-chromatin areas of this zone. Grains overlying the β-chromatin outside the peripheral zone were taken to represent ^3H-uridine incorporated into this region. A total of 30 EM autoradiographs obtained from different glands were analysed in this manner. The data on the total areas examined and the average grain densities observed in different regions are presented in table 1. As mentioned earlier, there is a high variability in the labelling density in different nuclei and as an example of this variability, it may be mentioned that in the different autoradiographs analysed, the labelling density in the β-heterochromatin as well as euchromatin areas ranged from a low value of only 21 grains/100 μm^2 to as much as about 120 grains/100 μm^2. Generally, however, there seems to be a correlated variation in the labelling densities of the beta heterochromatin and euchromatin areas of a nucleus.

The data presented in table 1 show that in none of the autoradiographs any silver grains were located over the α-heterochromatin, although the area of this region is large enough for the majority of grains overlie it, if it were labelled. In the same preparations the peripheral zone, with only a slightly larger area than the α-heterochromatin, has been seen to be labelled (35.5 grains/100 μm^2, table 1). The β-heterochromatin seems to be as active as the average euchromatin areas. The average grain density on the β-chromatin (47.2 grains/100 μm^2) is very close to that of the euchromatic regions (45.8 grains/100 μm^2). The lower number of grains on the peripheral zone compared with the β-heterochromatin may be due to absence of any incorporation in the α-chromatin; all grains in the peripheral zone are probably due to radioactivity in the adjoining β-heterochromatin but since this zone also includes an area of α-chromatin, the overall grain density may become lowered.

DISCUSSION

We have examined the ultrastructural organization of the chromocentre heterochromatin in salivary gland polytene nuclei of late third instar larvae of *Drosophila melanogaster*. Since sections of unsquashed glands have been studied, different sections pass at different planes of nuclei and as a result, different micrographs do not show the typical structure of the chromocentre region. This may make the study and a true identification of the chromocentre difficult. However, several distinctive features of the chromocentre region, like a higher electron density, a disorganized appearance with a total absence of chromomeric organization, proximity to the nuclear membrane etc., permit unmistakable identification of this region in any section.

Heitz [7] differentiated heterochromatin in the chromocentre region of salivary gland chromosomes of *D. virilis* into the "alpha"- and "beta"-heterochromatin on the basis of their degree of compactness and other cytogenetical properties. In *D. melanogaster*, however, distinct α- and β-heterochromatins cannot usually be visualized in ordinary squashed preparations of salivary gland chromosomes. In sections of unsquashed glands, two types of chromatin organization in the chromocentre could be clearly identified both under light and electron microscope. The centrally located single large mass of homogeneously dense chromatin has been termed the α-heterochromatin, while the rest of the network of dense chromatin masses surrounding the central block and extending to the bases of euchromatic chromosome arms is designated the β-heterochromatin. Sorsa [19] studying the chromocentre organization in ultrathin sections of squashed preparations of these chromosomes also obtained EM micrographs showing similar structure of the chromocentre heterochromatin, but the interpretation of the micrographs relating to the limits of the α- and β-heterochromatin seems to be slightly different from the one suggested here (see figs 14, 15 of Sorsa [19]). However, the present interpretation of the distinctness of the central block (α-heterochromatin) and the surrounding network of dense chromatin masses (β-heterochromatin) is also substantiated by our EM autoradiographic studies on the replicative organization within the chromocentre heterochromatin [11, 13]. It may be stated here that the use of the terms "alpha" and "beta" heterochromatins is only for the sake of nomenclature and does not necessarily imply the same significance as suggested in classical studies.

In the earlier study [19] on the ultrastructure of the chromocentre region, 600–700 Å wide loops made of 25–30 Å thick fibrils, projecting from the compact blocks of the chromocentre and showing occasional 'stickiness' with each other were described; true interconnections between the dense blocks were noted rarely. In our preparations, however, no such loops were seen, but bundles of 30 Å thick fibrils extending from one dense block to another were regularly noted. It is suggested that the loops and the 'stickiness' observed by Sorsa [19] probably resulted due to the technique employed.

Another important difference between the present observations and those of Sorsa [19] relates to the particles seen by us in the β-heterochromatin region. Nothing of this kind was noted by Sorsa, which again was probably due to acetomethanol fixation and squashing prior to electron microscopy.

We have seen the two types of particles in the chromocentre region of most of the glands from late third instar larvae. Particles of the smaller type have also been seen to be present in the β-heterochromatin area of the chromocentre region of salivary gland polytene nuclei of *D. hydei* (Lakhotia, unpublished). The abundance and an orderly arrangement (especially the larger type) of particles indicates that these are related to the chromatin areas in which they occur. It is certainly unlikely that the particles are products of some other loci but are left in the β-heterochromatin region during the process of sectioning. When present, these particles may be seen in all the nuclei of a given gland and also in all the serial sections of a given chromocentre region. Besides, the larger type of particles appear to be orderly placed in a specific part of β-heterochromatin and have not been seen in any other chromosomal regions of the nucleus. Furthermore, the α-chromatin lacks these particles totally. Taken together, these observations demonstrate that the β-heterochromatin in these nuclei is characterized by

the presence of two types of particles, the placement of which in this region is not an artifact. The cytochemical nature of these particles has not yet been studied but indirect information is available to suggest that these are ribonucleoprotein (RNP) in nature.

Observations made during the course of investigations on the replicative organization of the chromocentre region [11] in these nuclei, indicate that the particles in the β-chromatin do not contain any DNA. In these experiments, ^{3}H-thymidine was fed to larvae throughout their growth period and the incorporation examined by EM autoradiography. The point of interest in the present context is that both the types of particles in the β-heterochromatin always remained unlabelled by ^{3}H-thymidine, thus indicating an absence of DNA. On the other hand, the morphological similarity of these particles with the well known puff particles in the polytene chromosomes [1, 2, 21, 22, 24] makes it likely that the chromocentre particles are also RNP in nature.

The α-heterochromatin is free of any RNP-particles and has also not been seen to be labelled with ^{3}H-uridine. The limited sample size of the α-chromatin in the present autoradiographic analysis does not permit one to rule out the possibility of some transcriptive activity in this part, but this seems unlikely since the peripheral zone with a closely similar sample size has been seen to be labelled in these preparations. The non-labelling of the α-heterochromatin may, therefore, reflect inactivity rather than lack of detection of activity. It is significant in this context that the α-heterochromatin does not participate in the polytenic replications in salivary gland nuclei [11, 13] and this too would indicate the 'non-essential' nature of this part of the genome in salivary gland nuclei.

Our data show that the labelling of the β-heterochromatin is of the same order as in the average euchromatin regions. That this labelling reflects the incorporation of ^{3}H-uridine in the β-heterochromatin area itself is borne out by several considerations. Since the glands were sectioned without squashing, a displacement of label from other nuclear areas onto the chromocentre region may be ruled out; furthermore, duration of the pulse was rather short (5 min) and this makes a transport of labelled RNA from other sites of synthesis unlikely. All these suggest that the β-heterochromatic actively transcribes in the salivary gland cells in late third instar larvae of *D. melanogaster*. The two types of RNP-particles seen in this part seem to be related to this transcription, although we do not know if these particles are two or more separate transcription products.

Constitutive heterochromatin [4] is generally considered to be transcriptionally inactive [18]. In *D. melanogaster*, there are very few known genetic markers in the heterochromatic chromosome regions which form the chromocentre in polytene nuclei [5, 16]. The enrichment of repetitive DNA sequences in the centric heterochromatin [3, 6, 8, 10, 15], may also suggest that the chromocentre DNA would be inactive in transcription. The present evidence for transcriptionally active β-heterochromatin, therefore, is very interesting and understanding of its nature and significance would be important.

One of the known genetic locus in the heterochromatic region of the X-chromosome of *D. melanogaster* is the nucleolar organizer region [5, 16, 23]. Since the heterochromatic part of the X-chromosome is also involved in the chromocentre formation, it may be conceived that the transcription noted in the β-heterochromatin may actually reflect the activity of the ribosomal cistrons. This, however, seems unlikely. The β-heterochromatin was seen to be labelled throughout its area uniformly with no evidence for a

localized high degree of incorporation which would have been expected if the transcription was restricted to rRNA cistrons. Further, the grain density over nucleolus was much higher than on β-heterochromatin and, therefore, the magnitude of rRNA transcription in the β-chromatin, if any, must be very low. It may also be added that on the basis of in situ hybridization studies, Pardue et al. [14] concluded "that in salivary gland nuclei of *D. hydei* at late third instar the ribosomal cistrons are located almost exclusively within the body of nucleolus". Our studies on replication of chromocentre [11] and nucleolar DNA (unpublished data) of *D. melanogaster* salivary gland nuclei also favour the location of ribosomal DNA within the nucleolus.

Kram et al. [10] have provided evidence for the occurrence of unique or "spacer" sequences interspersed among the repetitive sequences of the centric heterochromatin of *D. melanogaster*; they have also mentioned that there is evidence for the existence of RNA complementary to the "spacer" DNA but not to the repetitive sequences. It would be of interest to examine the correlation of this reported [10] RNA with the transcription and the smaller type of RNP particles noted in β-heterochromatin in the present study. In *D. melanogaster*, Steffensen [20] has localized the cistrons for ribosomal proteins on the X-chromosome heterochromatin very close to the rRNA cistrons. It may be possible that while the rRNA cistrons in polytene nuclei are located within the nucleolus, the ribosomal protein cistrons remain in the β-heterochromatin where they may be seen as transcriptionally active. In this connection, it may be recalled that there is some suggestion that the larger type of RNP-particles described here may be located on the X-chromosome β-heterochromatin (see fig. 4*a*); if this is confirmed, it would be interesting to examine a possible relationship between these particles and the ribosomal protein cistron transcription.

Many aspects of *Drosophila* chromocentre heterochromatin still need to be studied in the light of recent advances in this field for a complete understanding of its organization and function. In view of the evidence presented here, the general concept of total "inactivity' of constitutive heterochromatin also needs to be reexamined.

S. C. L. gratefully acknowledges the Royal Commission for the Exhibition of 1851, London, for the award of an overseas scholarship, and the British Council for a travel grant. The study leave granted to S. C. L. by Gujarat University, India, is also thankfully acknowledged.

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Received October 23, 1973
Revised version received February 5, 1974