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Repetitive DNA Sequences in *Drosophila*

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Abstract. The satellite DNAs of *Drosophila melanogaster* and *D. virilis* have been examined by isopycnic centrifugation, thermal denaturation, and *in situ* molecular hybridization. The satellites melt over a narrow temperature range, reassociate rapidly after denaturation, and separate into strands of differing buoyant density in alkaline CsCl. In *D. virilis* and *D. melanogaster* the satellites constitute respectively 41% and 8% of the DNA isolated from diploid tissue. The satellites make up only a minute fraction of the DNA isolated from polytene tissue. Complementary RNA synthesized *in vitro* from the largest satellite of *D. virilis* hybridized to the centromeric heterochromatin of mitotic chromosomes, although binding to the Y chromosome was low. The same cRNA hybridized primarily to the α-heterochromatin in the chromocenter of salivary gland nuclei. The level of hybridization in diploid and polytene nuclei was similar, despite the great difference in total DNA content. The centrifugation and hybridization data imply that the α-heterochromatin either does not replicate or replicates only slightly during polytenization. Similar but less extensive data are presented for *D. melanogaster*. — In *D. melanogaster* cRNA synthesized from total DNA hybridized to the entire chromocenter (α- and β-heterochromatin) and less intensely to many bands on the chromosome arms. The X chromosome was more heavily labeled than the autosomes. In *D. virilis* the X chromosome showed a similar preferential binding of cRNA copied from main peak sequences.—It is concluded that the majority of repetitive sequences in *D. virilis* and *D. melanogaster* are located in the α- and β-heterochromatin. Repetitive sequences constitute only a small percentage of the euchromatin, but they are widely distributed in the chromosomes. During polytenization the α-heterochromatin probably does not replicate, but some or all of the repetitive sequences in the β-heterochromatin and the euchromatin do replicate.

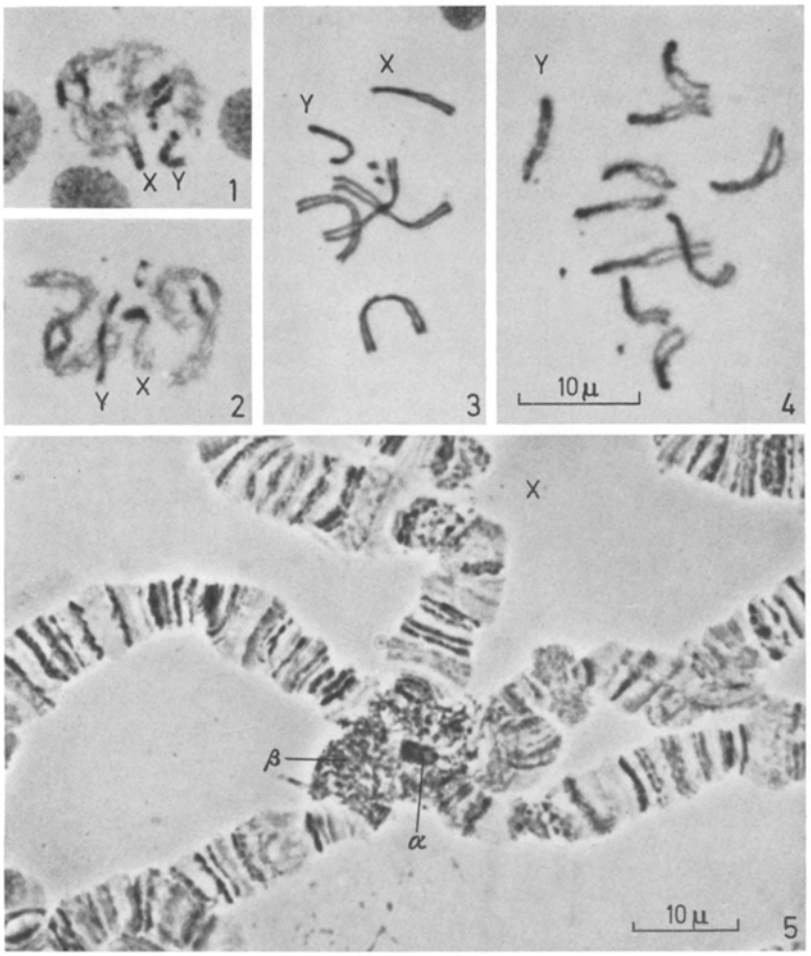
Introduction

In various species of *Drosophila* the mitotic chromosomes possess conspicuous heterochromatic regions next to the centromeres (Heitz, 1934a, b; Kaufmann, 1934; Cooper, 1959). The heterochromatic segments are compact and deeply staining during prophase, and the sister chromatids in these regions tend to remain closely apposed until anaphase (Figs. 1–4). In *D. melanogaster* the heterochromatin comprises the proximal third to half of the X chromosome, the entire Y chromosome, and the middle 20% of chromosomes 2 and 3. As shown by Heitz (1934a, b) even longer heterochromatic regions characterize the mitotic chromo-

somes of *D. virilis* (Fig. 4). In the giant polytene nuclei the heterochromatic regions are much reduced in relative length, and they are fused together to form the chromocenter (Fig. 5). Within the chromocenter the limits of individual chromosomes are difficult to identify. The Y chromosome, for instance, is either unrecognizable or reduced to a few indistinct bands associated with the proximal part of the X (Prokofyeva-Belgovskaya, 1937; Nicoletti and Lindsley, 1960). Detailed cytogenetic analysis in *D. melanogaster* has shown that the great majority of genes are confined to the euchromatic segments of the mitotic chromosomes, and that these in turn correspond to the regularly banded regions of the polytene chromosomes (reviewed in Beermann, 1962).

Heitz (1934a, b) early suggested that the heterochromatin fails to replicate during the formation of the polytene chromosomes, or replicates more slowly than the euchromatin. This hypothesis was strongly supported by the microspectrophotometric measurements of Rudkin (1964, 1969), Berendes and Keyl (1967), and Mulder, van Duijn, and Gloor (1968). Rudkin measured the amount of DNA in nuclei from developing salivary glands of *D. melanogaster* and demonstrated that the values do not fit a simple 2^n series. Instead he suggested that about 25% of the DNA in the diploid nucleus fails to replicate. Berendes and Keyl examined the DNA in larval ganglion cells of *D. hydei*, measuring the euchromatic and heterochromatic parts of each nucleus separately. They concluded that both euchromatin and heterochromatin increase in a geometric fashion, but that the heterochromatin undergoes only a few replications at most. Mulder, *et al.*, also studied *D. hydei*, and concluded that the heterochromatin ordinarily does not replicate more than once during polytenization in the salivary gland.

We describe here the localization of repetitive DNA sequences in the chromocenter of *Drosophila*, confirming the earlier reports of Rae (1970), Jones and Robertson (1970) and Hennig, Hennig, and Stein (1970). In addition we present biochemical and cytological evidence for differential replication of eu- and heterochromatin. Specifically we show that certain repetitive DNA sequences, isolated as satellites from CsCl gradients, are located in the heterochromatic portions of the mitotic chromosomes and in the chromocenters of the polytene nuclei. However, the satellite sequences are relatively much more abundant in diploid than in polytene nuclei. This differential abundance is demonstrated by two observations. First, the DNA isolated from diploid tissue displays the satellites when centrifuged to equilibrium in CsCl, whereas the satellites are almost undetectable in DNA isolated from polytene tissue. Second the amount of satellite DNA demonstrable by cytological hybridization in diploid nuclei is approximately the same as in polytene nuclei, despite the great difference in total DNA content.



Figs. 1–4. Neuroblast mitoses of *Drosophila melanogaster*. Magnification as indicated in Fig. 4. Figs. 1 and 2. Heterochromatic regions in prophase chromosomes. The entire Y, the proximal third to half of the X, and the centromeric regions of chromosomes 2 and 3 are more compact than the distal euchromatic segments. The greater part of chromosome 4 also appears to be heterochromatic. Somatic pairing of homologues is evident. Colchicine-hypotonic treatment. Giemsa stain after HCl hydrolysis. Fig. 3. Late prophase in a larval neuroblast of *D. melanogaster* treated with colchicine. Sister chromatids tend to remain paired in the heterochromatic regions, whereas they separate in the distal euchromatic regions. The Y chromatids remain paired along their entire length. Giemsa stain after HCl hydrolysis. Fig. 4. Colchicine mitosis in a larval neuroblast of *D. virilis*. The heterochromatic segments are relatively longer than in *D. melanogaster*. They comprise the proximal half of all the major chromosomes except the Y, which is totally heterochromatic. Giemsa stain after HCl hydrolysis

Fig. 5. The chromocenter of *D. virilis* from a salivary gland squash. The most compact region in the center (arrow) is the α-heterochromatin of Heitz. The surrounding granular area is the β-heterochromatin. Lactic-aceto-orcein, phase contrast

Materials and Methods

1. Preparation of DNA[1]. DNA has been prepared from adult *D. melanogaster* and *D. virilis* using the method of Laird and McCarthy (1968). In some cases the crude nuclear pellet was suspended in 0.05 M tris, 0.1 M EDTA, 0.5% Na lauroyl sarcosinate (Sarkosyl NL-97, Geigy) pH 8.4 to which NaCl or $NaClO_4$ was added to give a concentration of 1–2 M. After treatment with pancreatic RNase (100 μg/ml), T_1 RNase (330 units/ml) and amylase (100 μg/ml) the solution was extracted with phenol, and the DNA was precipitated with 2 volumes of 95% ethanol. The DNA was centrifuged, washed briefly with 70% ethanol, and dissolved in 0.1 × SSC. Saturated CsCl was added to give a final density of 1.70, and the solution was centrifuged in the Spinco 50 rotor at 18° for 20 hr at 42000 rpm or 65 hr at 30000 rpm. Ten-drop fractions were collected from the bottom of the centrifuge tube, which had been pierced with a 27 gauge needle. The $O.D._{260}$ of each fraction was determined in a spectrophotometer. Fractions which contained satellite DNA were pooled and recentrifuged one or more times to remove main peak DNA. The purity of the fractions was routinely estimated by analytical ultracentrifugation.

2. Analytical Ultracentrifugation. The buoyant density of DNA samples was determined by equilibrium centrifugation in CsCl, using the Spinco Model E ultracentrifuge. Samples were centrifuged at 44770 rpm for 18–20 hr at 20° C in the An-F rotor. In most cases 2° sector cells were used. Approximately 0.5–2.0 μg of DNA was dissolved in 0.4 ml of CsCl at a density of 1.70. DNA from *E. coli* ($\varrho = 1.710$) or *M. lysodeikticus* ($\varrho = 1.731$) was used as a density standard. Densities of the *Drosophila* DNA were calculated using the methods described by Szybalski (1968) and Mandel, Schildkraut, and Marmur (1968).

Alkaline centrifugation was carried out in a CsCl solution having an initial density of 1.74. NaOH was added to a concentration of 0.05 N. DNA of *M. lysodeikticus* ($\varrho = 1.789$) provided a density standard for the alkaline gradients (Vinograd, Morris, Davidson, and Dove, 1963).

The DNA from small tissue samples was examined by a procedure similar to that described by Pikó, Tyler, and Vinograd (1967). The imaginal discs and brains from 3–6 larvae or the salivary glands from 10–12 larvae were lysed in about 100 μl of tris-EDTA-Sarkosyl at pH 8.4. After one or two hours, satured CsCl was added to bring the density to 1.70, and the sample was placed directly into the centrifuge cell. During centrifugation the protein rises to the top of the gradient while high molecular weight RNA sediments. DNA (and in some cases carbohydrate) bands at its equilibrium density. Alkaline gradients were run in a similar manner, although with all but very small amounts of tissue the background optical density was high, presumably because of the hydrolysis of RNA.

3. Thermal Denaturation. Melting temperature determinations were carried out as described by Mandel and Marmur (1968). DNA samples were dissolved in SSC and were then heated in Teflon-stoppered cuvettes. The increase in optical density at 260 mμ was continuously monitored with a Gilford Model 2000 recorder. The temperature rise was approximately 0.5°/min. Reassociation of denatured DNA was studied by reducing the temperature to 60° and holding it there for various times. In the case of the satellite DNAs, reassociation was completed in the few minutes required with our apparatus to bring the temperature to 60°.

4. Preparation of Complementary RNA (cRNA). Molecular hybridization experiments were carried out using cRNA synthesized *in vitro* by *E. coli* RNA

1 *Abbreviations.* cRNA: complementary RNA synthesized *in vitro* with *E. coli* RNA polymerase; EDTA: ethylene diamine tetraacetate; SSC: 0.15 M NaCl, 0.015 M Na citrate, pH 7.0; t_m: melting temperature; tris: tris-(hydroxymethyl) amino methane.

polymerase (Burgess, 1969). Various DNA fractions were used as template. The isolation of the polymerase and details of the cRNA synthesis were described in an earlier paper from this laboratory (Pardue, Gerbi, Eckhardt, and Gall, 1970). The cRNA had a calculated specific activity of about 10^8 dpm/μg.

5. Cytological Hybridization. Cytological squash preparations were hybridized with cRNA according to procedures described earlier (Pardue, *et al.*, 1970; Gall and Pardue, 1971). In some cases we have omitted the alkali denaturation step, which often causes extensive cytological distortion. Instead we have treated tissue with 0.2 N HCl at 37° for various times (usually 30 min) in order to denature the DNA. Details are given in the figure legends.

Results

1. DNA from Diploid Tissue

We have examined the buoyant density in CsCl of DNA from larval brains and imaginal discs, tissues which contain predominantly diploid nuclei. In each case the tissue was dissected from a few larvae, lysed in detergent, mixed with an appropriate amount of saturated CsCl, and centrifuged to equilibrium without further processing. Under these conditions proteins rise to the top of the gradient, high molecular weight RNA sediments, and DNA bands at its equilibrium density.

In *D. melanogaster* the DNA from these larval tissues consisted of two fractions, a main band with a density of 1.702 and a minor satellite band with a density of 1.689 (Fig. 6a). The satellite has been reported before in DNA isolated from whole animals (Laird and McCarthy, 1968; Travaglini, Petrovic, and Schultz, 1968; Rae, 1970). The satellite constituted approximately 8% of the total DNA. This estimate was based on six determinations with a range of 7–11%. The higher estimates are known to be slightly in error due to underestimation of the main band (overloaded gradients with the U.V. absorption of the main band outside the linear range of the photographic emulsion). On heavily loaded gradients we have noted two faint bands at $\varrho = 1.669$ and $\varrho = 1.674$, whose nature we have not investigated. The band at $\varrho = 1.669$ may correspond to the poly dAT studied by Fansler, Travaglini, Loeb, and Schultz (1970). On most gradients the main band is slightly skewed toward the heavier side.

In *D. virilis* the satellites are more prominent. In addition to the main band at a density of 1.700 there are three sharply banding satellites with densities of 1.692, 1.688, and 1.671 (numbered I, II, and III in Fig. 6c). Together these satellites make up approximately 41% of the total DNA (I = 25%, II = 8%, III = 8%). The heterogeneity of *D. virilis* DNA was reported earlier by Schurin and Marmur (1961) and by Travaglini, Petrovic, and Schultz (1968). In both species we have seen the satellites in all samples of larval brains and imaginal discs examined.

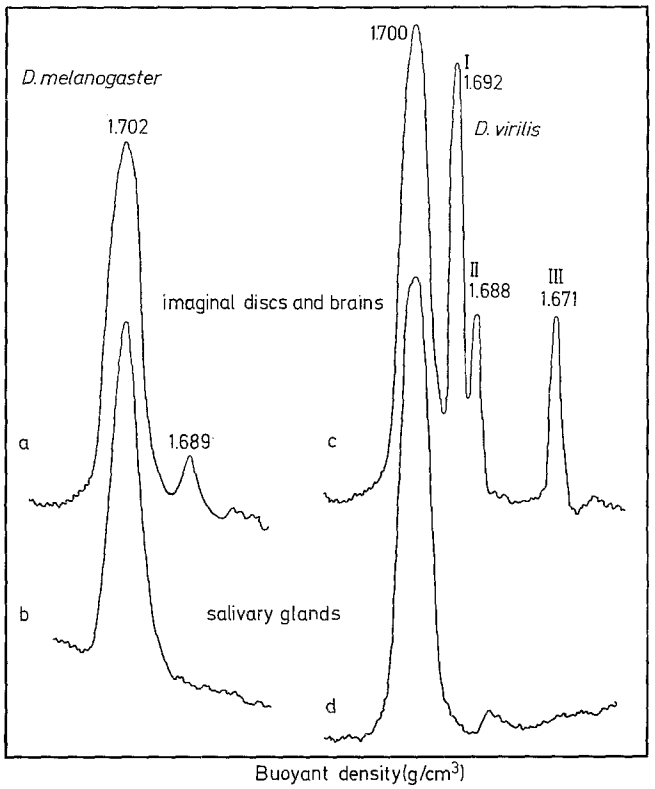


Fig. 6a–d. Banding patterns of *Drosophila* DNA after equilibrium centrifugation in neutral CsCl. In both *D. melanogaster* and *D. virilis* satellite DNA is present in the diploid tissue (brains and imaginal discs) but is not detected in the polytene tissue (salivary glands). Tissue samples from third instar larvae were dissolved in Sarkosyl detergent and placed directly into CsCl for centrifugation. a Imaginal discs and brains of *D. melanogaster*. b Salivary glands of *D. melanogaster*. c Imaginal discs and brains of *D. virilis*. d Salivary glands of *D. virilis*

The quantitative measurements demonstrate that the relative amounts of the satellites are constant in these diploid tissues.

2. DNA from Polytene Tissue

The situation in polytene tissue stands in striking contrast to that in diploid tissue. We have examined the DNA from larval salivary glands of both species and from larval guts of *D. virilis* (from which the gut contents and peritrophic membrane had been removed). In all cases the satellites were either undetectable or present in very much reduced amount (Fig. 6b, d). In order to check the possibility that satellite DNA

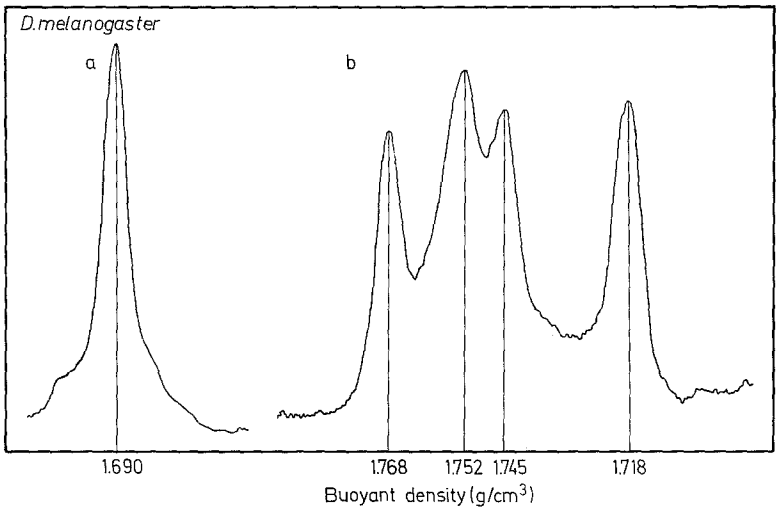


Fig. 7a and b. Banding pattern of *D. melanogaster* satellite DNA after equilibrium centrifugation. a Purified satellite in neutral CsCl showing a single narrow band along with more heterogeneous material. b The same sample centrifuged in alkaline CsCl, showing four bands which presumably represent the separated strands of two satellite species

might be selectively removed from the CsCl gradient by some substance in these tissues, we have lysed *D. virilis* salivary glands and imaginal discs in the same solution. Under these conditions the satellite DNA from the imaginal discs was readily apparent on the gradient.

Our cytological hybridization experiments demonstrate that satellite DNA is not completely absent from the polytene nuclei of the salivary glands. The reduction is so great, however, that the satellites are not detected at normal DNA loadings in the analytical centrifuge.

3. Characteristics of Satellite DNA

a) D. melanogaster. The satellite DNA of *D. melanogaster* was purified by successive isopycnic centrifugations in neutral CsCl. When examined in neutral CsCl in the analytical ultracentrifuge the purified satellite formed a narrow band at a buoyant density of 1.690 superimposed on more heterogeneous material (Fig. 7a). When the same DNA was centrifuged in alkaline CsCl, four separate bands were seen (Fig. 7b). These four bands presumably represent the separated strands of two different satellite DNAs. The strands of satellite DNAs frequently band at different buoyant densities in alkaline CsCl because of disparity in their G + T contents (Vinograd, Morris, Davidson, and Dove, 1963;

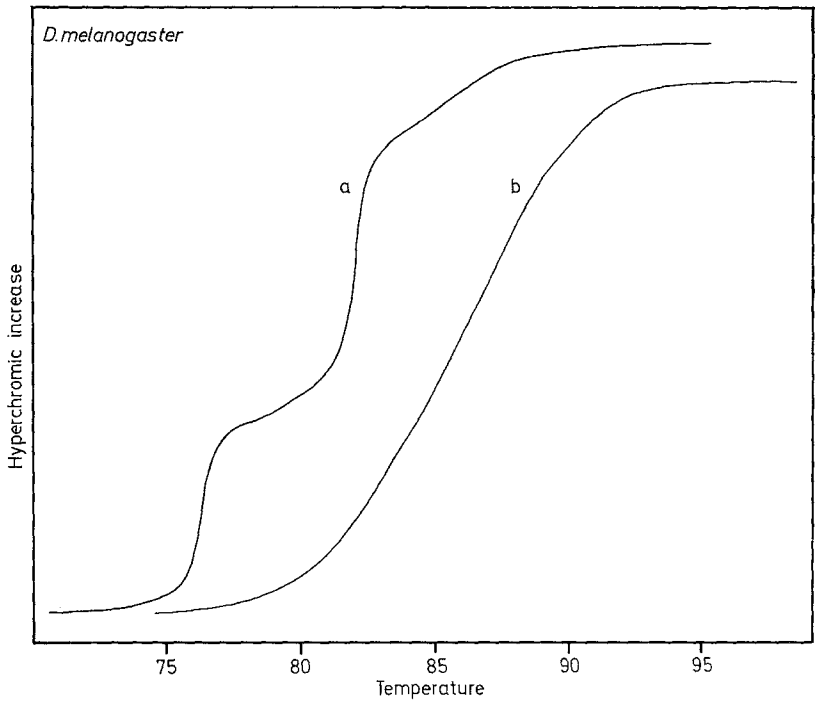


Fig. 8a and b. Thermal denaturation of *D. melanogaster* DNA in SSC. a Melting curve of the satellite DNA shown in Fig. 7. There are two sharp melting transitions at 76.2° and 81.9° along with a more gradual rise in optical density at higher temperatures (possibly representing contaminating main peak sequences). b Melting curve of main peak DNA from adult flies. The t_m is 85.8°, consistent with a G + C content of 40.2%

Flamm, McCallum, and Walker, 1967). Because the four bands are similar in size, we do not know which ones are complementary. When unfractionated imaginal disc DNA was centrifuged in alkaline CsCl the two lighter satellite bands were readily identified (Fig. 11a); the two heavier bands were obscured by the main peak DNA.

Further information on the satellites was provided by thermal denaturation experiments (Fig. 8a). A complex melting curve was obtained from the sample of satellite DNA shown in Fig. 7. There were two sharp melting transitions at 76.2° and 81.9° and a more gradual increase in optical density at still higher temperatures. The transition at 81.9° is close to that expected for a DNA having the buoyant density of the satellite pair (Marmur and Doty, 1962). It is possible that one satellite has a normal melting curve and the other has a biphasic curve with components both higher and lower than 81.9°. In addition there is

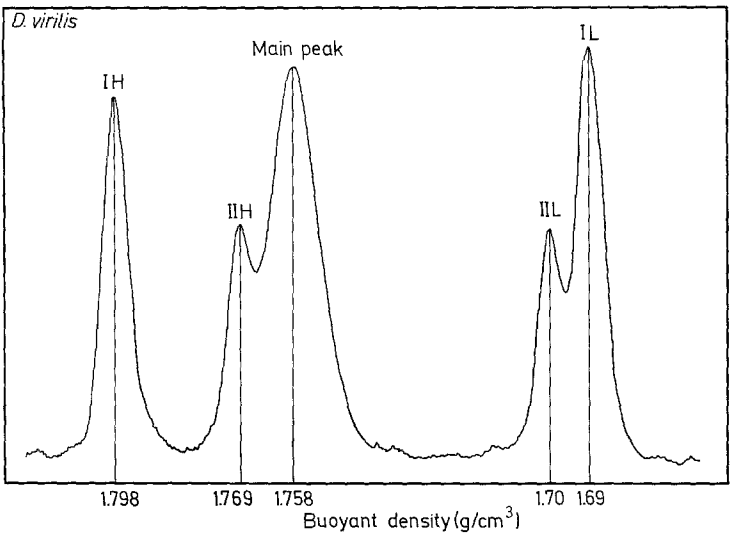


Fig. 9. Banding pattern of a DNA sample from *D. virilis* after equilibrium centrifugation in alkaline CsCl. This sample contained satellites I and II as well as contaminating main peak sequences. The identification of the heavy (*H*) and light (*L*) satellite strands was made on the basis of relative heights of the peaks. Buoyant densitites were estimated from a similar gradient in which *M. lysodeikticus* DNA ($\varrho = 1.789$) served as a standard. The density determinations may be somewhat in error at the light end of the gradient because of the great distance from the marker

probably contamination with main band sequences. The melting curve of main band DNA showed a gradual rise in optical density with a $t_m = 85.8°$ (Fig. 8b).

The reassociation of the mixed satellites was followed by reducing the temperature to 60° and plotting loss of optical density as a function of time. Approximately 60% of the hyperchromic increase had been lost in the 15 minutes required for the temperature in the cuvette to drop to 60°. Further incubation at 60° for 18 hr resulted in a slight additional loss of optical density. When reheated the DNA displayed two major melting components at approximately 72.8° and 78.5°. These t_m values were 3.4° lower than those seen during the original melt. The material that did not reassociate rapidly probably consisted of contaminating main peak sequences. From the concentration of DNA in this experiment (27 μg/ml) we can calculate that the $C_0 t_{1/2}$ of the reassociating material was less than 0.03.

b) D. virilis. The buoyant densities of *D. virilis* satellites I and II are too similar to permit easy purification of these two components on

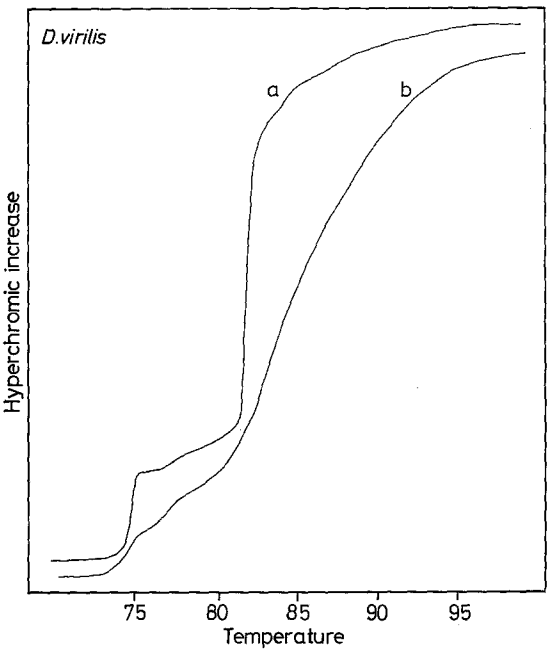


Fig. 10a and b. Thermal denaturation of *D. virilis* DNA. a A sample which consisted primarily of satellites I and II, with approximately half as much main peak contamination as in the sample shown in Fig. 9. Two sharp melting transitions are seen at 74.8° and 82.4°. The buoyant densities of the satellites ($\varrho = 1.688$ and $\varrho = 1.692$) predict melting temperatures of 80.8° and 82.4°. The significance of the observed transition at 74.8° is unknown. b Unfractionated *D. virilis* DNA. Transitions due to the satellites are superimposed on a broader melting curve characteristic of the main peak DNA

preparative CsCl gradients. However, by successive centrifugations the two satellites were separated from satellite III and from much of the main band DNA. Such an enriched satellite preparation was centrifuged in alkaline CsCl with the result shown in Fig. 9. From the relative amounts of DNA in each peak we may tentatively identify the most dense and least dense peaks as the separated strands of satellite I (IH and IL). Similarly the next lightest and next heaviest bands probably correspond to the strands of satellite II (IIH and IIL) and the middle peak represents the main band contamination.

A more complex pattern was obtained when bits of imaginal disc were lysed in detergent and centrifuged directly in alkaline CsCl (Fig. 11 b). The two strands of satellite I were easily recognized as the lightest and heaviest bands on the gradient. The second lightest band contained IIL and presumably one or both strands of satellite III. Strand IIH and an

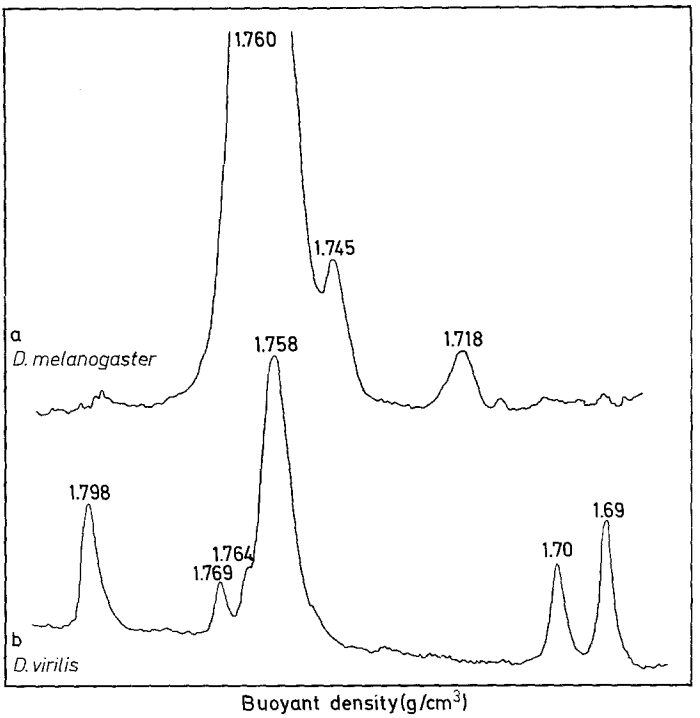
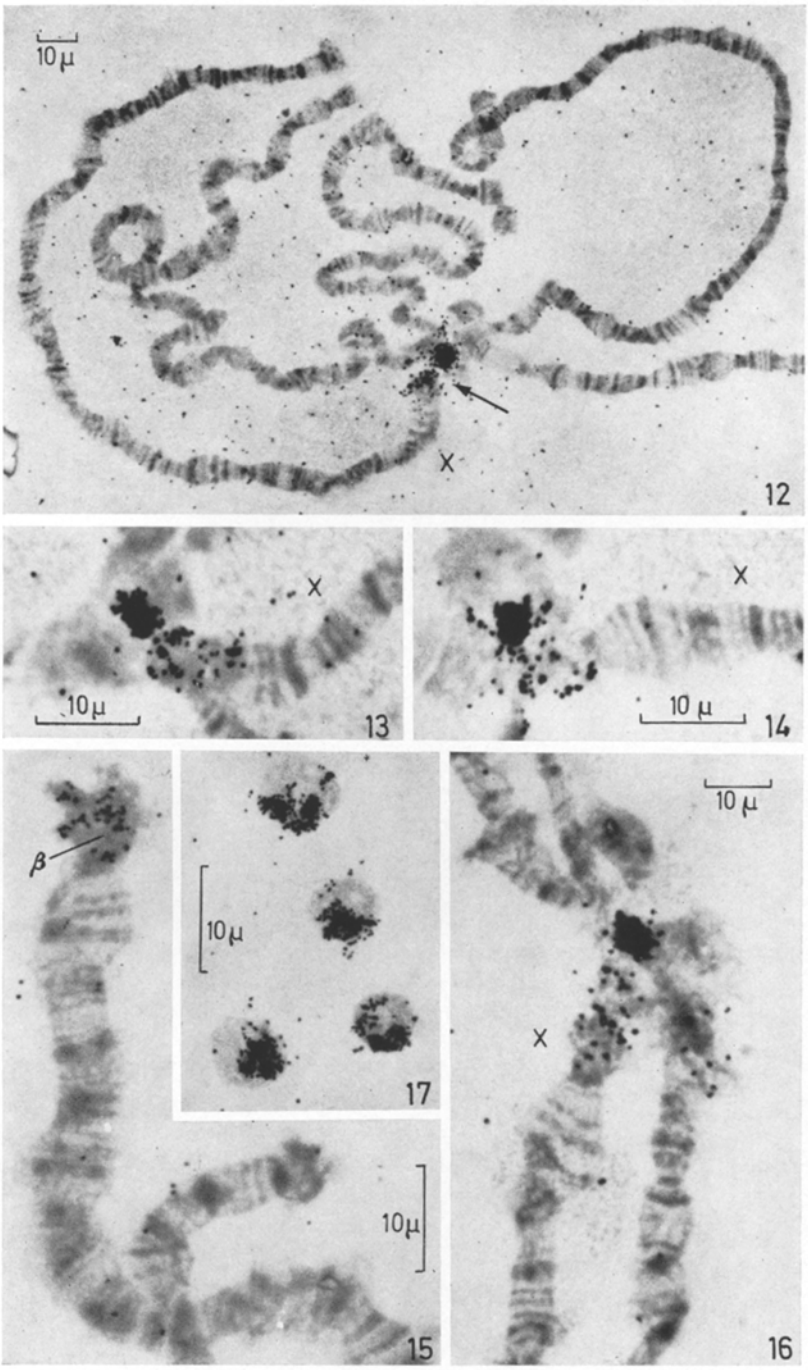


Fig. 11 a and b. Buoyant density banding patterns in alkaline CsCl of unfractionated DNA from diploid tissue of *D. melanogaster* (a) and *D. virilis* (b). For the *D. virilis* gradient, imaginal discs from 3 larvae were dissolved in Sarkosyl detergent and added directly to alkaline CsCl. For *D. melanogaster* a heavier loading was necessary in order to demonstrate the smaller satellite peaks. Imaginal discs from 30 female larvae were lysed in detergent, digested with pancreatic and T_1 RNase, precipitated with ethanol, and redissolved in alkaline CsCl. See text for description of bands

unidentified band (IIIH ?) constituted the two bands immediately to the left of the main peak.

The *D. virilis* DNA has been subjected to thermal denaturation (Fig. 10). A sample which contained satellites I and II plus some main peak contamination showed two sharp transitions at 74.8° and 82.4°, along with a broader transition at slightly higher temperatures. About 73% of the hyperchromic increase was lost during cooling to 60° ($C_0 t_{1/2}$ less than 10^{-2}). The material remelted in two stages with t_m's of 74.7° and 81.2°, only slightly below the original melt. DNA having the buoyant density of satellites I and II would be expected to melt at 80.8° and 82.4°. The significance of the observed transition at 74.8° is unknown.



Figs. 12–17

4. Cytological Hybridization

In order to determine the chromosomal location of the satellite DNA we have prepared cRNA from satellite DNA using RNA polymerase isolated from *E. coli*. We have then hybridized this cRNA to squash preparations of larval salivary glands (Pardue, Gerbi, Eckhardt, and Gall, 1970). The DNA samples used as template for the enzyme were isolated from alkaline CsCl gradients. They included the strand of lowest buoyant density from *D. melanogaster* and the strand of highest density from *D. virilis* (IH). These strands were chosen because they could be obtained in essentially pure form after only one preparative centrifugation (Figs. 7b, 9). In addition, a complement was prepared from a mixture of the three denser strands from *D. melanogaster*.

a) D. virilis. The cRNA prepared for the experiments with *D. virilis* salivary glands was copied from the heavier of the two strands of satellite I. In the polytene nuclei cytological hybridization was detected only in the chromocenter region (Figs. 12–14, 16, 18). By far the heaviest binding was seen directly over the densely staining mass designated α-heterochromatin by Heitz (1934b). Some silver grains were seen over other parts of the chromocenter. These could often be followed into the base of the X chromosome (Figs. 13–16). However, generalized labeling of the β-heterochromatin was not seen, even after longer exposures (3 months), nor were the euchromatic parts of the chromosomes labeled.

Figs. 12–17. *Drosophila virilis.* Giemsa stain. Figs. 12–16 Polytene chromosomes. Fig. 12 after cytological hybridization with cRNA synthesized from the heavier strand of satellite I. Labelling is restricted to the α-heterochromatin in the chromocenter and to a short region at the base of the X chromosome (arrow). The preparation was treated with 0.2 N HCl, 37° for 30 min and boiling H_2O for 1 min. The hybridization was carried out for 4 hrs at 66° with cRNA at a concentration of 3 μg/ml in 2 X SSC. Specific activity of cRNA about 10^8 dpm/ml. Exposure 54 days. Figs. 13 and 14. Chromocenter and proximal region of the X chromosome of *D. virilis*, hybridized as in Fig. 12 except that boiling water was omitted. Label is restricted to the α-heterochromatin and to the β-heterochromatin of the X. Exposure 7 days. Fig. 15. An X chromosome of *D. virilis* which was squashed away from the chromocenter, hybridized with cRNA to satellite IH as in Figs. 13 and 14. Label is restricted to the β-heterochromatin at the proximal end of the chromosome (arrow). Exposure 7 days. Fig. 16. Chromocenter and proximal regions of the polytene chromosomes of *D. virilis*, hybridized with cRNA to satellite IH as in Figs. 13 and 14. Label restricted to the α-heterochromatin and to the proximal heterochromatin of the X. Exposure 7 days. Fig. 17. Four diploid nuclei from a salivary gland, hybridized with cRNA to satellite IH. In such diploid nuclei label is located over the eccentrically placed heterochromatin. Note that the label intensity is similar in diploid and polytene nuclei (Figs. 13, 14, 16) suggesting that the α-heterochromatin fails to replicate during polytenization. These nuclei and the chromosomes shown in Figs. 15 and 16 were located close to one another on the same slide. Hybridization as in Figs. 13 and 14. Exposure 7 days

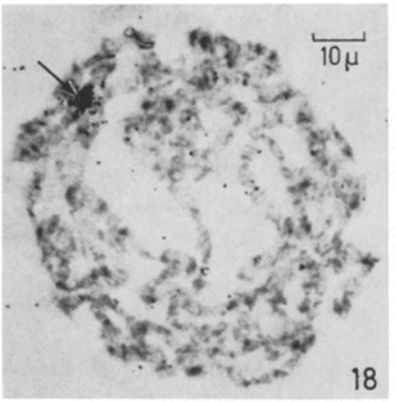
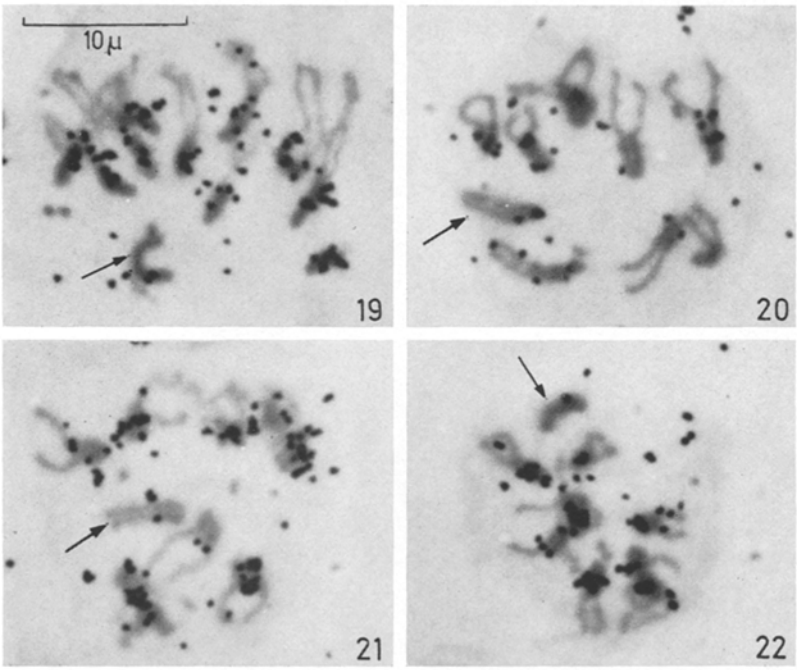


Fig. 18. A medium-sized polytene nucleus from a salivary gland of *D. virilis*. This nucleus was taken from the same preparation as Figs. 15–17, and demonstrates that the amount of hybridization in the α-heterochromatin (arrow) is independent of the size of the nucleus. Exposure 7 days. Giemsa stain

In addition to the giant polytene nuclei, salivary glands contain a small area of diploid imaginal nuclei just posterior to the duct region (Bodenstein, 1950). The intensity of cRNA binding to these nuclei is most remarkable: the heterochromatic part of each diploid nucleus is labeled as strongly as the chromocenter of the largest polytene nuclei on the same slide (Fig. 17). The chromocenters in the smaller polytene nuclei in the anterior part of the gland have a similar level of label (Fig. 18). To the extent that quantitative conclusions can be drawn from cytological hybridization experiments, one can say that the amount of cRNA binding is independent of the level of polyteny, over the entire thousand-fold range characteristic of *Drosophila* salivary gland nuclei (Swift and Rasch, 1954; Pettit, Rasch, and Rasch, 1967). This observation strongly suggests that satellite I fails to undergo replication during the polytenization process.

Preliminary observations have been made on the binding of satellite cRNA to mitotic chromosomes from larval neuroblasts. In the preparations so far examined binding was clearly seen over the heterochromatic regions of the X chromosome and of the autosomes. The Y chromosome, however, appeared to be relatively deficient in label (Figs. 19–22). Satellite I of *D. virilis* is, therefore, widely distributed in the genome. The α-heterochromatin of the chromocenter is a composite structure contributed by all of the chromosomes with the possible exception of the Y.

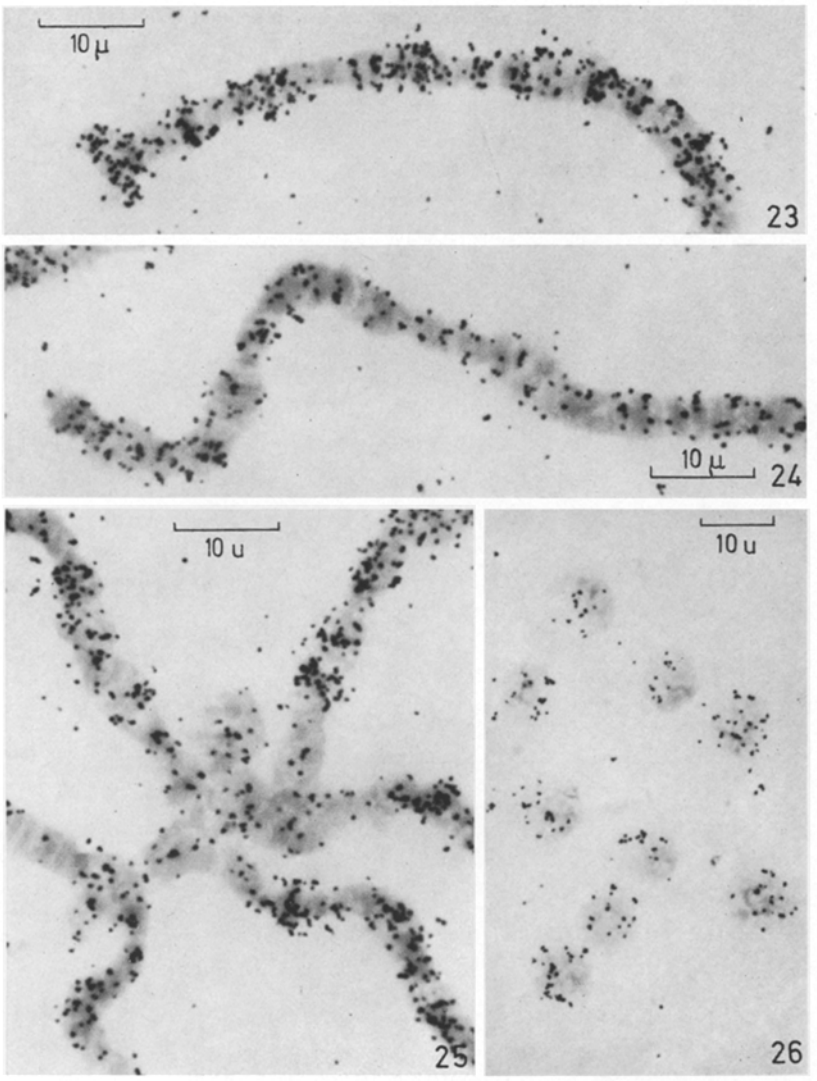
An examination has also been made of certain repetitive sequences from the main peak DNA. A sample of unfractionated *D. virilis* DNA (M.W. $> 5 \times 10^6$ daltons) was denatured by boiling (150 μg/ml in $0.1 \times$ SSC) and was then allowed to reassociate for 2 hr at 64° in 2 X SSC ($C_0t \sim 3$). The sample was centrifuged to equilibrium in neutral CsCl and the main



Figs. 19–22. Chromosomes from diploid larval neuroblasts of a male *D. virilis*, hybridized with cRNA to the heavy strand of satellite I. Silver grains are localized for the most part over the heterochromatic regions of the autosomes (and presumably the X as well, although the X is not consistently identifiable). By contrast the entirely heterochromatic Y chromosome (arrows) is less heavily labeled than any other member of the set. Chromosomes treated with 0.2 N HCl at 37° for 30 min. Hybridization for 6 hrs. at 65° at a cRNA concentration of 3 μg/ml in 2X SSC. Specific activity of cRNA about 10^8 dpm/μg. Exposure 14 days. Stained with Giemsa. Magnification as indicated in Fig. 19

peak was collected for transcription by *E. coli* polymerase. The transcribed DNA was, therefore, free of satellites I, II, and III, but presumably still contained "moderately repetitive" sequences still attached to the "unique" DNA.

The cytological hybridization of this cRNA is shown in Figs. 23–26. In salivary gland nuclei the label was found at many loci along the polytene chromosomes. Casual inspection showed, however, that the label was not randomly distributed, nor was the intensity of labeling simply related to the banding pattern. For instance, some prominent bands were weakly labeled, and vice versa (Fig. 23). An interesting feature was the relatively heavy label along the X chromosome compared to that on the



Figs. 23–26. Chromosomes of *D. virilis*, hybridized with cRNA synthesized from main peak DNA sequences from which satellites I, II, and III had been excluded. The chromosomes were denatured with 0.07 N NaOH for 2 min., and were then hybridized for 15 hrs. at 66° with cRNA at a concentration of 0.1 µg/ml in 2 X SSC. Specific activity of the cRNA was approximately 10^8 dpm/µg. Exposure 42 days. Giemsa stain. Fig. 23. Distal portion of the X chromosome, showing label in discrete bands at many loci. Note that the intensity of label is greater here than in the autosome shown in Fig. 24. The heavier labeling of the X was a consistent finding in all nuclei. Fig. 24. Distal portion of an autosome from the same nucleus as Fig. 23. Fig. 25. Chromocenter and proximal regions of the polytene chromosomes. Note the comparatively light labeling in the chromocenter, including both α- and β-heterochromatin. Fig. 26. Diploid nuclei from the same slide as Figs. 23–25. Label is largely restricted to the euchromatic part of each nucleus, and the total amount of label per nucleus is less than 1% of that seen over the polytene nuclei. This observation implies that repetitive sequences in the euchromatin replicate during polytenization

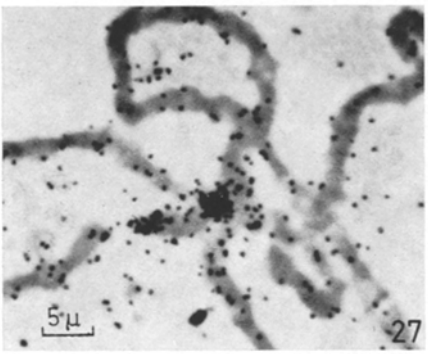


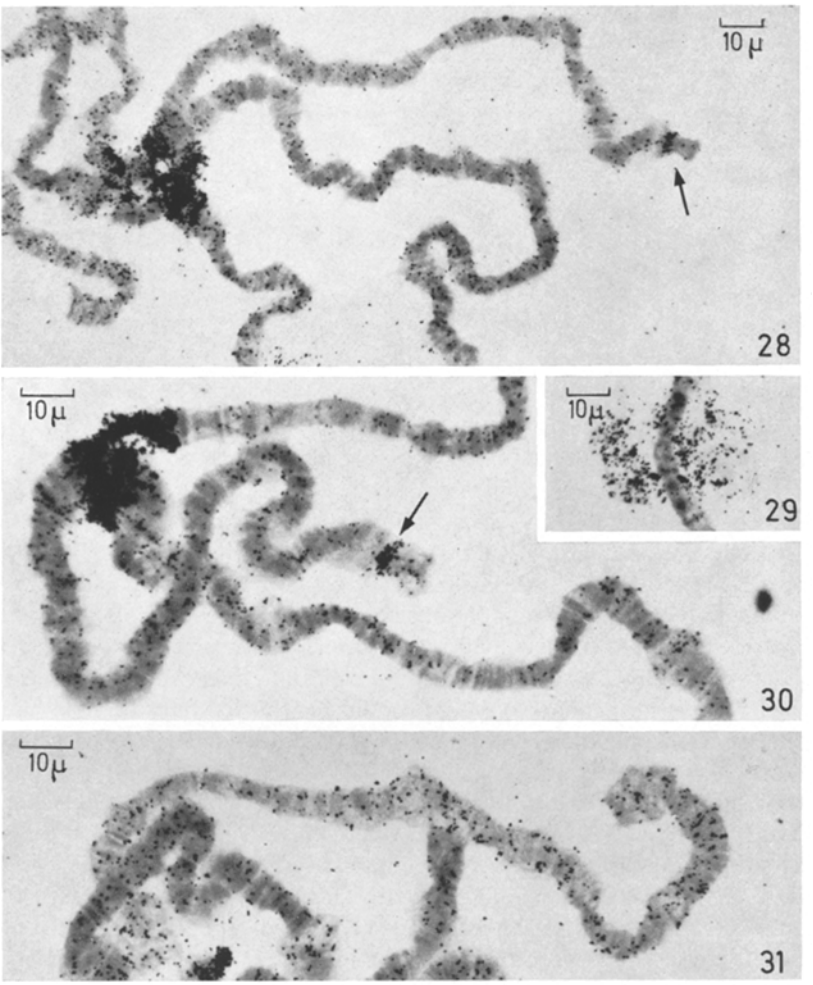
Fig. 27. Polytene chromosomes from a salivary gland nucleus of *D. melanogaster*, hybridized with cRNA synthesized from the lightest of the four satellite strands. Label is concentrated in the chromocenter. This series of slides showed unusually high levels of binding to the cytoplasm. Preparation treated with 0.2 N HCl for 45 min at 37°, and then hybridized for 6 hr with cRNA in 2 X SSC at a concentration of 1.4 µg/ml. Specific activity of cRNA approximately 10^8 dpm/µg. Exposure 56 days. Giemsa stain

autosomes (Figs. 23, 24). The chromocenter (both α- and β-heterochromatin) was a region of comparatively weak labeling (Fig. 25).

The diploid nuclei in this experiment labeled quite differently from those hybridized with cRNA copied from satellite I. The label was largely restricted to the euchromatic part of the nucleus (Fig. 26). Furthermore, the total number of silver grains over each diploid nucleus was less than 1% of that over the polytene chromosome groups on the same slide. This observation demonstrates that the sequences detected in this experiment undergo replication during the polytenization process.

b) D. melanogaster. The cytological hybridization experiments with *D. melanogaster* salivary glands present a picture essentially similar to that in *D. virilis*. For examination of the satellite DNA in *D. melanogaster* two types of cRNA were prepared. One used the least dense of the four satellite strands as template, the other used a mixture of the three heavier strands (including undoubtedly some main peak contamination). In cytological hybridization experiments both types of cRNA bound most heavily to the chromocenter region in polytene nuclei (Fig. 27). Label was also seen over the nucleolus and randomly along the chromosome arms. Because labeling outside of the chromocenter region was variable in intensity, and all slides in this series showed strong cytoplasmic binding, we hesitate to consider the labeling of nucleolus and chromosome arms as specific. Although further studies of the *D. melanogaster* satellites are clearly needed, it seems safe to conclude that satellite sequences are found in the centromeric heterochromatin. As in *D. virilis*, binding to diploid nuclei on the same slides was essentially as strong as to the largest polytene nuclei.

Information on the overall distribution of repetitive sequences can be obtained by hybridizing with cRNA copied from the total DNA of the organism (Jones and Robertson, 1970) or from the repetitive sequences



Figs. 28–31. Polytene chromosomes of *D. melanogaster* hybridized with cRNA synthesized from unfractionated *D. melanogaster* DNA. Preparation denatured with 0.07 N NaOH for 2 min, hybridized 15 hr at 66° with cRNA at 0.3 μg/ml in 2X SSC. Exposure 29 days. Giemsa stain. Fig. 28. Intense labeling occurs over the entire chromocenter region. Heavy label also characterizes a region near the end of one autosome (arrow). Some label occurs at many loci along the chromosome arms. Fig. 29. Nucleolus from the nucleus shown in Fig. 28, squashed away from its usual association with the chromocenter. Heavy label is seen throughout the nucleolus. Fig. 30. Part of another nucleus from the same preparation as Fig. 28. Heavy label in the chromocenter and near the end of one autosome (arrow), light label along the chromosome arms. Fig. 31. The X chromosome from the nucleus shown in Fig. 30. In male larvae such as this, the X can often be recognized by its lighter staining. Note that it is more heavily labeled than the autosomes despite its hemizygous state. This labeling pattern may indicate a higher proportion of repetitive sequences in the X than in the autosomes

alone (Rae, 1970). We have prepared cRNA using unfractionated *D. melanogaster* DNA as template. Such cRNA is expected to contain sequences complementary to many parts of the genome. When used in cytological hybridization experiments, only the more repetitive sequences will hybridize and therefore be detected on the autoradiographs (Gall and Pardue, 1971). When applied to *D. melanogaster* chromosomes, this cRNA gave the hybridization picture seen in Figs. 28–31. The strongest label occurred over the chromocenter (both α and β-heterochromatin), but significant binding was seen over the nucleolus and along the chromosome arms. Particularly heavy label occurred on a band near the end of one autosome (Figs. 28, 30). As in the experiment with *D. virilis*, the X was the most heavily labeled chromosome. The excess of labeling on the X was evident in squash preparations from male larvae, which carry the X in the hemizygous condition (Fig. 31).

Discussion

The centrifugation studies reported here demonstrate that the satellite DNAs of *D. melanogaster* and *D. virilis* are a regular and conspicuous feature of the DNA derived from predominantly diploid tissue (larval brain and imaginal discs). On the other hand, DNA from predominantly polytene tissue (larval salivary gland and larval gut) fails to show satellite bands when centrifuged at similar concentrations (Fig. 6). Physical studies show that the three major satellites of *D. virilis* and the satellite pair of *D. melanogaster* consist of homogeneous, repetitive DNA. These data include the narrow bands formed in neutral CsCl, the strand separation on alkaline CsCl gradients, the sharp melting transitions, and the rapid reassociation of strands after denaturation.

It was already known that certain repetitive DNA satellites in other organisms are located in the centromeric heterochromatin. These include the mouse (Pardue and Gall, 1969, 1970; Jones, 1970), the fly, *Rhynchosciara* (Eckhardt, 1970; Eckhardt and Gall, 1971), and the salamander, *Plethodon* (Macgregor and Kezer, 1971). We therefore suspected that the same might be true for *Drosophila*, and that the absence of satellite in polytene tissues reflected the underreplication of the heterochromatic regions. Our cytological hybridization experiments show that satellite I of *D. virilis* is located exclusively in the chromocenter of polytene nuclei and in the centromeric heterochromatin of mitotic chromosomes (with the possible exception of the Y chromosome). The amount of this satellite DNA in a diploid nucleus appears to be as great as that in a polytene nucleus. The simplest interpretation is that this DNA fraction does not replicate at all during the polytenization process. The centrifugation results imply that the same is true of satellites II and III,

although as yet we do not have confirmation by cytological hybridization. Similar observations were made on *D. melanogaster* using cRNA synthesized from the compound satellite.

The satellite DNA of *D. melanogaster* was described by Laird and McCarthy (1968), who thought that it might be non-nuclear because of its varying amount in different preparations. Rae (1970) also noted this variability and speculated that the satellite might be viral in nature. We suggest that the variability is due to differential extractability of DNA from diploid and polytene tissue, or to varying numbers of polytene and diploid nuclei in the material used for DNA preparation (a difference that might be quite marked, say, between larvae, pupae, and adults). Travaglini, Petrovic, and Schultz (1968) also studied the *D. melanogaster* satellite. They concluded that it is mitochondrial in origin. They based their conclusion in part on the observation that satellite was absent from DNA isolated from salivary gland nuclei. Our studies suggest that their sample lacked satellite because it was from salivary glands, not because it was from nuclei. It should be noted that nothing in our study precludes the possibility that *D. melanogaster* mitochondrial DNA has the same density as the nuclear satellites. Thus the DNA of this density seen by Travaglini in unfertilized eggs may well be ascribable to mitochondria.

Schurin and Marmur (1961) described a single very large satellite ($\varrho = 1.690$) in DNA isolated from adult *D. virilis*. The satellite was missing from larval DNA. It is possible that their larval DNA was derived preferentially from polytene tissues.

The repetitive DNA of *Drosophila* has been studied in several laboratories. Laird and McCarthy (1969) presented optical data on the reassociation of denatured DNA from *D. melanogaster*, *D. simulans*, *D. funebris*, and *Sarcophaga bullata*. They found only a small percent of repetitive sequences in these four species, ranging from 5% in *D. melanogaster* and *D. simulans* to 8% in *Sarcophaga* and 12% in *D. funebris*. The estimate for *D. melanogaster* referrred to sequences reassociating at a C_0t above 1. Satellite DNA was present in the sample studied by Laird and McCarthy (their Fig. 5a), but in unfractionated DNA the small drop in optical density due to reassociation of the satellite would be difficult to distinguish from the loss in hyperchromicity ordinarily seen as the sample is cooled from 100° to 60°. Rae and Swift (1971) have also examined the reassociation of *D. melanogaster* DNA, using both optical density changes and hydroxyapatite binding as criteria of reassociation. They estimated that about 13% of *D. melanogaster* DNA belongs to the moderately repetitive class. In their samples satellite DNA was present in only small amounts (about 3%), and it was not included in their estimate.

Dickson, Boyd, and Laird (1971) have examined the renaturation of DNA from embryos, pupae, and larval salivary glands of *D. hydei*. They

found that the salivary gland DNA contains less than 5% fast renaturing sequences instead of the 20% present in embryo and pupal DNAs. The underrepresentation of the fast renaturing sequences in the salivary gland presumably reflects their localization in the centromeric heterochromatin.

In *D. melanogaster* Rae (1970) and Jones and Robertson (1970) have shown by cytological hybridization that repetitive sequences are located in the chromocenter of salivary gland nuclei. Rae used cRNA copied from the total rapidly reannealing DNA, isolated by hydroxyapatite chromatography. He also prepared cRNA using as template various DNA fractions isolated from CsCl gradients. All his cRNA samples bound most heavily to the chromocenter, particularly in the α-heterochromatin. He also described binding to the chromosome arms, including a heavily labeled region near the tip of the left arm of chromosome 3. The binding which we observed near the tip of an autosome (Figs. 28, 30) is undoubtedly in the same region, although the cytological preservation in our slides was not adequate for arm identification. Jones and Robertson (1970) used cRNA synthesized from the total DNA. They detected hybridization in the chromocenter region and in the chromosome arms, with the strongest label restricted to a small area within the chromocenter. They also noted the relatively strong annealing to small nuclei, which they assumed to be derived from fat body, but they did not relate this observation to the underreplication of the heterochromatin. Neither Rae nor Jones and Robertson reported the relatively heavier labeling of the X chromosome. In the study of Jones and Robertson, as well as in our observations on cRNA made from total DNA, it is not certain how much of the observed hybridization is due to satellite sequences. Since in one experiment Rae specifically removed the lighter parts of his gradient before using the DNA for *in vitro* transcription, his result referred primarily or exclusively to main peak sequences. It appears, therefore, that the chromocenter of *D. melanogaster*, like the heterochromatic regions of *Rhynchosciara* (Eckhardt, 1970; Eckhardt and Gall, 1971) contains repetitive sequences from the main peak as well as from the satellites.

This conclusion is supported by the fact that the pair of satellites constitutes only 8% of the DNA in diploid tissue, whereas the heterochromatic regions include at least 20% of the mitotic length, and Rudkin's photometric data show that some 22–30% of the DNA fails to replicate during polytenization. Perreault, Kaufmann, and Gay (1968) first suggested that the majority of the heterochromatin must have a base composition similar to that of the total DNA. They based their conclusion on the melting characteristics of DNA isolated from various *D. melanogaster* karyotypes, including XO, XX, XY, XXY, and XYY.

In a recent study, Hennig, Hennig and Stein (1970) have examined the cytological localization of satellite sequences in several members of

the *D. hydei* group. They found that some of the satellites are located in the centromeric heterochromatin, but that others may be scattered throughout the chromosome arms.

Our data suggest that the satellites in both *D. virilis* and *D. melanogaster* fail to replicate during polytenization. However, not all repetitive sequences are subject to this replication control. As shown by comparison of Figs. 25 and 26 repetitive sequences at many loci along the chromosome arms belong to the category of replicating DNA. The total amount of such DNA in the genome is probably very small, however, as Dickson, Boyd, and Laird (1971) have shown that DNA from salivary glands contains only a few percent of repetitive sequences (most of which must be in the chromocenter). The repetitive sequences in the euchromatin constitute, therefore, a widely distributed, but very small class of DNA, probably less than 1% of the total.

The characteristics of the β-heterochromatin are still poorly defined. We know that it contains a large fraction of the repetitive sequences in polytene nuclei, as judged by cytological hybridization (Rae, 1970; Jones and Robertson, 1970; our Figs. 28–31). Unlike the α-heterochromatin, however, there is evidence that the β-heterochromatin undergoes replication. First, thymidine incorporation occurs in this part of the nucleus (Plaut, 1963; Swift, 1964; Tulchin, Mateyko, and Kopac, 1967). Second, the mass of DNA in the chromocenter is many times the diploid DNA amount, even though it is a small fraction of the total polytene DNA. Rudkin (1964) estimated that 3% of the X chromosome is heterochromatin in the polytene nucleus. Third, in the hybridization experiments using cRNA copied from unfractionated DNA, binding to the chromocenter was considerably higher than to diploid nuclei on the same slide. That is, a differential was easily detected even though the amount of label in the α-heterochromatin of the two kinds of nuclei was presumably the same. The available evidence suggests, therefore, that at least a part of the β-heterochromatin belongs to the class of replicating repetitive sequences. A corollary to this conclusion is that the relative amounts of α- and β-heterochromatin are different in diploid and polytene nuclei, a prediction made originally by Heitz (1934) and emphasized by Fujii (1942).

A diagram of a mitotic and a polytene chromosome is given in Fig. 32 with tentative suggestions concerning the distribution of repetitive sequences and their replicative behavior during polytenization.

That no simple statement about replication of the heterochromatin is possible is underscored by the behavior of the genes coding for ribosomal RNA (rDNA). Classical cytogenetic studies localized the nucleolus in the middle of the heterochromatin on the X, and on the short arm of the Y (Kaufmann, 1934; Cooper, 1959). More recently it was shown by Ritossa

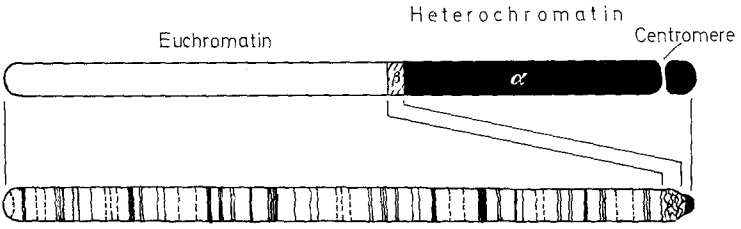


Fig. 32. Comparison of mitotic and polytene chromosomes of *Drosophila*, based on data from *D. virilis* and *D. melanogaster*. Three regions can be recognized on the basis of DNA composition and replicative behavior. 1) α-heterochromatin: highly repetitive sequences (satellites), probably not replicated during polytenization. 2) β-heterochromatin: a region in the mitotic chromosome containing repetitive sequences of which at least some are replicated during polytenization. 3) euchromatin: largely unique sequences, but contains a small percentage of widely distributed repetitive sequences; both unique and repetitive sequences are replicated during polytenization

and Spiegelman (1965) and Ritossa, Atwood, Lindsley, and Spiegelman (1966) that the rDNA is found at or near the nucleolus organizer. In salivary gland nuclei of *D. hydei*, Pardue, Gerbi, Eckhardt, and Gall (1970) showed that rDNA could be detected by cytological hybridization within the body of the nucleolus. The level of hybridization was considerably higher than would have been observed had the rDNA not replicated during polytenization. In a quantitative hybridization experiment Gerbi (1971) demonstrated that the rDNA of *Rhynchosciara* constituted the same fraction of the total DNA in larval salivary glands as in adult carcasses. These observations indicate that the ribosomal cistrons replicate at a rate comparable to that of the bulk of the DNA, and are therefore not under the same control as the adjacent satellite regions. In view of the known replicative independence of the rDNA in oocytes (Gall, 1968, 1969; Brown and Dawid, 1968; Evans and Birnstiel, 1968) one might postulate that the level of rDNA in polytene nuclei is regulated by cellular factors other than the number of cistrons in the diploid genome. The situation might be comparable to that in the oocytes of wild-type and anucleolate heterozygotes of *Xenopus*, which produce equivalent amounts of extrachromosomal rDNA (Perkowska, Macgregor, and Birnstiel, 1968). Replicative independence of the ribosomal cistrons in polytene tissue might provide a basis for the phenomenon of gene magnification described by Ritossa and Scala (1969) and Tartof (1971).

Our studies indicate that the sex chromosomes are special with respect to their content of repetitive sequences. First, the Y chromosome of *D. virilis* seems to be relatively deficient for satellite I, although the

X certainly is not. We had earlier reported failure to detect satellite sequences on the Y chromosome of the mouse (Pardue and Gall, 1970). Second, the X chromosome, in addition to the satellite sequences which are limited to the centromeric heterochromatin, displays an unexpectedly high level of hybridization with cRNA copied from total DNA, in both *D. melanogaster* and *D. virilis*. Selective transcription of special X chromosome sequences by the *coli* RNA polymerase could explain this higher hybridization. On the other hand, the X chromosome may contain a higher proportion of repetitive sequences than the autosomes.

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