Highly Repeated DNA in *Drosophila melanogaster*

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A major fraction (~80%) of the highly repeated DNA sequences from the *Drosophila melanogaster* genome has been isolated as well-defined satellites by buoyant density-centrifugation in CsCl gradients containing DNA-binding antibiotics. Four major satellites of densities 1.672 g/cm³, 1.686 g/cm³, 1.688 g/cm³ and 1.705 g/cm³ (in CsCl) were purified and characterized with regard to their amounts in the genome, denaturation-renaturation characteristics and their mode of organization. Several minor satellites were found with one (1.699 g/cm³) apparently representing linkage molecules resulting from covalent joining of large blocks of the major satellites.

Analysis of yields of the 1.705 g/cm³ satellite from DNA of different molecular weights suggested a mean segment length of approximately 10⁶ bases. Since this satellite accounts for between 4 and 5% of the genome, we deduced it to have approximately eight major sites in chromosomes. These data taken together with the existence of "linkage" molecules are discussed in relation to the possibility of a chromosome-specific arrangement of satellite DNA.

1. Introduction

The most highly repeated DNA in eukaryotes usually consists of short nucleotide sequences organized in tandem arrays. Because of this arrangement and the simple nature of their repeated sequence, these DNA species often have distinctive buoyant densities and can be resolved as satellites in CsCl gradients. A highly repeated DNA may have a density similar to the bulk of the DNA (a cryptic satellite; Walker, 1968) or similar to other repeated species (Skinner et al., 1970; Skinner & Beattie, 1973; Gall et al., 1971; Endow et al., 1975). In such cases the satellites may be resolved in buoyant gradients containing a DNA binding agent specific for certain base-pairs or sequences (e.g. Ag⁺ or Hg²⁺). We have used the antibiotics actinomycin D and netropsin sulphate to isolate four classes of highly repeated DNA from *Drosophila melanogaster*: of these DNA species one is a cryptic satellite and two have nearly identical densities in neutral CsCl gradients.

We have also used actinomycin D/CsCl gradients to examine the length and organization of the tandem arrays containing highly repeated sequences. Previous studies have reported a highly repeated sequence from *D. melanogaster* which was organized in short regions (2000 to 9000 bases) interspersed amongst more complex DNA (Botchan et al., 1971; Kram et al., 1972). This DNA constituted 8% of the genome.

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and was located in the centric heterochromatin. In contrast, the four different species described in this report exist in tandem arrays over 225,000 bases in length and make up over 16% of the genome (3 to 5% each). These long tandem arrays are pericentromeric in origin (Peacock et al., 1973). Our estimates of the length of these satellite regions indicate that each satellite occurs in only a few sites (<20) in the haploid genome. Our evidence further suggests that these long arrays are not interspersed in more complex DNA but that one satellite sequence is often adjacent to other satellites. While each species appears homogeneous in neutral and alkaline CsCl gradients, both thermal denaturation and filter hybridization experiments indicate that the major contaminants of the satellites are other satellite species. In one case a covalent attachment of two different species is demonstrated. The adjacent location of these different satellites within the *Drosophila* genome might be expected, since they are all located in heterochromatin and since they constitute the bulk of the DNA of these regions.

2. Materials and Methods

(a) Isolation and fractionation of total DNA

Developing embryos of *D. melanogaster* (Canberra wild-type) of mean age 9 h (25°C) were collected and washed in 0.7% NaCl, 0.01% Triton X-100, Packard. The embryos were dechorionated by treatment with sodium hypochlorite (1-2% available chlorine) for 2 min and then washed again in 0.7% NaCl, 0.01% Triton X-100.

Dechorionated embryos (100 ml packed volume, 50,000 embryos/ml) were disrupted at 0°C with a Dounce homogenizer (B pestle) in 0-25 mM sucrose, 30 mM Tris-HCl (pH 7-5), 10 mM-EDTA, 2-5 mM-CaCl₂ (200 ml) and filtered through nylon mesh (50 μm). Nuclei were sedimented from the filtrate by centrifugation (1000 g for 10 min). The nuclear pellet was washed 3 or 4 times by centrifugation from homogenization buffer until the supernatant was clear. The nuclei were resuspended in 10 mM Tris·HCl (pH 8-4), 1 mM-EDTA and lysed by addition of sodium lauryl sarcosinate (Sarkosyl N97; Geigy) to 1%; solid CsCl was then added to a final density of 1.70 g/cm³ and the solution centrifuged to equilibrium (Ti60 rotor, 44,000 revs/min, 20°C). One ml fractions were collected by pipette from the top of each gradient. Three fractions on either side of the viscous DNA region were pooled with the viscous fractions and dialysed against 10 mM-Tris·HCl (pH 8-4), 1 mM-EDTA. The average yield was 150 to 200 μg of DNA/ml of packed embryos. Recovery of total DNA was 2 to 3 times lower (relative to that recovered in CsCl gradients) when chloroform/isoamyl alcohol, phenol or a Cs₂SO₄ gradient were used to purify DNA from nuclei.

Total nuclear DNA was diluted to a concentration of 100 to 250 μg/ml and 30 μg of actinomycin D were added per 100 μg of DNA. Solid CsCl was then added to a final density of 1.66 g/cm³ and the sample centrifuged to equilibrium (Ti60 rotor, 44,000 revs/min, 20°C). Fractions were collected from the bottom of the tube and the DNA fractions located by u.v. absorbance. The regions indicated in Fig. 1 were pooled and dialysed against 10 mM-Tris·HCl (pH 8-4), 1 mM-EDTA. In some experiments the actinomycin D was extracted before dialysing by shaking pooled (undiluted) fractions 3 times with an equal volume of isopropanol that had been equilibrated with a saturated aqueous solution of CsCl. After dialysis, actinomycin D was further removed from the main band and regions I to III DNA by making the solution 5 mM in NaCl and extracting 3 more times with isopropanol equilibrated with a saturated solution of NaCl. A third method useful only for small amounts of DNA (<50 μg) was to mix the dialysed DNA with an equal volume of cold 8 M-NaClO₄ with vortexing and then precipitating the DNA by addition of 0·1 vol. 3 M-sodium acetate (pH 5-6) and 2 vol. ethanol. After 2 h at −20°C, DNA was recovered by centrifugation (16,000 g for 30 min). Removal of actinomycin D was monitored by measuring absorption at 440 nm.

DNA from the region of peak 1 in actinomycin D/CsCl gradients was repurified by centrifugation to equilibrium in a CsCl gradient (1.70 g/cm³, 44,000 revs/min, 20°C). The major
peak fractions around the density 1.705 g/cm³ and the lighter material were pooled separately.

DNA in region II of an actinomycin gradient was further fractionated by addition of netropsin sulphate (0.1 mg/ml solution, 160 µg/100 µg DNA) and centrifugation to equilibrium in CsCl (1.62 g/cm³, 35,000 revs/min). Regions of the gradient indicated in the text were pooled, extracted and dialysed as described above. Removal of netropsin sulphate was monitored by measuring absorption at 310 nm.

DNA in the peak III region was also purified by recentrifugation in either a CaCl or in a netropsin sulphate/CsCl gradient. Two recentrifugations were usually required to remove material of main band density.

(b) Preparation of labelled satellite DNA

Purified satellite DNA was used as template for Escherichia coli DNA polymerase I using either ³H or (±³²P)-labelled nucleoside triphosphates at 20 µM each in 50 mM-potassium phosphate (pH 7.4), 5 mM-MgCl₂, at 14°C for 8 to 12 hr. Pancreatic DNAase (10 to 20 mg/ml) was added to introduce primer termini. When acid-precipitable label reached a plateau (2 to 8 hr, with up to 25% of the nucleotides replaced), the radioactive product was isolated by phenol extraction, Sephadex G75 chromatography and precipitation with ethanol.

(c) Hybridization procedures

Nitrocellulose filters of 7 mm diameter were cut from larger 45 mm filters loaded with total Drosophila DNA (Gillespie & Spiegelman, 1965). Each small filter carrying 13-1 µg of DNA was preincubated in 0.02% Ficoll (Pharmacia), 0.02% polyvinyl pyrrolidone, 4.7 mM-NaClO₄, 0.001 M-Tris-HCl (pH 8.4) at 35°C for 1.5 hr to reduce non-specific DNA adsorption (Denhardt, 1966). Labelled satellite and E. coli DNA (4 µg in 4.7 mM-NaClO₄, 0.001 M-Tris-HCl, pH 8.4) were then added to the preincubation medium (0.24 ml final vol./filter), the mixture was heated to 80°C for 1 min to denature the DNA and then incubated at t₀ = 20 deg C for 2 hr. The filters were removed and washed 6 times for 10 min in 10 ml of 6 x SSC (SSC is 0.15 M-NaCl, 0.015 M-sodium citrate, pH 7), with shaking. Filters were dried by suction and heated at 60°C for 30 min and counted. Control experiments indicated 10 to 15% DNA loss during hybridization and washing; appropriate corrections were made in saturation analyses. Calculation of the amount of DNA on a filter complementary to a radioactive DNA was determined by extrapolation of the inverse plot of a saturation curve as described by Bishop et al. (1969). The amount of a satellite DNA competing for hybridization of radioactive satellite DNA to total DNA on filters was also determined by the inverse plot of such competition data (Bishop et al., 1969).

To determine the tₙ₀ value of hybrids formed, a filter with radioactive DNA hybridized was serially transferred to solutions at appropriate temperatures and the loss of radioactivity from the filter was monitored by following the increase in released counts. The tₙ₀ value of the hybrid 1.705 g/cm³ on the filters was found to be within 1 deg C of the tₙ₀ of native 1.705 g/cm³ in the same solvent.

(d) Analytical ultracentrifugation

Analytical actinomycin D/CsCl gradients were prepared by mixing DNA solutions (5 to 20 µg/ml in 1 mM-EDTA, 10 mM-Tris-HCl, pH 8.4) with actinomycin D (16.7 µg/ml final conc.) prior to the addition of solid CsCl to a final density of 1.66 g/cm³. Analytical gradients containing netropsin sulphate (5 µg/ml) usually contained 5 to 10 µg DNA/ml and solid CsCl added to a final density of 1.62 g/cm³. Samples were centrifuged to equilibrium at 44,000 revs/min for 18 hr at 25°C in a Beckman model E ultracentrifuge equipped with monochromator and a photoelectric scanner. Densities were calculated assuming a linear gradient and using Micrococcus luteus DNA as a standard (1.731 g/cm³ in neutral gradients and 1.799 g/cm³ in alkaline CsCl (Szybalski & Szybalski, 1971)). In the presence of antibiotics, densities were calculated by reference to the isoconcentration position of the gradient (Jfft et al., 1961). In the presence of actinomycin D, synthetic poly[d(A·T)] was also used as a density standard (1.679 g/cm³). All buoyant density species discussed in this paper were DNA as judged from scans at 260 nm and 280 nm.
(c) Thermal denaturation and renaturation analyses

Thermal denaturation and renaturation kinetics were measured optically and recorded on a Gilford 2400 automatic multiple sample absorbance spectrometer. Temperature was controlled with a programmed Haake circulating bath filled with ethylene glycol. Temperature was measured by a platinum resistor mounted in an adjacent cuvette within the holder and automatically recorded along with the absorbance.

DNA samples were precipitated with ethanol from 4·0 M-NaClO₄ (to remove antibiotics), redissolved in the appropriate solvent and dialysed against the same. The samples were then centrifuged at 16,000 g for 10 min to remove particulate material, and dissolved gas removed by a partial vacuum. DNA samples for renaturation were sheared in a Virtis homogenizer at 40,000 revs/min for 10 min, at 0 to 4°C, and then treated as above. Thermal denaturation was carried out with a temperature increase of 0·5 deg. C per min. Optical density changes were corrected for thermal expansion. Renaturation rates were measured in 0·1 X SSC by immersing the quartz cuvette in boiling water, cooling to the renaturation temperature for 1 min and transferring the cuvette to a prewarmed cuvette holder. Renaturation in 4·7 M-NaClO₄ was performed by first heating the DNA above the melting temperature and then cooling slowly (approx. 0·5 deg. C/min).

The renaturation kinetics were analysed by least-squares fit of the data by the modified second-order equation

\[ C/C_0 = (1 + KC_{eq}/N)^{-N} \]

(Morrow, 1974). The best values of both \( K \) and \( N \) were determined by fitting the \( C/C_0 \) versus \( C_{eq} \) values directly by the least-squares methods outlined by Cleland (1967). When the data were plotted in the modified second-order form \( (C_{eq}/C)^{1/N} \) versus \( C_{eq} \) on a linear scale, the resulting curves were linear throughout the reaction when the best value of \( N \) was used. The standard errors of the estimates of both \( K \) and \( N \) are given, again calculated according to Cleland (1967). Due to the uncertainty as to the dependence of \( K \) on length of satellite DNA (Hutton & Wetmur, 1973) or on salt concentration in the range used (0-0195 M-Na⁺), no correction has been made for these variables. The kinetic complexities of main band and total DNA renatured in SSC were calculated according to Wetmur & Davidson (1968).

(f) Electron microscopy of DNA

DNA was spread for electron microscopy by the method of Davis et al. (1971) and measurements were made relative to a standard magnified to the same extent. Photographs were taken at magnification of 12,800× using a Philips 200 electron microscope.

(g) Tissue culture

Line 2 cells (Schneider, 1972) were grown in Schneider’s medium. In the experiments where these cells were used (Table 2) cells in log phase were labelled by the addition of 100 µCi of [³H]thymidine (5 Ci/mmol), or by the addition of 5 µCi of [¹⁴C]thymidine (62 mCi/ mmol) for 4 h. The cells were harvested by centrifugation (2000 revs/min for 10 min), washed in 0·75% NaCl and resuspended in buffer (0·25 M-sucrose, 0·03 M-Tris (pH 7·5), 1 mM-EDTA, 2·4 mM-CaCl₂). Sarkosyl was added to 0·5%, and saturated CsCl layered underneath the sample. Actinomycin was added to a final concen of 40 µg/ml, and the density adjusted to 1·66 g/cm³ for actinomycin D gradients. The tubes were centrifuged for 60 h at 39,000 revs/min in a no. 40 rotor (Spinco). Fractions were collected and 50-µl portions spotted onto glass-fibre discs, dried and counted in a Packard liquid scintillation counter. The channels were set so that the spillover from the ³H channel into the ¹⁴C channel was negligible, and that from ¹³C into ³H was 10%. The ratio of ³H to ¹⁴C counts was 10:1. The [¹³C]DNA was sheared, prior to CsCl addition, through a 26-gauge needle. Samples were withdrawn and spread immediately for electron microscopy by the method described by Davis et al. (1971). A mass average molecular weight was calculated.

3. Results

(a) Isolation of four highly repeated DNA fractions

DNA isolated from nuclei of developing D. melanogaster embryos shows two discrete satellites in CsCl gradients (1·672 and 1·688 g/cm³) in addition to the main
band (1·701 g/cm³) (Gall et al., 1971; Peacock et al., 1973). A number of DNA-binding antibiotics were tested for their ability to further fractionate the DNA. Among those tested were actinomycin D, netropsin sulphate, distamycin, chromomycin, phleomycin, mithramycin, nogalamycin, anthramycin, daunomycin and olivomycin (Kersten et al., 1966). Actinomycin D and netropsin sulphate proved to be the most effective; distamycin was similar to, but not quite as effective as, netropsin sulphate in resolving the satellites. When the total nuclear DNA is analysed in a CsCl gradient containing actinomycin D, the bulk of the DNA shifts from 1·701 g/cm³ to 1·62 to 1·63 g/cm³, while some fractions remain at densities close to that of their original densities in CsCl gradients. These satellites revealed by the use of actinomycin D were divided into three regions according to their position in the gradient (Fig. 1).

![Graph showing the density distribution of DNA with actinomycin D.](image)

**Fig. 1.** Actinomycin D/CsCl gradients resolve 4 satellite species of *D. melanogaster* DNA. The regions labelled I, II, III and main band correspond to the fractions separated in a preparative gradient. The labels on the peaks indicate the buoyant density of these DNA fractions in the absence of any antibiotics. Region III contains the 1·688 g/cm³ density satellite in addition to a broad peak of main band density material.

Region I yielded a single peak having a density of 1·705 g/cm³ in CsCl gradients when analysed after removal of actinomycin D (Fig. 2). The DNA in this fraction was not homogeneous as indicated by the skew trace. Recentrifugation did yield an apparently homogenous fraction with a density of 1·705 g/cm³. The remaining DNA sequences, from the skewed region, are further analysed below.

The DNA in region I bound relatively little actinomycin D in the initial purification step since its density in actinomycin D/CsCl gradients is 1·703 g/cm³ compared to

![Graph showing the density distribution of DNA with actinomycin D and M. luteus DNA.](image)

**Fig. 2.** The 1·705 g/cm³ satellite shows a skew towards the lighter side. The DNA of region I of an actinomycinD/CsCl gradient appears as a skewed peak with a density of 1·705 g/cm³ in an analytical CsCl gradient. *M. luteus* DNA has been added as a standard.
1.705 g/cm³ in CsCl. Region II contains DNA which, like that in region I, binds relatively little actinomycin D. The two major DNA species in these fractions band at densities of 1.672 and 1.686 g/cm³, respectively, in CsCl gradients. Analyses of the total DNA from this region in netropsin sulphate/CsCl gradients increases the resolution between 1.672 and 1.686 g/cm³ satellites and shows additional DNA species to be present (Fig. 3(a)). The three fractions from netropsin sulphate/CsCl gradients when analysed separately give a species of density 1.672 g/cm³, one of 1.686 g/cm³, and a complex fraction with at least three species of densities 1.702, 1.690 and 1.674 g/cm³ (Fig. 3).

![Graph showing DNA banding patterns](image)

**Fig. 3.** Netropsin sulphate/CsCl gradients further resolve the satellites of the actinomycin D II region. (a) DNA of the actinomycin D/CsCl region II recently isolated from netropsin sulphate/CsCl gradient and showing the regions pooled; (b) DNA of netropsin sulphate/CsCl gradient region I analysed in CsCl equilibrium gradient; (c) DNA of netropsin sulphate/CsCl gradient region II; (d) DNA of netropsin sulphate/CsCl region III.

Region III of the actinomycin D/CsCl gradient, appearing as a shoulder on the main band, contains another major satellite of density 1.688 g/cm³, which was purified by repeated centrifugation in CsCl gradients. The 1.688 g/cm³ species bound more actinomycin D than other satellites as judged by its density shift.

An analysis of DNA recovered between the main fractions separated above revealed several other minor satellite species (Fig. 4(a) and (b)). Most of the DNA between region I and II of the actinomycin-containing gradients consists of the 1.672, 1.686 and 1.705 g/cm³ species but each peak is asymmetric (Fig. 4(a)), suggesting covalent association of each with other species. The DNA between regions I and II of the netropsin sulphate-containing gradient was of particular interest (Fig. 4(b)). While
these DNAs all have densities different from the major satellites, they may be composed of major sequences in covalent association.

The satellites isolated in these antibiotic-containing gradients can also be obtained in Ag⁺/Cs₂SO₄ gradients (Peacock et al., 1973; Endow et al., 1975) but we find that the resolution is not as great. All of the major satellites, except the 1·688 g/cm³ species, appeared to be selectively lost from DNA isolated by extractions with 1 M-NaClO₄/chloroform/isoamyl alcohol following published procedures (Kram et al., 1972). The satellite species found in actinomycin D/CsCl gradients could also be found in rapidly renatured DNA isolated on hydroxyapatite.

(b) Amounts of highly repeated DNA in the genome

The quantities of the various satellite DNA species present in total nuclear DNA are estimated in Table 1. A number of different methods of estimation were used, since any one method was not entirely satisfactory for all the satellites. For example, the 1·672 g/cm³ satellite estimate using hybridization may be low, due to intrastrand hybridization (discussed later). The amount of this satellite, however, could be accurately determined from a melting profile of total DNA (Blumenfeld & Forrest, 1971). Estimates from yields on isolation may be low due to losses involved in removing bound antibiotics or high if the species was not completely pure. The respective amounts of satellites examined ranged between 2·6 and 5·3% of the total DNA. These quantities place an upper limit of about 8 × 10⁶ bases for the number of bases in any one satellite sequence, assuming a content of 165 × 10⁶ bases for the haploid genome of D. melanogaster (Rasch et al., 1971; Laird, 1971).

(c) Length of highly repeated DNA regions

During experiments where quantities of various satellites were being estimated, it became clear that the lengths of highly repeated regions might be very long, since yields were independent of initial molecular weights of the DNA. A detailed analysis showed that the same amounts of pure satellite (either 1·705, 1·686 or 1·672 g/cm³)
### Table 1

**Amounts of various satellite DNAs in the Drosophila genome**

<table>
<thead>
<tr>
<th>DNA fraction (g/cm³)</th>
<th>Yield upon isolation (%)</th>
<th>Saturation hybridization (%)</th>
<th>Competition hybridization (%)</th>
<th>Thermal denaturation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Main band</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.701</td>
<td>78.0</td>
<td>4.6</td>
<td>5.3</td>
<td></td>
</tr>
<tr>
<td>1.705</td>
<td>4.2</td>
<td>4.6</td>
<td>4.6</td>
<td></td>
</tr>
<tr>
<td>1.688</td>
<td>2.6</td>
<td>3.8</td>
<td>3.4</td>
<td></td>
</tr>
<tr>
<td>1.672</td>
<td>5.2</td>
<td>3.8</td>
<td>2.9</td>
<td>5.0</td>
</tr>
</tbody>
</table>

The yields upon isolation were determined as the proportion of the indicated fraction of the total DNA recovered from an actinomycin D/CeCl gradient (including the DNA between the major fractions). The relative amounts of 1.672 and 1.686 g/cm³ species were determined from a subsequent n Ergin sulphate/CeCl gradient, and these fractions multiplied by the amount of DNA in region II of the actinomycin D/CeCl gradient. The amounts determined by hybridization of [³H]DNA to total DNA on filters are described in Materials and Methods. The amount of 1.672 g/cm³ DNA can be accurately determined from thermal denaturation profiles of total DNA, since its $t_m$ is sufficiently distinct from the bulk of the DNA (Blumenfeld & Forrest, 1971).

could be recovered independently of the length of the DNA up to 15,000 bases (Peacock et al., 1973), suggesting that the satellite sequences were arranged in blocks longer than 15,000 bases. We have repeated these experiments isolating DNA 45,000 bases in length and have determined the amount of 1.705 g/cm³ sequences present in DNA not in the peak seen at 1.705 g/cm³ to be 13% of the total 1.705 g/cm³ sequences. If the 1.705 g/cm³ sequences in non-1.705 g/cm³ DNA are assumed to be due to covalent association of 1.705 g/cm³ species to adjacent sequences at the ends of 1.705 g/cm³ chromosomal regions, then we can estimate the average size of the regions to be at least 45,000/0.13 bases = 346,000 bases† (see Spadari & Ritossa, 1970).

† If a satellite region of length $m$ base-pairs is bounded on either side by DNA of greatly different density in an actinomycin D/CeCl gradient, then the size of the satellite region can be approximated from the yield of satellite DNA isolated from total DNA fragments of length $n$ base-pairs using the following assumptions.

(1) All the DNA is fragmented to a constant size (in base-pairs) which is less than the size of the satellite region ($m$ base-pairs).

(2) All fragments from the end of a satellite region which contain adjacent DNA sequences will not appear at satellite density.

(3) DNA fragmentation is random, so that from each end-point of any region there are an equal number of fragments containing no adjacent DNA, one base-pair of adjacent DNA, 2 base-pairs of adjacent DNA, 3 base-pairs, etc., to $n-1$ base-pairs of adjacent DNA and a correspondingly decreasing amount of satellite DNA sequences. The total number of base-pairs in "impure" fragments is then:

$$\frac{1}{n} \sum_{i=0}^{n-1} i = \frac{n - 1}{2} \simeq \frac{n}{2} \text{assuming } n \gg 1.$$  

Assuming each satellite region will have 2 ends, a total of $n$ base-pairs of satellite from the region $m$ base-pairs long will be lost. The fractions of satellite recovered at satellite density will be $f = (m - n)/m$, which gives the region size $m = n/(1 - f)$. Relaxation of both assumptions (1) and (2) to include a distribution of fragment sizes and partial recovery of end fragments of predominantly satellite composition results in a similar relation: the region size $m$ is proportional to the fragment size $n$ and inversely proportional to $(1 - f)$ as long as $n < m$. 

Additional estimates of the length of chromosomal satellite regions were obtained using tissue culture cells. Radioactive DNA of very high molecular weight can be more readily obtained from culture cells than from embryos. The use of labelled DNA provides simple determinations of recovery of particular satellites (Table 2). The amount of DNA recovered in the 1.705 g/cm³ region of an actinomycin D/CsCl gradient was reduced by 17% (i.e. from 87% to 70% of the 1.705 g/cm³ sequences) when the size of the DNA molecules was increased from 4500 bases to 225,000 bases.

**Table 2**

Satellite recoveries from sheared and unsheared DNA

<table>
<thead>
<tr>
<th>Actinomycin D/CsCl gradient</th>
<th>Counts in 1.705 g/cm³ peak</th>
<th>Counts in 1.672 and 1.686 g/cm³ peak</th>
<th>Total counts in gradient</th>
</tr>
</thead>
<tbody>
<tr>
<td>[³H]DNA, average length 225,000 bases</td>
<td>87,671 (3.8%)</td>
<td>123,294 (5.4%)</td>
<td>2.29 x 10⁶</td>
</tr>
<tr>
<td>[¹⁴C]DNA, average length 4500 bases</td>
<td>4291 (4.8%)</td>
<td>6171 (5.5%)</td>
<td>93,277</td>
</tr>
</tbody>
</table>

Details of the experiment are given in Materials and Methods. Note that if [¹⁴C] and [³H]-labelled DNAs were sheared together, the proportion of DNA in the 1.705 g/cm³ band was found to be the same for both samples.

These data extend the lower limit for the average size of satellite regions to 225,000/0.30 = 750,000 bases. Such a long region size would place an upper limit of approximately ten sites for this species in the haploid genome (assuming 8 x 10⁶ bases total 1.705 g/cm³ DNA genome). The amount of DNA isolated in region II of an actinomycin D gradient is the same from DNA molecules 225,000 bases long and 4500 bases long, suggesting regions for 1.672 g/cm³ and 1.686 g/cm³ species are at least as large as those of 1.705 g/cm³ and possibly larger. The interpretation is not as straightforward as for 1.705 g/cm³ because of the more complex nature of this part of the gradient (see Fig. 3), however a direct verification was possible for 1.686 g/cm³ in gradients of DNA from tissue culture cells (Goldring et al., 1975). The above size estimate of 1.705 g/cm³ sequences applies to the bulk of 1.705 g/cm³. There may however exist a small fraction (<10%) which is not organized in large blocks, since the yield of 1.705 g/cm³ satellite does not alter detectably with DNA varying in molecular weight from 4500 to 45,000 bases. If this is correct, the calculations shown can only give a lower estimate for the size of the postulated, large blocks of 1.705 g/cm³.

(d) Covalent attachment of different classes of highly repeated DNA

Since the major satellites of *D. melanogaster* are all located in similar positions (the centromeric regions; Peacock et al., 1973), they may be located adjacent to each other. Evidence for a covalent linkage between two satellites was obtained from a detailed analysis of 1.705 g/cm³ DNA recovered at an initial stage of purification. Region I from an actinomycin D/CsCl gradient when in a CsCl gradient showed a peak of density 1.705 g/cm³ with a pronounced skew (Fig. 2). The DNA in the skewed portion
of the gradient was divided into fractions and analysed on analytical CsCl gradients. The detailed appearance of these fractions varied from experiment to experiment, depending on the way fractions were pooled. Generally, however, a peak of 1.705 g/cm³ was seen with a broad shoulder at about 1.699 g/cm³ (Fig. 5). No discrete peaks of 1.672 g/cm³ or 1.686 g/cm³ satellite DNA were discernible in this region. In contrast, when the skew fractions were analysed by thermal denaturation or by hybridization, both 1.672 and 1.686 g/cm³ sequences could be detected in addition to 1.705 g/cm³ sequences (Table 3) at levels which should have been detectable in the analytical centrifuge if they represented simple contaminants. Our interpretation of these data is that the molecules banding in the skewed portion of the 1.705 g/cm³ are 1.686 and/or 1.672 g/cm³ sequences covalently linked to 1.705 g/cm³ sequences. The thermal denaturation profile (data not shown) of this material also showed an unidentified broad melting transition after the 1.705 g/cm³ transition. These molecules may reflect an arrangement of 1.705 g/cm³ sequences within non-satellite DNA.

![Graph showing DNA centrifugation](image)

**Fig. 5.** Centrifugation of DNA in the skewed portion of the main 1.705 g/cm³ peak in region I. A broad shoulder with a density of 1.699 g/cm³ is seen in addition to the 1.705 g/cm³ peak. No clear peaks at densities 1.672 g/cm³ and 1.686 g/cm³ are discernible.

<table>
<thead>
<tr>
<th>DNA sequences associated with the 1.705 g/cm³ satellite</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA fraction</td>
</tr>
<tr>
<td>g/cm³</td>
</tr>
<tr>
<td>-------------</td>
</tr>
<tr>
<td>1.672</td>
</tr>
<tr>
<td>1.686</td>
</tr>
<tr>
<td>1.705</td>
</tr>
<tr>
<td>Main band†</td>
</tr>
</tbody>
</table>

The skewed peak obtained from region I of an actinomycin D/CsCl gradient was divided into 2 fractions, 1 adjacent to the 1.705 g/cm³ peak and 1 more distal. These DNAs were applied to nitrocellulose filters and the content of each DNA species was determined by hybridization of [³H]DNA at saturation as described in Materials and Methods. Hybridization of main band was carried out to a Cₘ of 1000, based on the concentration of total DNA in solution.

† The 1.688 g/cm³ species and other less highly repeated DNAs were included in the main band proportion.
(e) Intrinsic homogeneity of satellite DNA

The satellite fractions discussed above (1.672, 1.686 and 1.705 g/cm³) were obtained as homogeneous fractions as determined in neutral and alkaline CsCl gradients. Each is a single band at neutral pH which resolves into two equal bands in alkali (Peacock et al., 1973; Table 4). To determine sequence homogeneity, thermal denaturation profiles were examined in 0.1 x SSC or 4.7 M-NaClO₄. As shown in Figure 6 for 0.1 x SSC, each denaturation profile consisted predominantly of one sharp transition, with the apparent cross-contamination of satellites discussed in the preceding section. The sharp transitions observed indicated that a homogeneous sequence was responsible for the major transition. This was verified by allowing the denatured satellites to renature and then to denature them again. When the renaturation was performed

| Table 4 |
| Buoyant densities of satellite DNA in CsCl gradients |
| --- | --- | --- | --- | --- | --- |
| CsCl | Native satellites | Complementary strands | | | |
| | CsCl + 20 µg | CsCl + 5 µg | Alkali | Heavy | Light | Heavy | Light |
| | actinomycin | netropsin | | | | | |
| | D/ml | sulphate/ml | | | | | |
| 1.672 | 1.671 | 1.594 | 1.744 | 1.712 | 1.705 | 1.667 |
| 1.686 | 1.680 | 1.632 | 1.769 | 1.713 | 1.729 | 1.686 |
| 1.688 | 1.640 | 1.642 | 1.750 | 1.741 | | | |
| 1.705 | 1.703 | 1.690 | 1.758 | 1.751 | | | |
| Main band | 1.62-1.63 | 1.670 | | | | | |
| 1.701 | | | | | | | |
| Mitochondrial | 1.647 | | | | | | |
| 1.680 | | | | | | | |

All densities (all values are g/cm³) are from analytical gradients performed as described in Materials and Methods using M. luteus DNA as a standard (1.731 g/cm³ in neutral and 1.789 g/cm³ in alkaline CsCl) and ρ₀ = 1.19 × 10⁻⁵ g mn. sec. (Hoff et al., 1961). Since the complementary strands of the 1.672 and 1.686 g/cm³ satellites can be isolated preparatively, they have been given in neutral as well as alkaline pH.

Fig. 6. Thermal denaturation profiles of satellite DNAs show predominantly single transitions. Each satellite DNA at an A₂₆₀nm of 0.3 to 0.550 in 0.1 x SSC was denatured as described in Materials and Methods. The optical density measurements were then normalised to 1.000 at 25°C.
in high salt (4.7 M-\text{NaClO}_4, 0.001 M-\text{Tris-HCl, pH 8.0}), double-stranded DNA formed as soon as the temperature was lowered below the \( t_m \) of the original native DNA. The duplexes formed had nearly the same thermal stability and sharp transition as the original DNA (Table 5). Renatured 1.705 g/cm\(^3\) DNA had the same thermal stability (within 0.5 deg. C) as the original native form whether renaturation was carried out as described in high salt or slowly in 0.1 \( \times \) SSC. This suggested a very high degree of sequence homogeneity within the 1.705 g/cm\(^3\) satellite. The 1.686 g/cm\(^3\) species showed a \( t_m \), 3 deg. C lower after renaturation in high salt but a 7 deg. C difference when renatured in 0.1 \( \times \) SSC. Renatured 1.672 g/cm\(^3\) DNA showed a 1 deg. C lower \( t_m \) after renaturation in high salt and a 5 deg. C lower \( t_m \) after renaturation in 0.1 \( \times \) SSC.

**Table 5**

**Thermal stability of native and renatured satellite DNAs**

<table>
<thead>
<tr>
<th>Satellite (g/cm(^3))</th>
<th>Solvent</th>
<th>( t_m ) native (°C)</th>
<th>( t_m ) renatured (°C)</th>
<th>( \Delta t_m ) (deg. C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.705</td>
<td>4.7 M-\text{NaClO}_4</td>
<td>55</td>
<td>55</td>
<td>0</td>
</tr>
<tr>
<td>0.1 ( \times ) SSC</td>
<td>68</td>
<td>68</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.686</td>
<td>4.7 M-\text{NaClO}_4</td>
<td>43</td>
<td>40</td>
<td>3</td>
</tr>
<tr>
<td>0.1 ( \times ) SSC</td>
<td>66</td>
<td>56</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.672</td>
<td>4.7 M-\text{NaClO}_4</td>
<td>68</td>
<td>56</td>
<td>7</td>
</tr>
<tr>
<td>0.1 ( \times ) SSC</td>
<td>63</td>
<td>56</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.688</td>
<td>0.1 ( \times ) SSC</td>
<td>51</td>
<td>46</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>64</td>
<td>64</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>68.5</td>
<td>64</td>
<td></td>
<td>4.5</td>
</tr>
</tbody>
</table>

The fact that renaturation in high salt consistently resulted in a more thermostable product was most likely due to differences in the temperature of renaturation. In high salt, most renaturation occurred within 5 deg. C of the \( t_m \) (i.e. under very stringent conditions), while renaturation in 0.1 \( \times \) SSC occurred at the standard \( t_m - 25 \) deg. C.

When satellite DNAs renatured in 0.1 \( \times \) SSC were analysed in CsCl gradients, their densities were shifted slightly from their original density and the extent of the shift paralleled the decrease in thermal stability. Renatured 1.705 g/cm\(^3\) DNA, which had the same thermal stability as native 1.705 g/cm\(^3\) DNA, also showed no change in density. Renatured 1.686 g/cm\(^3\) species, however, shifted to 1.692 g/cm\(^3\) and renatured 1.672 g/cm\(^3\) DNA shifted to 1.687 g/cm\(^3\). Buoyant analyses of satellites renatured in high salt were not performed.

**f** Renaturation rates of satellite DNA

Since all classes of satellite DNA renatured very rapidly in SSC, accurate relative rates of renaturation could be obtained optically only by renaturation at low ionic strength. In 0.1 \( \times \) SSC (0.0195 M-\text{Na}^+\) the satellites all renatured with kinetics that approximated the modified second-order rate equation, \( C/C_0 = (1 + KC_0/(N)^{-N}) \) (Fig. 7). In this equation, \( N = 1 \) if the species renatures according to an ideal second-order rate which is constant throughout the reaction. Empirically it has been found that reaction kinetics of sheared DNA measured optically slow down during the
Fig. 7. Renaturation kinetics of satellite DNA. Satellite DNA was denatured and renatured in 0.1 × SSC as described in Materials and Methods and the C/C₀ value was calculated from the percentage decrease of the optical density from the maximum optical density at time zero to the optical density before denaturation. The smooth lines show the theoretical fitted curves of the form C/C₀ = (1 + KC₀t/N)⁻¹, the values of K and N for each curve are: 1.672 g/cm³, K = 1.03 ± 0.04 mol⁻¹ s⁻¹ (N = 0.87 ± 0.02, 2500 bases, 30°C); 1.686 g/cm³, K = 2.83 ± 0.16 mol⁻¹ s⁻¹ (N = 0.93 ± 0.03, 2600 bases, 30°C); 1.688 g/cm³, K = 0.22 ± 0.01 mol⁻¹ s⁻¹ (N = 0.42 ± 0.02, 2300 bases, 43°C); and 1.705 g/cm³, K = 10.5 ± 0.1 mol⁻¹ s⁻¹ (N = 0.55 ± 0.01, 1600 bases, 42°C).

course of the reaction and are reflected in a value of N = 0.42 (Morrow, 1974). This is believed to be due to a slower reaction of single-stranded tails on partially renatured molecules (Smith et al., 1975). The value of N for the 1.705 g/cm³ satellite is significantly above 0.42 and for the 1.672 and 1.686 g/cm³ species is close to 1.0. Randomly sheared satellite DNA might be expected to behave in an ideal second-order fashion because once two strands are renatured with each other over any part of their length, they may cyclize in a rapid first-order reaction.

The 1.705 g/cm³ species is seen to renature the most rapidly (Fig. 7) and it is also the most homogeneous when measured by thermal stability (Fig. 6). Relative to a value of 1.0 for 1.705 g/cm³, the kinetic complexities of the other satellites are 3.8 for 1.686 g/cm³, 10.4 for 1.672 g/cm³ and 46.8 for 1.688 g/cm³ assuming no length dependence of K. If K varied as L⁻⁰.⁵ or L⁻¹ (Wetmur & Davidson, 1968; Hutton & Wetmur, 1973) then these relative values would increase 20% or decrease 16%, respectively. Absolute kinetic complexities for each satellite would require, in addition to knowledge of the dependence of K on length, the dependence of K on low ionic strengths, on the degree of mismatch on base composition and on temperature.

When total nuclear DNA is allowed to reassociate in 0.1 × SSC, the duplex formation observed can be attributed entirely to highly repeated sequences. The initial rate was 3.59 × 10⁻⁹ ± 0.11 × 10⁻⁹ 1 mol⁻¹ s⁻¹ (at 37°C, based on total DNA
concentration). Actinomycin D/CsCl main band DNA reannealed only at one-sixth of this rate (0.47 \times 10^{-9} \pm 0.03 \times 10^{-9} 1 \text{ mol}^{-1} \text{s}^{-1}), suggesting that over 84% of the highly repeated DNA has been removed by the purification procedure utilizing actinomycin D/CsCl buoyant density gradients.

When either total DNA or the main band fraction were annealed in SSC at 60°C, their kinetics were similar. Neither was linear in the initial part of the reaction, while a second-order reaction rate calculated from later portions of the reaction gave an average \( K_2 = 0.023 \pm 0.002 \text{ l mol}^{-1} \text{s}^{-1} \) (\( L = 3700 \) bases, \( N = 0.42 \pm 0.02 \)) for main band DNA and \( K_2 = 0.025 \pm 0.002 \text{ l mol}^{-1} \text{s}^{-1} \) (\( L = 3200 \) bases, \( N = 0.42 \pm 0.02 \)) for total DNA. These rates correspond to complexities of \( 1.02 \times 10^{11} \) daltons and \( 0.87 \times 10^{11} \) daltons, respectively, which agrees with earlier estimates for the \textit{Drosophila} genome (Laird, 1971).

4. Discussion

We have studied the highly repeated DNA in \textit{D. melanogaster} and isolated well-defined satellite sequences using CsCl gradients containing DNA-binding antibiotics. The four major highly repeated DNA species (1-672, 1-686, 1-688 and 1-705 g/cm\(^3\)) all band at densities greater than the bulk of the nuclear DNA in actinomycin D/CsCl gradients. The satellites thus share a common feature, namely, a low frequency of GpC sequences (the binding site for actinomycin D) relative to main band DNA, and show related sequence patterns (Brutlag & Peacock, 1974; Endow \textit{et al.}, 1975). The studies reported here document isolation techniques used for purifying specific repeated DNA sequences, the inherent purity of these DNA fractions, the levels at which they are present in the genome, and their general organization within the genome.

While each of these satellites appears homogeneous in both neutral and alkaline CsCl gradients, the thermal stability of renatured satellite DNA does indicate a limited degree of nucleotide mismatch in the 1-672, 1-686 and 1-688 g/cm\(^3\) species. The degree of nucleotide mismatch depends strongly on the conditions of renaturation. Renaturation close to the \( t_m \) results in a much lower degree of mismatch than renaturation at \( t_m - 25 \) deg. C, probably because a much longer region of interstrand homology would be needed at higher temperatures for an effective nucleation event. The ability of satellite DNA to renature more accurately near its \( t_m \) is consistent with the mismatches formed at \( t_m - 25 \) deg. C being distributed regularly along the DNA and being forced into register at higher temperature. A regular distribution of nucleotide mismatches could result from a single repeating sequence altered in a regular fashion or a mixture of two distinct but closely related sequences which could cross-hybridize. Sequence analyses of the 1-705 and 1-686 g/cm\(^3\) species have suggested they are homogeneous (Endow \textit{et al.}, 1975), however, we have found minor closely related sequences present in the 1-686 g/cm\(^3\) satellite which could result in mismatches upon hybridization with the major sequence (Brutlag & Peacock, 1975).

The kinetic complexities of satellite DNAs indicate that they have short repeating subunits consistent with sequencing data. If we assume an absolute complexity of five base-pairs for the 1-705 g/cm\(^3\) satellite (based on a partial sequence analysis; Endow \textit{et al.}, 1975), then the 1-686 g/cm\(^3\) species would have a complexity of 19 base-pairs. One major repeating sequence ten nucleotides in length and two minor repeat sequences have been reported for this satellite (Brutlag & Peacock, 1975;
Endow et al., 1975). Cross hybridization between these minor sequences might cause both the decreased thermal stability as well as a slowing of the renaturation of 1.686 g/cm³ DNA. The complexity of 1.688 g/cm³ species would be 234 base-pairs relative to the 1.705 g/cm³ complexity. A repeating unit of 365 base-pairs has been detected in this DNA by restriction enzyme analysis (Manteuil et al., 1975) and T₁ RNAase digests of complementary RNA have indicated a much higher complexity of 1.688 than either 1.705 or the 1.672 g/cm³ satellites (Carlson & Brutlag, unpublished results). The relative kinetic complexity of the 1.672 g/cm³ species appears to be 52 base-pairs, which is not consistent with sequence analysis (Peacock et al., 1973; Brutlag & Peacock, 1975; Endow et al., 1975). Sequence analysis has shown two repeating species present in this DNA (5'A-A-T-A-T 3' and 5' A-A-T-A-T-A-T 3'; Brutlag & Peacock, 1975). Moreover, under the conditions of renaturation, a significant fraction of the isolated complementary strands of this satellite would exist in transient self-annealed hairpin structures. This intrastrand structure could decrease the rate of renaturation causing an anomalously high complexity. In general, the physical properties of the satellites reported here, renaturation kinetics, buoyant densities and strand separation are consistent with sequence analysis.

The satellite DNA species were all found to be of comparable representation in the genome, each amounting to approximately 4% of the total nuclear DNA and together accounting for 84% of the highly repeated DNA sequences. This quantity of highly repeated DNA (20% of the genome) is somewhat higher than has been previously reported (for a review see Laird, 1973). Several factors contribute to this discrepancy. The method of preparation of DNA is an important variable, since we have shown that treatment with hot sodium perchlorate results in the loss of at least three of the four D. melanogaster satellites and others have reported selective losses of A+T rich DNA in phenol extractions (Skinner & Triplett, 1967). The tissue used as the source of nuclei for the DNA preparation is also an important variable, as polyteny nuclei are common in Drosophila and highly repeated DNA is under-represented (as a result of underreplication, see Laird, 1973) in such nuclei. The source of nuclei we have used is six to ten-hour embryos, where the frequency and levels of polyteny are very low. Finally, in using renaturation kinetics to estimate levels of repeated sequences, an appropriate choice of renaturation temperature is important because of the wide range of tₘ values of the different repeated sequences (Fig. 6). Although there is no evidence for the possibility of differences between stocks, we should note that the Drosophila stock used in this laboratory is Canberra wild-type, whereas many other laboratories have used Oregon R.

The amount of highly repeated DNA we have found within D. melanogaster approximates the amount of heterochromatin seen cytologically in the genome (Cooper, 1959). In situ hybridization studies (Rae, 1970; Gall et al., 1971; Peacock et al., 1973) have shown that the highly repeated DNA sequences are localized in heterochromatin. Since the in situ studies showed that specific satellites are not localized to a single site in the complement, it was important to determine the lengths in which the highly repeated DNAs occurred in the chromosome. In this way it would be possible to distinguish between models where the respective sequences were in small blocks (Kram et al., 1972) or in large homogeneous blocks. Our estimates (Table 2) placed a lower limit of approximately 750,000 bases for the in vivo sizes of the 1.705, 1.686 and 1.672 g/cm³ sequences. This estimate does not necessarily contradict the previous data of Kram et al. (1972) for "harr" DNA, because the total DNA in their study did
not contain or contained greatly reduced amounts of the satellites considered in detail here. The satellite DNA considered by Kram et al. (1972) appears to be localized in region III of the actinomycin D/CsCl gradient (Fig. 1) as discussed by Hearst et al. (1974). We have found the yield of one of the satellites (1.695 g/cm³) from region III of an actinomycin D/CsCl gradient to be dependent on molecular weight (Peacock & Appels, manuscript in preparation). This result probably reflects a condition where the repeated sequences are dispersed among more complex DNA, as is postulated by Kram et al. for "harr" DNA. It should be emphasized that the above discussion applies to only a small fraction of the highly repeated sequences in the genome, the majority of satellite sequences are arranged in large homogeneous blocks.

Some of our data show the above blocks of satellite DNA to be contiguous. The 1.705 g/cm³ satellite after isolation in actinomycin D/CsCl gradients consistently showed an asymmetrical peak skewed toward lighter densities when rerun in CsCl (Fig. 2(a)). Rebanding of the skewed portion sometimes showed a broad peak at approximately 1.699 g/cm³ (e.g. Fig. 5), but gave no indication of the 1.672 and 1.686 g/cm³ sequences which were present (Table 3). We therefore infer that the 1.705 g/cm³ sequences in this DNA fraction are covalently joined to 1.672 and/or 1.686 g/cm³ sequences, and presumably represent the regions of junction between contiguous blocks of these different satellites. More complex DNA, not identifiable as satellite sequence DNA, was also present. Thus the "1.699 g/cm³" DNA from the skewed region of the 1.705 g/cm³ satellite may represent not only a mixture of linkage molecules recovered from sites where large blocks of different satellite sequences are joined but also molecules where there is dispersion of the 1.705 g/cm³ satellite amongst non-satellite DNA. Despite this complexity, it seems probable that the sequence homogeneity of the various satellites involved in covalent associations is comparable to that found within the large blocks of sequences of each particular DNA species. This interpretation of the data is consistent with detailed in situ hybridization studies which show satellites localized near each other in heterochromatin (Peacock, Appels & Steffensen, manuscript in preparation).

The covalent linkage of blocks of different highly repeated sequences raises the possibility that various combinations of such blocks may characterize the different chromosomes of the complement. Characteristic combinations of satellites for the heterochromatin of each chromosome can be directly visualized by in situ hybridization, to mitotic chromosomes, of the satellites reported here (Peacock, Appels & Steffensen, manuscript in preparation). These arrays may therefore be involved in homologous chromosomal recognition processes such as meiotic pairing or in the maintenance of specific chromosomal positions in interphase nuclei. On the other hand, the fact that there are segments of any one of the satellites on more than one chromosome of the complement implies that the sequence blocks may also function in processes that do not distinguish between homologues but in which a more general association of heterochromatin regions is important; for example, chromocentral formation in the early stages of meiosis in the egg mother cells (Dävring & Sunner, 1973,1976), and in cells with polytene nuclei.

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