The Organization of Highly Repeated DNA Sequences in Drosophila melanogaster Chromosomes

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Each chromosome of the Drosophila melanogaster genome may contain only one DNA molecule. Molecules of lengths corresponding to the DNA content of entire chromosomes have been detected (Ravenoff and Zimm, 1973). This simplicity of basic molecular organization of chromosomal DNA emphasizes that sequence arrangement within the DNA must underlie complexities evident in cytological and genetic analyses of the chromosomes. A major cytological complexity is the distinction between condensed heterochromatin and less condensed euchromatin in interphase and prophase stages of mitosis and meiosis. Localization of highly repeated and satellite DNA species to the heterochromatin (Rae, 1970; Gall et al., 1971) has provided a correlation between sequence differentiation of DNA at a molecular level and chromatin differentiation at a cytological level. A finer level of organization has been suggested for centromeric heterochromatin by Kram et al. (1972), who concluded that the highly repeated sequences are interspersed with less repeated sequences.

Another level of chromosomal organization is observed in polytene nuclei in which the genetically active euchromatin has a banded appearance. Analysis of this particular form of organization has posed a fundamental question in the problem of genome organization. Combined genetic and cytological observations have led to the conclusion that only one function is associated with each band (Judd et al., 1972; Hochman, 1971; Lifschytz, 1971) despite each band containing enough DNA, on the average, to code for 20–30 proteins (30,000 base pairs). One solution to the problem is that there are 20–30 tandem repeats of a single gene in each band (Thomas, 1970, 1971). Lee and Thomas (1973) have detected tandemly repeated sequences in the genome of D. melanogaster by the formation of circular molecules after exonuclease resection of linear DNA fragments. They have concluded that the repeats of any one sequence are clustered into a restricted region of the chromosome and that these regions occur largely in the euchromatin. This latter point depends, in part, on their observation that DNA fragments from polytene or diploid nuclei form circles at the same frequency, even though euchromatin DNA is greatly amplified relative to heterochromatin DNA in polytene nuclei (Rudkin, 1969). These conclusions are in conflict with renaturation analyses which indicate that 75–85% of nuclear DNA sequences are present in only a single copy per genome (Laird, 1971; Wu et al., 1972).

In our analysis of the organization of the Drosophila melanogaster genome we have concentrated on the isolation and characterization of the highly repeated DNA species. We have isolated four major (and one minor) satellites representing 80% of the highly repeated sequences. They occur in only a small number of long homogeneous blocks located primarily in the heterochromatin. These satellites comprise 18% of the genome and account for over half the tandemly repeated sequences as determined by circle formation following exonuclease resection.

Isolation of Satellite DNA Species

The DNA content of polytene nuclei of larval salivary glands does not reflect the haploid genome in the proportions of the component DNA species (Rudkin, 1969; Gall et al., 1971), and it is likely that other differentiated tissues have varying DNA constitutions. We have therefore standardized our source of DNA, and throughout this paper the term total DNA refers to DNA extracted from embryos of D. melanogaster (Canberra wild-type) with a mean age of 8 hr.

DNA was extracted from isolated nuclei by equilibrium centrifugation in a CsCl gradient, this method (Fig. 1) being chosen to minimize the possibility of preferential loss of certain classes of DNA (Skinner and Triplett, 1967; Laskowski, 1972). Equilibrium centrifugation of nuclear DNA in a
CsCl gradient shows a main band of buoyant density of 1.701 g/cc with two satellite bands of densities 1.688 and 1.672 g/cc (Fig. 1). Similar analysis of total embryonic DNA yields one additional satellite at 1.680 g/cc (Fig. 1). Isolation of DNA from preparations of purified mitochondria shows that the 1.680 satellite is mitochondrial DNA (Fig. 1).

The finding of two light nuclear satellites is in agreement with the result obtained by Blumenfeld and Forrest (1971) and Lee and Thomas (1973). Gall et al. (1971) isolated one light satellite, probably corresponding to our 1.688 band, but commented that two additional light satellites could be detected in overloaded gradients. Earlier reports that the light satellites are of cytoplasmic origin (Laird and McCarthy, 1969; Botchan et al., 1971) are likely to have been in error. The identification of the 1.680 band as mitochondrial DNA is in agreement with recent reports (Polan et al., 1973; Bultmann and Laird, 1973). In DNA extracted from 8-hr embryos, we did not find evidence of a heavy satellite as reported by Lee and Thomas (1973), however, DNA from younger embryos does show a heavy satellite (1.718 g/cc) of uncertain origin.
Antibiotic-CsCl gradients. Equilibrium centrifugation of Drosophila DNA in the presence of certain antibiotics increases the resolution of the satellites mentioned above and leads to the detection of other discrete DNA species. These antibiotics bind to specific base sequences under conditions of high salt (Zimmer and Luck, 1972; Kersten et al., 1966; Brown et al., 1971). Total DNA is resolved into six peaks in an actinomycin D-CsCl gradient (Fig. 2).

The buoyant densities of these DNA species were determined following isolation from preparative gradients and removal of bound actinomycin D. Actinomycin-1 is a single peak in CsCl gradients with a density of 1.705 g/cc (Fig. 3); this satellite is not discernible in a CsCl gradient of total DNA, since it is obscured by the main band. The actinomycin-2/3 region is composed of two satellites of densities 1.686 and 1.672 g/cc (Fig. 3). Actinomycin-4 is absent from nuclear DNA, and since mitochondrial DNA (1.680 g/cc) bands in this position in actinomycin D-CsCl gradients, this peak is probably of mitochondrial origin. Actinomycin-5 has a density of 1.688 g/cc (Fig. 3), and it seems likely that the 1.688 band in regular CsCl gradients of nuclear DNA is a composite of this satellite and the 1.686 species. No satellites can be distinguished when the main band, actinomycin-8, is analyzed in CsCl; it shows only a single symmetrical peak of density 1.701 g/cc (Fig. 3).

Complete purification of the 1.688, 1.686, and 1.672 fractions is not possible from a single actinomycin D-CsCl gradient. An additional centrifugation in CsCl in the presence of netropsin sulfate, another antibiotic, is necessary to resolve these DNA species. Netropsin has an affinity for A-T base pairs (Zimmer et al., 1971), whereas actinomycin D

![Figure 2. Isolation of satellite DNA. Total DNA (10 μg/0.6 ml) prepared from 8-hr embryos (Fig. 1) in 10 mM Tris-HCl, 1 mM EDTA (pH 8.4), 10 μg actinomycin D was brought to a density of 1.66 g/cc with CsCl and the sample analyzed as in Figure 1. For preparative isolation of the satellites, nuclear DNA (100 to 250 μg/ml) was mixed with actinomycin D (30 μg/100 μg of DNA) in 10 mM Tris-HCl, 1 mM EDTA (pH 8.4) and CsCl added as above. The solution was centrifuged at 44,000 rpm for 4 hr in either a Ti50 or a Ti60 rotor and fractions collected from the bottom of the tube. Actinomycin D can be removed by extracting the pooled fractions three times with isopropanol saturated with a saturated aqueous solution of CsCl. DNA from the Actinomycin-2/3 region (50 to 100 μg/ml) was mixed with netropsin sulfate (160 μg/100 μg of DNA) in 10 mM Tris-HCl, 1 mM EDTA (pH 8.4), and CsCl added to a density of 1.63 g/cc. The gradients were centrifuged, collected, and the netropsin sulfate extracted exactly as for actinomycin D. The removal of actinomycin D was monitored by absorption at 440 nm and netropsin sulfate at 310 nm. A fourth satellite from region 5 of the actinomycin-CsCl gradient can be further purified by centrifugation in the presence of netropsin sulfate as above.]

![Figure 3. Buoyant density analysis of isolated actinomycin D-CsCl satellite fractions. DNA from region 1 (1.9 μg), region 2/3 (2.4 μg), or region 6 (3.5 μg) of a preparative actinomycin D-CsCl gradient (Fig. 2) was mixed with a marker (0.9 μg of M. luteus DNA) in 10 mM Tris-HCl, 1 mM EDTA (pH 8.4), and CsCl to a density of 1.698 g/cc. The solution was centrifuged and analyzed as in Figure 1.]
preferentially binds to GpC sequences. Preparative centrifugation of the actinomycin-5 region with netropsin resolves the 1.688 satellite from main band (Fig. 3). Centrifugation of the actinomycin-2/3 region not only gives an adequate separation of the 1.686 and 1.672 satellites, but also discloses a minor component, netropsin-1. Buoyant density analysis indicates this latter fraction is comprised of at least three discrete bands with densities close to that of main-band DNA (1.690, 1.702, 1.704 g/cc). We have not yet attempted to separate the components of this satellite.

Fractionation of total DNA with Ag⁺-Cs₂SO₄ gradients (Jensen and Davidson, 1966) reveals the same satellite species but does not achieve the resolution given by the actinomycin-netropsin method.

**Characterization of the Nuclear Satellite Species**

The 1.705 satellite. It is possible that this satellite corresponds to fraction I of a Hg⁺⁺-Cs₂SO₄ gradient reported by Blumenfeld and Forrest (1971) to have a buoyant density of 1.704 g/cc. Our recoveries of this satellite from both Ag⁺-Cs₂SO₄ and actinomycin D-CsCl gradients indicate that it constitutes 4.2-4.7% of the total nuclear DNA. Saturating hybridization of 1.705 DNA to total nuclear DNA indicates that it represents 4.6% of the genome, and competition hybridization experiments lead to estimates ranging from 4.8 to 5.8%. The G + C content of in vivo ³²P-labeled 1.705 DNA, extracted from tissue culture cells, is 38.4%, compared to 45.8% predicted from its buoyant density (Schildkraut et al., 1962).

Alkaline CsCl centrifugation of the 1.705 satellite yields two equal bands of densities 1.758 and 1.751 g/cc (Fig. 4). The buoyant density of renatured DNA, following either thermal or alkaline denaturation, is 1.705 g/cc. The strand separation in alkali and the equivalence of densities of native and renatured DNA suggest that the satellite is a homogeneous, highly repeated DNA species. The thermal denaturation profile is compatible with this interpretation, showing a steep, monophasic increase in optical density with a Tₘ of 68°C in 0.1 x SSC (the Tₘ of total nuclear DNA is 74°C in 0.1 x SSC). Furthermore, the Tₘ of renatured 1.705 DNA is identical to that of the native satellite. Kinetics of renaturation yields a Cₚv/ₚₐ value of the order of 2 x 10⁻⁵ mole · sec/liter, suggesting a basic repeat sequence of about 10 nucleotides (Britten and Kohne, 1968). Electron microscope analysis of renatured 1.705 DNA shows mainly branched and circular structures.

The highly repeated nature of this satellite is confirmed by the relatively simple pattern obtained upon pyrimidine tract analysis (Fig. 5a). The tracts of major representation are C₁, T₁, T₂, C₃T₁, C₅T₂, and C₇T₃; the molar proportions are 3:12:3:1:2:1, respectively, suggesting a basic repeat of 33 nucleotides.

The 1.686 satellite. Saturation hybridization shows that this satellite comprises 3.8% of the genome. The yield from actinomycin and netropsin gradient isolation is somewhat lower at 2.6%. Homogeneity is indicated by the melting profile,
with a $T_m$ of 61°C in 0.1 x SSC, and by the clear separation of heavy and light strands in alkaline CsCl (Fig. 4). Although renaturation is not as rapid as that observed with the 1.705 satellite, the C1 of the 1.672 satellite is still less than $10^{-8}$ mole · sec/liter. The $T_m$ of re-}

Figure 5. Pyrimidine tract analysis of satellite DNA. Each satellite (4 μg) was incubated with pancreatic DNase (20 ng/ml), E. coli DNA polymerase (30 μg/ml), and all four [α-32P]-deoxynucleotide triphosphates of identical specific activities (each 20 μCi/μ mole) in 0.05 x potassium phosphate, pH 7.4, 5 mM MgCl₂ at 14°C for 8-12 hr. At the termination of the reaction, the radioactive DNA was isolated by phenol extraction, G-50 Sephadex column chromatography, and ethanol precipitation of the eluted fraction. The radioactive DNA was dissolved in water, carrier DNA added (200 μg of calf thymus DNA), and hydrolyzed to pyrimidine oligonucleotides (Burton and Peterson, 1960). The chromatographic separation and analysis of the radioactive pyrimidine oligonucleotides were performed as described by Southern and Mitchell (1971).

extensive base mismatching has occurred. This is also indicated by the broadened peak and increased buoyant density (1.692 g/cc) of renatured DNA.

Direct determination of base composition shows that buoyant density does not accurately predict the G + C content. Analysis of in vivo labeled
[32P]DNA shows A, T, G, and C in the proportions 40.7%, 31.4%, 10.3%, and 11.9%, respectively, whereas the buoyant density predicts 26% G + C. Chromatography of pyrimidine tracts shows a pattern which is remarkably similar to that of the 1.705 satellite (Fig. 5b). The major tracts are present in molar ratios of 3C$_1$; 2T$_1$; 1C$_2$; 1T$_2$, indicating a repeat sequence of 19 nucleotides. The light strand contains two of the C$_1$ tracts and five of the T tracts; the other tracts being derived from the heavy strand.

**The 1.672 satellite.** The so-called dAT satellite of Drosophila was first claimed to be of cytoplasmic origin (Fansler et al., 1970), but more recently, Blumenfeld and Forrest (1971) concluded that it is nuclear and suggested that it is largely confined to the Y chromosome. Our data support a nuclear origin of this satellite, and our estimates of the amount of this component, based on yield (5.2%) and saturation hybridization (3.8%), are comparable to the value of 3.8% obtained by Blumenfeld and Forrest in Hg$^{+}$-Cs$_2$SO$_4$ gradients. Base composition analysis of 32P-labeled 1.672 DNA reveals only 7% G + C. An identical value can be derived from the pyrimidine tract analysis in which the cytidylate residues are present as the minor components C$_1$ and C$_1$T$_2$ (Fig. 5c). The extreme simplicity of the distribution of the major pyrimidine tracts (3 T$_1$; 1 T$_2$) suggests that the basic repeat of this satellite could be as simple as AATA.

Another sequence consistent with these data is the longer repeat AATAATAATTA, placing the simpliest sequence adjacent to a permutation of that sequence. Other arrangements of the AAT and AT TTA TA sequences are also possible. Data are not yet available to discriminate conclusively between these possibilities. However, the presence of the minor pyrimidine tracts C$_1$T$_2$ and T$_2$ and the absence of the C$_1$T$_1$ and T$_1$ tracts show that single base changes of the pentamer sequence are present as minor components. This indicates that an alternating arrangement of the AAT and AT sequences predominates. Were this the case, we would expect that separated strands of this pentamer sequence could self-associate to form duplex structures with one base pair out of every five mismatched—an A-A base pair in one strand and a T-T pair in the other. Renaturation experiments confirm that intranstrand duplex formation can occur and is favored by low salt concentration and low temperature. The two strands separate in alkaline CsCl with densities of 1.713 and 1.745 g/cc (Fig. 4) and are readily isolated in preparative gradients. Intranstrand duplexes have melting temperatures $17^\circ$ and $26^\circ$ lower than that of the renatured interstrand duplex ($46^\circ$ in 0.1 x SSC). Since the $T_m$ of the renatured duplex is $5^\circ$ lower than native satellite ($51^\circ$ in 0.1 x SSC), some mismatching must occur even under conditions favoring intranstrand duplex formation.

**Other nuclear satellites.** The 1.688 species shows strand separation in alkaline CsCl (1.750 and 1.741 g/cc, Fig. 4) and can be classified as the fourth major satellite (about 4% of the D. melanogaster genome. On the other hand, the netropein-1 satellite constitutes only about 1% of the nuclear DNA and contains at least three species. Although characterization of these satellites is incomplete, evidence that they are highly repeated comes from analysis of circle formation (see below). A summary of the physico-chemical properties of the major satellites is given in Table 1.

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**Table 1. Characteristics of Major DNA Species**

<table>
<thead>
<tr>
<th>Buoyant Density (g/cc) in CsCl</th>
<th>Buoyant Density (g/cc) in Actinomyein D-CsCl</th>
<th>Buoyant Density (g/cc) in Netropein-CsCl</th>
<th>Buoyant Density (g/cc) in Alkaline CsCl</th>
<th>G + C Content (%)</th>
<th>$T_m$(°C) Native DNA</th>
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<tr>
<td>1.705</td>
<td>1.701</td>
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<td>1.686</td>
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<td>1.595</td>
<td>1.713</td>
<td>1.745</td>
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<tr>
<td>Residual main band</td>
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<td>1.669</td>
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<tr>
<td>Total nuclear DNA</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Mitochondrial DNA</td>
<td>1.680</td>
<td>1.647</td>
<td>1.735</td>
<td>1.740</td>
<td>21.7</td>
</tr>
</tbody>
</table>

* Densities calculated relative to isoneutron position as described by Ifs et al., 1961.
  * Determined by enzymic hydrolysis of [32P]DNA from tissue culture and of [32P]DNA synthesized as described in Figure 5.
  * Determined in 0.1 x SSC, except for mitochondrial DNA which was in 1 x SSC.
Organization of Highly Repeated Nuclear DNA

Length of satellite blocks. The isolation of nuclear DNA in CsCl gradients, without any special precautions to avoid hydrodynamic shear, results in DNA molecules with a mean length of 20 μ (4 × 10⁶ daltons). When this DNA is separated into satellite and main-band fractions in an actinomycin-D-CsCl gradient, the mean lengths of both the satellite and main-band molecules are the same. Shearing does not increase satellite yields. 1.705 DNA is isolated with 95% efficiency from total DNA of molecular weight of either 2 × 10⁹ daltons or 1.6 × 10⁹ daltons. Similar high yields of the 1.672 and 1.686 species are obtained from high molecular weight DNA. These data, and the fact that the 1.686 species can be separated from main-band DNA, suggest that most of each of the major satellites does not exist as short segments interspersed among unique sequences (cf. Kram et al., 1972).

Since each satellite exists in long, homogeneous blocks, each must occur in a small number of sites in the genome. An upper limit of 100 sites per genome can be estimated on the basis of the mean length of isolated satellite molecules (20 μ), assuming that each major satellite comprises 4% of the genome. The actual number of satellites must be fewer than 100, as the satellite blocks in the chromosome must be longer than the length of the isolated molecules. If the 5% of the 1.705 satellite which remains in the main band is due to ends of satellite regions covalently joined to main-band sequences, then a block length of 200 μ is indicated. This would mean that each satellite could occupy as few as 10 sites in the genome.

Chromosomal location of satellites. Cytological hybridization results are consistent with the conclusion that each of the satellites occurs in only a few places in the genome. [³H]RNA complementary to each of the satellites shows chromocentric localization when annealed to polytene chromosome preparations (methods modified after Gall et al., 1971). In addition, the 1.705, 1.686, 1.672, and netropsin-1 satellites anneal to a single band in the 21 C-D region of the left arm of chromosome 2 (the probable band is the D1-2 doublet). Rae (1970) reported that RNA complementary to a CsCl main-band DNA fraction hybridizes to the chromocenter and to a band in the distal region of the left arm of chromosome 3. Gall et al. (1971) also reported specific labeling of a distal autosomal band by RNA complementary to satellite DNA. We find that analysis and photography of chromosomes prior to hybridization adds precision to localization in radioautograms. eRNA made on a main-band DNA template (actinomycin-6 region) gives chromocentric labeling and also shows extensive labeling along the chromosome arms. Some preparations of satellite cRNAs result in labeling along chromosome arms which is not evident if the cRNA is initially purified by hybridization to, and elution from, the appropriate satellite DNA immobilized on a nitrocellulose filter. Hybridization of 1.705 cRNA to the chromocenter and 21 C-D can be eliminated by the inclusion of a 100-fold excess of unlabeled 1.705 DNA. Heterologous DNA (1.872 satellite or E. coli) has no effect.

Examination of chromosomes which have been dislocated from the others by squeezing indicates that the 1.705 and 1.686 sequences occur in the centromeric region of the large autosomes as well.

<table>
<thead>
<tr>
<th>Table 2. Frequency of Circle Formation by Fragments of Nuclear DNA Fractions</th>
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</thead>
<tbody>
<tr>
<td>DNA Species</td>
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<tr>
<td>Resection</td>
</tr>
<tr>
<td>Total nuclear</td>
</tr>
<tr>
<td>Residual main band</td>
</tr>
<tr>
<td>1.705</td>
</tr>
<tr>
<td>netropsin-1</td>
</tr>
<tr>
<td>1.686</td>
</tr>
<tr>
<td>1.672</td>
</tr>
<tr>
<td>E. coli</td>
</tr>
</tbody>
</table>

Each row in the table indicates a different experiment.

DNA was sheared in a Virtis homogenizer (30,000 rpm for 10 min in 2.5 M NaCl at 0°, Peyeritz et al., 1972). The DNA was dialyzed overnight in 10 mM Tris-HCl, 1 mM EDTA (pH 8.4). Exonuclease digestion was carried out in 300 μl of 67 mM Tris-HCl (pH 7.9), 6.7 mM MgCl₂, 0.1 mM dithiothreitol at 37°C using a saturating level of exonuclease III (300 units). A control sample (zero digestion) was removed immediately and made 2 x SSC, allowed to anneal at 65°C for a minimum of 2 hr, and then cooled slowly to room temperature. The extent of digestion was monitored by absorption at 260 nm (% digestion = A - (A₀ - 0.877)/A₀ as in Thomas et al., 1970). Samples were removed after 15% digestion and annealed as for the control. DNA was prepared for electron microscopy as in Figure 6. The relative frequencies of classes of molecules were scored on the fluorescent screen in randomly chosen grid fields. While these frequencies are number averages, no obvious difference in length of circular and linear molecules was noted.
as in the X and chromosome 4. This conclusion assumes that hybridization to a separated chromosome is not due to contaminating sequences from the other chromosomes. On the other hand, the 1.672 satellite appears to be restricted to chromosome 2. In three nuclei in which the second chromosome was separated from the chromocenter, 1.672 eRNA hybridized only to the chromocentric region of chromosome 2 and the 21 C-D region. Blumenfeld and Forrest (1971) presented data suggesting that the 1.672 satellite is present in high concentration on the Y chromosome. Since the specific hybridization of 1.672 eRNA to chromosome 2 was observed in male cells, our results do not support this suggestion; however, we cannot rule it out, as the location of the Y chromosome in these split chromocenters is unknown. Hybridization to mitotic or meiotic chromosomes should clarify this point.

Circle formation by nuclear DNA. Lee and Thomas (1973) compared circle formation of DNA fragments from diploid nuclei with that from polytene salivary gland tissue in which satellite DNA is underrepresented. They found the same frequency of circular molecules in each sample, suggesting that circle formation is not unique to satellite DNA fractions. This question can be approached more directly by comparing circle formation in the highly repeated species of DNA and the residual main band. Samples of total nuclear DNA, the five purified satellites, and the residual main band, sheared to a mean length of 1.5 μ, were resected to an extent of 15% with exonuclease III and annealed. These conditions are those reported by Lee and Thomas (1973) to maximize circular molecule formation. In our experiments, a majority of circular molecules are simple rings, but more complex forms such as lariats, double rings, and poly-rings were also found in all samples. The proportion of circular molecules was determined by electron microscopy (Table 2).

Two major facts emerge from the experiments. The frequency of circular molecules in the residual main-band fragments is less than half that found in total nuclear DNA. The frequency of circle formation by fragments of each satellite is much greater than in total nuclear DNA (70% compared to 22%). All fractions show significant frequencies of circle formation when annealed without prior exonuclease resection (cf. Pyeritz et al., 1971).

Characterization of Mitochondrial DNA

DNA isolated from purified D. melanogaster mitochondria has a buoyant density of 1.680 g/cc and

Figure 6. Electron microscopy of partially denatured mitochondrial DNA. Mitochondrial DNA was dialyzed against 0.05 M sodium phosphate, pH 7.5. Denaturation buffer 0.05 M sodium phosphate, 10% formaldehyde (pH 7.8), was brought to 42°C for 5 min and 1/2 volume of the DNA solution was added (0.25 μg). The solution was incubated for 10 min, cooled quickly in ice, and left 10 min. It was then spread on 0.25 M ammonium acetate using the technique of Davis et al. (1971). DNA molecules were picked up on parlodian coated grids and rotary shadowed with platinum. Photographs are at a magnification of ca. 42,500.
corresponds to the actinomycin-4 region of actinomycin D-CsCl gradients of whole DNA (Fig. 1). It can be isolated in a supercoiled form in ethidium bromide-CsCl gradients. Open circles have a contour length of 5.98 ± 0.04 μ (using 5X 174 RF as a standard, 1.69 μ, Benbow et al., 1972). In alkaline CsCl, the two DNA strands can be resolved and have densities of 1.740 and 1.735 g/cc (Table 1). These observations agree with those recently published by Polan et al. (1973) and Bultmann and Laird (1973). The thermal denaturation profile is trisphasic with minor components melting at 65°C (15%) and 71°C (15%) and a major component melting at 78°C (70%) in SSC.

This melting profile is indicative of intramolecular heterogeneity which is confirmed by visualization of early melting regions. At 42°C (see melting conditions in legend to Fig. 6), a single region, amounting to 15% of the molecule, is denatured. In some molecules, additional short, melted regions are evident (Fig. 7). The major denatured region increases to 25% of the molecule at 46°C (Fig. 6b). The G + C content of mitochondrial DNA is extremely low (21.7%, by enzymatic hydrolysis of [32P]DNA). The early melting region should have a G + C content close to zero based on its T_m (Mandel and Marmur, 1968). This AT-rich region can be detected as a separate peak in CsCl gradient centrifugation of sheared molecules (0.8 μ). The information content of the AT-rich region must be extremely low, reducing the potential genetic information of mitochondrial DNA.

Analysis of pyrimidine tracts of the whole mitochondrial DNA shows that 74% of the nucleotide pairs are found in sequences similar to those of the 1.672 and 1.686 satellites (T_t, T_x, C, C, T_x). Cytochemical hybridization initially suggested a relation to nuclear satellite DNA because eRNA made on a mitochondrial DNA template hybridized to the chromocenter and 21 C-D. However, when the eRNA was purified by hybridization to, and elution from, mitochondrial DNA, hybridization was not observed. Artifactual hybridization may have resulted from eRNA made against trace amounts of contaminating nuclear satellites, preferential transcription of particular nucleotide sequences in the mitochondrial DNA, or nontemplate-specific RNA synthesis. Filter hybridization shows no evidence for shared sequences among nuclear satellites and mitochondrial DNA. Nevertheless, the concordance of pyrimidine tract patterns suggest the possibility of a common origin of the AT-rich region and the AT-rich nuclear satellites.

**Discussion**

**Properties of satellites.** Our procedures have separated several highly repeated fractions from the total DNA of the *D. melanogaster* genome. The extent of repetition of the pentanucleotide 1.672 sequence can be estimated at 1.2 × 10^6 copies, whereas the 1.686 and 1.705 species, with their longer basic repeats, are represented by 3.2 × 10^6 and 1.8 × 10^6 copies, respectively. We cannot at present make precise estimates of the number of copies of the 1.686 or netropsin-I sequences. These five isolated DNA species together account for approximately 18% of the genome, and we estimate from renaturation kinetic analysis of the residual main-band DNA that an additional 3–4% of the genome is highly repeated (C^n+1/2 < 10^-2). These, as yet unisolated, sequences (or sequence) have buoyant densities within the range of main-band DNA, both with and without bound antibiotics. Their failure to separate from main band could be either because they bind antibiotics to the same extent as main-band sequences or because of their arrangement in the genome; if these sequences were in short segments interspersed between unique DNA, we could not have expected to separate them with our present techniques. Renaturation kinetics of residual main band also show a significant fraction of moderately repeated DNA (C^n+1/2 < 0.7–10), which may correspond to the DNA studied by Bonner and Wu (1973).

Pyrimidine tract analyses of the 1.686 and 1.705 species are similar. Two of the principal tracts, T_t
and $T_m$'s are those found for the 1.672 satellite, and the other tracts can be derived from the $^5\text{AATAT}^5\text{TTATA}$ pentamer by single base changes, e.g., $C_4T_3$ is generated by a single transversion. In addition, the minor sequence variability observed within a satellite can be explained by single base substitutions. The frequency of these occasional sequence alterations varies among satellites. For example, the $\Delta T_m$ (11°C in 0.1 × SSC) of native and renatured 1.686 DNA suggests that a substantial amount of mismatching occurs upon renaturing. On the other hand, both renatured and native 1.705 DNA have identical $T_m$'s. This contrast is also shown in the buoyant densities of native and renatured molecules of these two satellites; 1.705 DNA renatures to a density of 1.705 g/cc, whereas renatured 1.686 DNA has a density of 1.692 g/cc. The *D. melanogaster* satellites may thus be related by sequence duplication and single base pair substitution. Gall (this volume) has described a simpler case of relatedness in the three satellites of *Drosophila virilis*, where each has a repeat unit of seven nucleotides and where two differ from the third by a single base change. Sequence similarity of the different repeated DNA satellites within a species may have both evolutionary and functional implications.

We have also evaluated the similarity of the different satellites in hybridization experiments which show only a 2–3% level of competition between the 1.672, 1.686, and 1.705 satellites. In spite of the correspondence of the pyrimidine tracts, the sequence differences are sufficient to prevent cross-hybridization. A lack of interaction between the satellite sequences is also indicated by the cytological hybridization result in which hybridization of 1.705 cRNA is competed by 1.705 DNA but not by 1.672 DNA. These results, using purified cRNA, lend credence to the cytological localization data, arguing that radioautography demonstrates the distribution of each satellite species and does not result from nonspecific annealing as was illustrated by the nonpurified mitochondrial cRNA.

Cytological hybridization permits estimation of the amount of various DNA species in particular chromosome regions. Gall et al. (1971) reported that the amount of hybridization at the chromocenter, using RNA complementary to the 1.690 satellite, is independent of the level of polyteny and that an equal amount of cRNA hybridizes to the heterochromatin in diploid nuclei. They noted that this is in accord with Rudkin's (1964) photometric data, which show that the majority of centric heterochromatin does not undergo replication in the polytene nucleus. RNA complementary to a 1.705 template and purified by hybridization to 1.705 DNA, hybridizes to presumptive diploid nuclei, the chromocenter, and the 21 C-D region of polytene nuclei to yield averages of 12, 20, and 6 grains, respectively. Assuming equal hybridization efficiency, this suggests that the amount of 1.705 DNA in the polytene chromocenter is only twice that found in diploid nuclei. Furthermore, if the 1.705 sequences in 21 C-D are laterally polytene to the extent of 1000 copies, the six grains over this region correspond to 2$\mu_m$ of 1.705 DNA in the unit chromatid strand. This calculation assumes that the 12 grains over a diploid nucleus represent 4000 $\mu_m$ of 1.705 DNA (4% of an unreplicated diploid genome). We can therefore estimate that there are approximately 200 repeats of the 1.705 sequence in a unit chromatid in the 21 C-D region.

**Chromosome organization.** Our results differ from previously published studies of highly repeated nuclear DNA in *D. melanogaster* in a number of ways:

1. We have identified both a greater number and a greater amount of highly repeated DNA species. Identification of the 1.705 and netropain-1 satellites is not possible in regular CsCl gradients—these have been isolated with the use of antibiotics or metal ions. Separation of the 1.686 and 1.688 species also depends upon their differential affinities for antibiotics. It seems likely that these two species correspond to the two components of the 1.690 satellite isolated by Gall et al. (1971) by repeated CsCl centrifugation. The four bands they found in alkaline CsCl gradients (1.768, 1.752, 1.745, 1.718 g/cc) correspond to the alkaline densities we find for the separated strands of the 1.688 (1.750, 1.741 g/cc) and 1.686 (1.769, 1.714 g/cc) species. The use of Cs$_2$SO$_4$ gradients for DNA isolation results in more efficient recovery of all satellites than methods employing organic solvent depurination steps.

2. We have found it possible to isolate the highly repeated species as high molecular weight DNA ($4 \times 10^7$ daltons) in contrast to the report of Kram et al. (1972). They concluded that the rapidly renaturing DNA sequences occur in stretches of between 1.5–6 $\times 10^4$ daltons and are interspersed with less rapidly renaturing DNA occurring in blocks of 2–4 $\times 10^4$ daltons. We find that satellites (1.705, 1.686, and 1.672) are largely depleted if DNA is isolated according to their procedure. It is possible that the highly repeated DNA remaining in our residual main band (3–4% of the genome) is interspersed with less repeated sequences, however, the satellites we have isolated, representing 80% of the total highly repetitious DNA, exist in extremely long blocks. The melting profiles of the 1.705, 1.686, and 1.672 satellites show a minor level (2–10%) of crosscontamination, so that it is
possible that the long homogeneous stretches of these repeated species actually adjoin each other in the centromeric heterochromatin. However, we have no direct evidence for their adjacent location.

(3) We have found that the removal of a major proportion of the highly repeated sequences (the isolated satellites) reduces the ability of nuclear DNA fragments to form circles. The reduction from 22% circular fragments in total DNA to 10–11% in residual main band is accounted for by the removal of 18% satellites with their 70% circle-forming ability. These data are also consistent with the finding that the satellite sequences exist in long blocks. The 10–11% of circular molecules formed by residual main band represent 8.5% of the total DNA of the genome. Since 3–4% of the residual main band is highly repeated, a further 5% of the circular molecules of total DNA possibly stems from this source. This would mean that one quarter of the observed 22% cyclization is not ascribable to highly repeated sequences. These remaining 5.5% circular molecules could be derived from those fragments containing moderately repeated DNA (some may be derived from the ribosomal DNA sequences which we know to remain in the residual main-band). An analysis of polytene nuclear DNA in terms of its component DNA species may resolve the conflict between our finding that the majority of the circular fragments of nuclear DNA are highly repeated sequences and the result that polytene nuclear DNA and diploid nuclear DNA form the same number of circles (Lee and Thomas, 1973).

Summary and Conclusion

Twenty-two percent of Drosophila melanogaster nuclear DNA is highly repetitive. Eighty percent of these sequences (18% of the genome) have been isolated as four major satellites, each being homogeneous, and one complex minor satellite. The lightest satellite (1.672 g/cc) may have a basic repeat of only five nucleotides. Two of the other satellites (1.705 g/cc and 1.686 g/cc) have longer repeat units but appear by pyrimidine tract analysis to be related to each other and to the 1.672 sequence. The mitochondrial genome contains sequences resembling the nuclear satellites. However, none of the satellites compete with each other or with mitochondrial DNA in hybridization experiments.

The various satellites have been isolated as homogeneous high molecular weight molecules, and the data suggest that each occurs in very few places in the chromosome complement. Cytological hybridization localizes all satellites to the chromocenter. In addition, at least three of the four major satellites (1.705, 1.686, and 1.672) are represented in the 21 C-D region on the left arm of chromosome 2. Since (a) the highly repeated sequences represent 22% of the genome and are centromerically located, and (b), about 28% of the mitotic complement, is composed of heterochromatin (after Cooper, 1950), it seems probable that there is a correspondence between heterochromatin and repeated DNA in the Drosophila melanogaster genome.

Since a major proportion of the fragments of the genome which can form circles are attributed to the highly repeated DNA, it seems unlikely that a large fraction of the euchromatic region of the genome contains tandemly repeated sequences. Therefore, an assumption of 20–30 repeats of a particular sequence cannot be a general solution to the one band-one function paradox.

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