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Cleavage patterns of Drosophila melanogaster satellite DNA by restriction enzymes.

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ABSTRACT

The five satellite DNAs of Drosophila melanogaster have been isolated by the combined use of different equilibrium density gradients and hydrolyzed by seven different restriction enzymes; Hae III, Hind II + Hind III, Hinf, Hpa II, EcoR I and EcoR II. The 1.705 satellite is not hydrolyzed by any of the enzymes tested. Hae III is the only restriction enzyme that cuts the 1.672 and 1.686 satellites. The cleavage products from either of these reactions has a heterogeneous size distribution. Part of the 1.688 satellite is cut by Hae III and by Hinf into three discrete fragments with M.W. that are multiples of 2.3×10^5 daltons (∿350 base pairs). In addition, two minor bands are detected in the 1.688-Hinf products. The mole ratios of the trimer, dimer and monomer are: 1:6.30 : 63.6 for 1.688-Hae III and 1 : 22.0 : 403 for 1.688-Hinf. Circular mitochondrial DNA (ρ = 1.680)is cut into discrete fragments by all of the enzymes tested and molecular weights of these fragments have been determined.

INTRODUCTION

There are approximately 10×10^{10} daltons of DNA per haploid D. melanogaster genome (1,2). At least 18% of the total DNA is highly repetitive ($C_0t < 1.0$) (3,4). In a neutral CsCl equilibrium density gradient, the native total D. melanogaster DNA is resolved into four peaks. The major component is main band DNA with buoyant density ρ = 1.701 and there are three minor components with ρ = 1.672, 1.680 and 1.687, respectively (5-7). In Actinomycin D (Act D)-CsCl density gradient (4) the 1.687 species is further resolved into two components (ρ = 1.686 and 1.688) and another heavy satellite (ρ = 1.705) is separated from the main band. Four of these satellites (1.672, 1.686, 1.688 and 1.705), each comprising 2 to 4% of the total DNA, have been shown to be highly repetitive (4). The 1.680 satellite is of cytoplasmic origin and has been identified as mitochondrial DNA (8).

The sequences and arrangements of these satellites have been partially characterized. The highly repeated satellite DNAs are

localized in centric heterochromatin (7,9) as well as in the chromocenter of the polytene chromosomes and in a band of the left arm of chromosome 2 (10). Their sequences have been analyzed both by pyrimidine tract analysis (4) and by sequencing of the products made by E. coli RNA polymerase or DNA polymerase with the satellites as templates (11,12,13). Since the satellite DNAs of many species are found to contain simple short sequences tandemly repeated in long blocks, it has been suggested that the satellites are generated by a slow multiplication of the basic repeat followed by a rapid saltatory replication of a several hundred base pair unit (14). Unequal crossing over may also have played a role in the amplification of the nucleotide sequences. Alternatively, there are data indicating that random base mutations may continuously destroy the homogeneity of satellite sequences (14).

We have probed the Drosophila melanogaster satellites by using the type II restriction enzymes. These are the enzymes that will recognize specific base sequences on DNA and make double strand cleavages there. The recognition base sequences of many restriction enzymes have been determined (15). Study of the digestion products of an individual satellite DNA by different restriction enzymes provides information about the distribution of restriction sequences on the satellite DNA. If a satellite is composed of a homogeneous tandem array of a short repeating unit, it cannot be cut by a restriction enzyme unless the repeating unit itself contains the restriction sequence. If sequence heterogeneity has been introduced to a certain extent by random mutations, the satellite DNA might be found to be cut by a restriction enzyme in an irregular way. Higher order sequence arrangement such as that of the bovine satellite I (16) may also be detected if the appropriate restriction enzymes are used.

In this work, the restriction products of the five satellite DNAs of Drosophila melanogaster cleaved by seven restriction enzymes were analyzed on agarose or polyacrylamide gel. The positions of DNA fragments were determined from the fluorescence of the DNA-bound ethidium bromide (17). Their molecular weights were determined with respect to some standard phage or virus DNA fragments of known length. The relative mole ratios of DNA fragments were calculated from their molecular weights and the peak areas obtained

from the tracing of the photograph negatives with a Joyce-Loebl microdensitometer. In order to correct for the non-linearity of the film response, the microdensitometer sensitivity, and possibly the ethidium bromide binding, all of the peak areas have been calibrated on a curve constructed by using the experimental peak areas and known molecular weights of the λ bacteriophage DNA fragments generated by EcoR I cleavage.

MATERIALS AND METHODS

DNA Extraction. Drosophila melanogaster embryonic DNA was isolated from 20 hour fertilized eggs as described in reference 18. The fractions containing DNA obtained from the Cs_2SO_4 gradient centrifugation were dialyzed against 0.01M Tris, 0.001M EDTA buffer (pH 7.0) for two days with several changes.

Preparative CsCl Gradient. Routinely, 12 ml of 100 µg/ml DNA solution was adjusted to ρ = 1.70 gm/cc by adding CsCl (Harshaw, radio tracer grade) and put into one polyallomer 60 Ti tube. Several tubes were centrifuged in the 60 Ti rotor at 42,000 rpm and 20°C for 60 hours. After equilibrium, 0.15 ml fractions were collected from the bottom of each tube. The absorbance at 260 nm for each fraction was measured and the fractions were divided into several pools according to the figures shown in results. Each pool was dialyzed against the Tris-EDTA buffer for later analytical centrifugation or other preparative gradients.

Preparative Cesium Formate Gradient. Solid cesium formate (Harshaw, optical grade) was added to a 25 µg/ml DNA solution to adjust the density to 1.745 gm/cc. Solutions were then centrifuged in an angular 50 rotor at 40,000 rpm, 20°C for 72 hours. The fractions were pooled and dialyzed against the Tris-EDTA buffer. Cesium formate was used for the sake of improved resolution over CsCl (19).

Act D-CsCl Gradient. The 1.687 satellite obtained from the cesium formate gradient was put into an Act D-CsCl gradient according to the following procedure : 25 µg/ml DNA solution was mixed with a calculated amount of solid CsCl and agitated for 10 minutes. Concentrated Act D solution was added to give a final Act D concentration of 30 µg/ml. The final density was 1.66 gm/cc. The DNA solution was centrifuged in a 60 Ti rotor at 38,500 rpm, 20°C for 70 hours. The fractions were collected and absorbances at

260 nm were measured. Pooled fractions were extracted with 8-octanol several times until the yellow color was gone. They were then dialyzed against the Tris-EDTA buffer.

Slightly different conditions were used to isolate 1.705 satellite. A volume of 15 ml of a solution which contained 30 µg/ml DNA, 30 µg/ml Act D and CsCl added to a density of 1.63 gm/cc was centrifuged in a 60 Ti rotor at 42,000 rpm, 20°C for 60 hours.

Analytical CsCl Density Gradient. A 0.5 ml sample containing 5 µg/ml DNA was adjusted to a density of 1.70 gm/cc with solid CsCl. The DNA solution was centrifuged at 42,000 rpm, 20°C for 22 hours. M. lysodeikticus DNA whose buoyant density has been measured to be 1.733 was used as a marker, and the calculation of the samples buoyant densities was performed as previously described (19). A buoyancy gradient constant of 9.35×10^{-10} $g \cdot sec^2/cm^5$ was used for all measurements.

Mitochondrial DNA Isolation. Supercoiled mitochondrial DNA was isolated according to Botchan (8).

Restriction Enzyme Reactions. For the enzymes Hae III, Hinf, Hpa II and EcoR II, the reactions were carried out at 37°C in 0.01M Tris (pH 7.6), 0.01M $MgCl_2$ and 0.1%(w/v)DTT (dithiothreitol) for 5 hours. EcoR I endonuclease reaction was performed at 37°C in 0.1M Tris (pH 7.5), 0.05M NaCl and 0.01M $MgCl_2$. Hind II + Hind III reaction was in 0.01M Tris (pH 7.6), 0.01M $MgCl_2$ and 0.04M NaCl. In most cases 1 µg of DNA in a total volume of 50 µl was used. The reactions were terminated by adding 40% sucrose, 0.5M EDTA solution to make the final concentrations 8% sucrose and 0.01M EDTA.

1% Agarose Gel Electrophoresis. 1% agarose gel containing 0.6 µg/ml of ethidium bromide was formed according to Sharp et al. (17) between two glass plates clamped on a slab gel apparatus with an agarose dam preformed at the bottom to prevent the leaking of the agarose. A 12 tooth lucite comb was inserted into the agarose and the whole gel was allowed to harden at room temperature for one hour. The Tris-Acetate buffer E containing 0.6 µg/ml ethidium bromide was then added to both the upper and lower tanks and the comb pulled out. The slab gel thus formed has twelve evenly spaced 0.48 cm x 0.48 cm wells. The slab dimensions were 14 cm x 12.7 cm x 0.48 cm. The gel was

equilibrated with the buffer for at least 20 minutes before the sample loading.

Electrophoresis was carried out at 45 mA (constant current, voltage = 40 volts at the beginning) for 3 to 5 hours at room temperature. A pumping system (Buchler, polystatic pump) was used to exchange the buffers in the two tanks to avoid pH differences. The dye bromophenol blue was used as an indicator. Electrophoresis was stopped when the dye moved to a position 1 cm to 3 cm away from the bottom.

The gel slab was laid on a U.V. lamp (U.V. Products, Inc.) and photographed with a 250 Polaroid Land camera. An organge glass filter was used to cut off light below 560 nm. For microdensitometric tracing purposes, film with negatives (Polaroid Land film, type 105) was used. Film speed was set at 75 and the lighting selector was on "outdoor and flash." Film was exposed for 1 minute 50 seconds and developed in 12% sodium sulfite solution for 10 minutes. Longer or shorter exposure times were sometimes used to visualize the fainter or stronger bands on the gel.

4% Polyacrylamide Slab Gel. The 4% polyacrylamide gel was formed according to Loening (20) in the same slab gel apparatus used to make the agarose slab gel. Electrophoresis was performed at 60 mA (40 volts at the beginning), 20°C for about 9 hours. A pumping system was also used as in the agarose gel electrophoresis. Gels were stained in a buffer solution containing 0.6 μg/ml ethidium bromide for 2 hours and photographed as described above.

2.5% Polyacrylamide - 0.5% Agarose Slab Gel. The preparation procedures are essentially the same as those by Zeiger et al. (21)

Analysis of Gel Patterns. For molecular weight determination, restriction enzyme DNA fragments of known M.W. from phage or virus DNA were co-electrophoresed with samples on the same slab gel. Usually λ DNA-EcoR I fragments (22) and SV40 DNA-Hind II + Hind III (23) fragments were used as markers for the 1% agarose gel electrophoresis. PM2 DNA-Hae III fragments (24) or SV40-Hind II + Hind III fragments were used for 4% polyacrylamide or 2.5% polyacrylamide-0.5% agarose gel analysis. The negative films of gels were printed into 4 to 5 times enlarged positive pictures. Distances from wells of different DNA fragments were measured. A log M.W. vs. distances plot was constructed using the M.W. and relative distances of migration of the standard DNA fragments.

The M.W. of each sample fragment was determined from the molecular weight calibration curve.

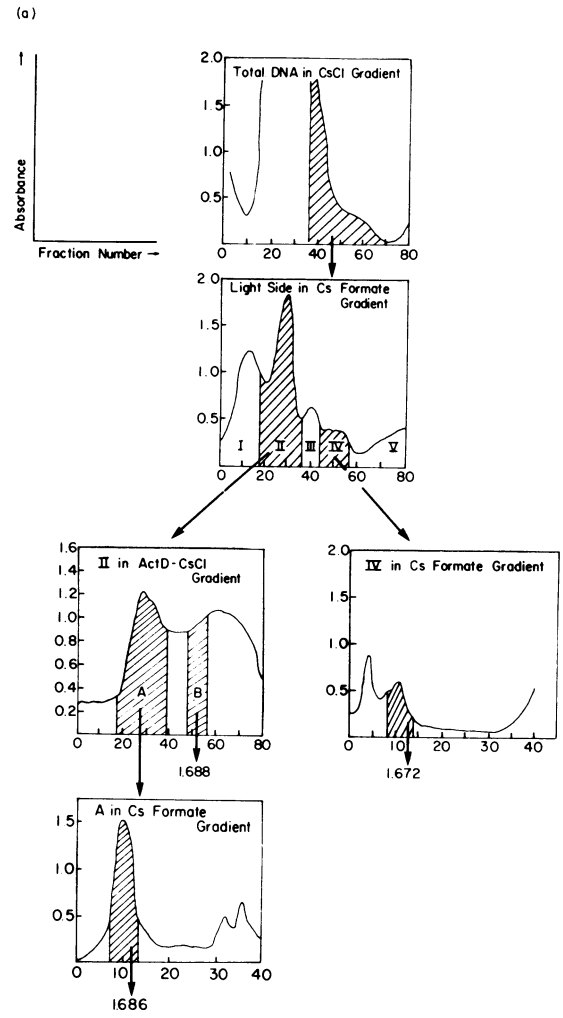
The relative amounts of the sample DNA fragments were determined using a calibration curve which was established using λ DNA-EcoR I fragments. Known amounts of λ cI 857 DNA were digested with the enzyme EcoR I and electrophoresed on 1% agarose gel. The gel was photographed and the negative was analyzed in a Joyce-Loebl microdensitometer. All of the sharp peaks of various λ DNA-EcoR I fragments on the tracing paper were cut and weighed. These experimentally measured paper weights and the proportions of the λ DNA fragments relative to a standard band were used to establish the calibration curve in Fig. 7.

After the calibration curve was plotted, the relative proportions of sample fragments were obtained according to the following procedure: (1) The negative of the sample gel was traced (see Fig. 6 for example). (2) A zero fluorescence base line was drawn for the trace of each sample. (3) The background fluorescence line was continued across the base of each peak. (4) The area under each peak was divided by vertical lines 3 mm apart (the average half-width of the λ DNA-EcoR I fragment peaks used for the calibration curve). (5) The weight of each 3 mm wide strip was determined. (6) The amount (proportion relative to the standard λ fragment) of DNA in each strip was then obtained from the λ DNA-EcoR I fragment calibration curve. (7) Knowing the total amount of DNA in each strip and the percent of DNA in each strip due to background, the proportion relative to the standard λ DNA-EcoR I fragment was determined.

RESULTS

Satellite Isolation. All the preparative density gradients were performed as described above. A general procedure for isolating the 1.672, 1.686 and 1.688 satellites is schematized in Fig. 1a. A single Act D-CsCl gradient of the total *D. melanogaster* DNA is sufficient to get 1.705 satellite with less than 5% contamination by other species (Fig. 1b).

The analytical CsCl profiles of the isolated 1.672, 1.686, 1.688, 1.705 and supercoiled mitochondrial DNAs are shown in Fig. 2. As can be seen, 1.672, 1.686 and 1.705 have less than 5% contamination by other DNA. 1.686 satellite might be present to a large extent in the 1.688 preparation. Mitochondrial DNA is contaminated



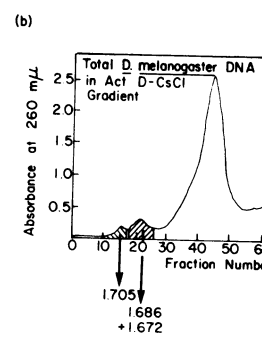


Figure 1. Preparation of satellite DNAs 1.672, 1.686, 1.688 and 1.705. (a) Successive gradients used to isolate 1.672, 1.686 and 1.688. (b) Act D-CsCl gradient used to isolate 1.705. The pooled DNA parts (indicated by included lines: ////// or \\\\\\ ) from the last gradient were dialyzed into the Tris-EDTA buffer (pH 7.0) and analyzed in a Model E analytical ultracentrifuge. Their analytical tracings are shown in Fig. 2.

by 20% main band DNA. The 1.672, 1.686, 1.688 and 1.705 have a double stranded M.W. of approximately 12 x 10^6 daltons.

Restriction of the Mitochondrial DNA (1.680). The partially purified mitochondrial DNA was subjected to endonuclease cleavage by the restriction enzymes Hae III, Hind II + Hind III mixture, Hinf, Hpa II, EcoR I and EcoR II. Reactions were complete after 5 hours of digestion because longer time of incubation and addition of more enzymes did not change the band pattern of 1.680 fragments on 1% agarose gel electrophoresis (Fig. 3a) or the relative peak

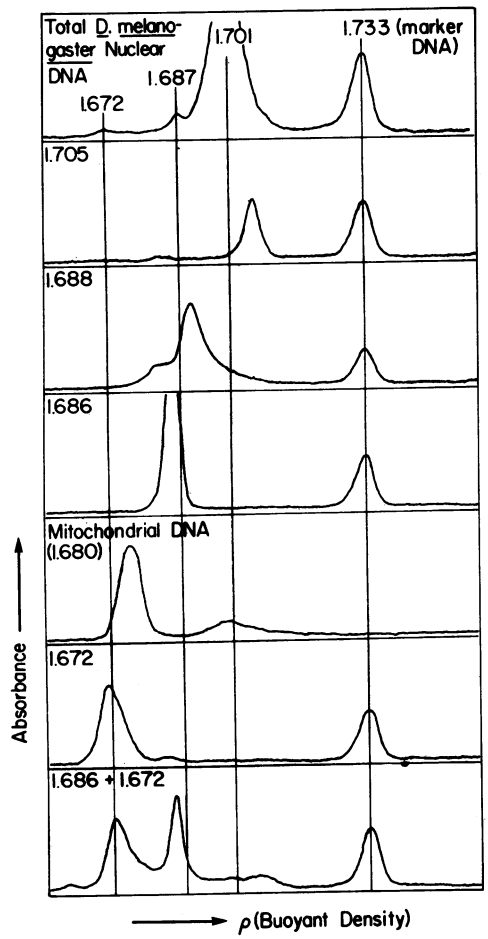


Figure 2. Bouyant densities of various satellite DNAs are determined im Model E at a speed of 42,000 rpm. M.lysodeikticus DNA with a bouyant density 1.733 gm/cc was used as a marker. For comparison, the profile of the total D.melanogaster nuclear DNA is also shown at the top.

areas of the microdensitometric tracings of each sample column (except the 1.680-EcoR II). The molecular weight of all the fragments were obtained from the calibration curve in Fig. 3b and are listed in Table 1.

One of the enzymes, Hinf, cut 1.680 and gave some bands migrating into the region of poor resolution on 1% agarose gel. A 4% polyacrylamide gel electrophoresis showed that the three low M.W. fragments on 1% agarose gel can be resolved into 7 bands (Fig. 4). Assuming that the second low M.W. band on the 4% polyacrylamide gel is composed of two fragments on the basis of its intensity, 1.680-Hinf gave a total of 11 restriction fragments. The 4% polyacrylamide gel electrophoresis of the 1.680 restricted by other enzymes showed no extra bands other than those seen on the 1% agarose gel.

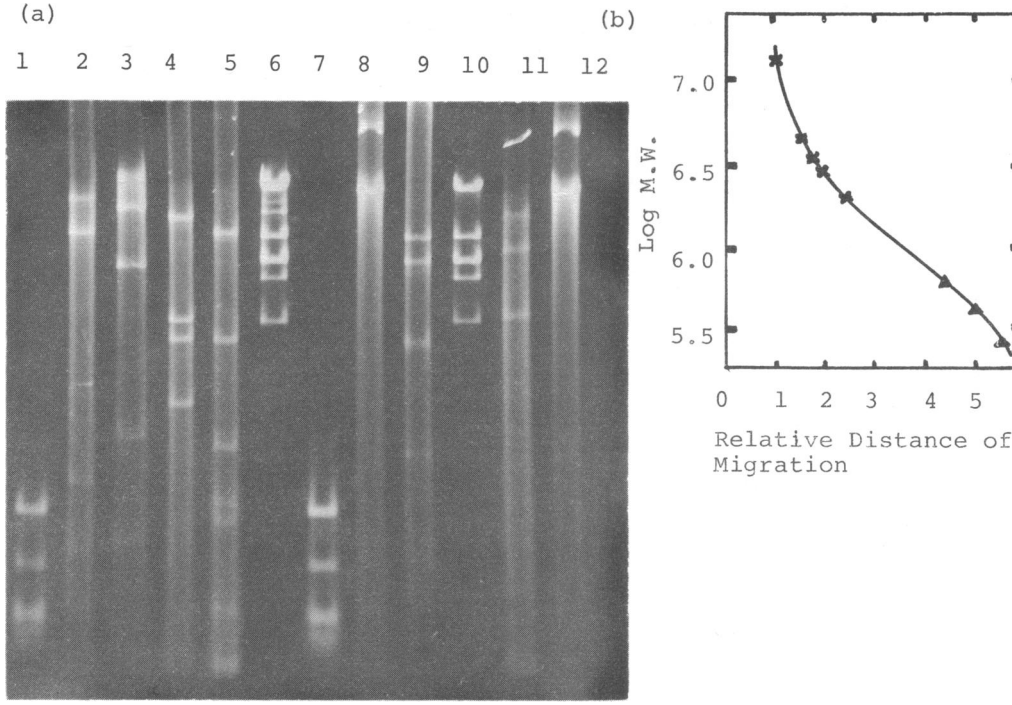


Figure 3. (a) 1% agarose gel electrophoresis of restricted 1.680 fragments. λ-EcoR I fragments and SV40 - Hind II + Hind III fragments were used as markers. Samples in different wells are 1) SV40-Hind II + Hind III; 2) 1.680-EcoR II; 3) 1.680-EcoR I; 4) 1.680-Hpa II; 5) 1.680-Hinf; 6) partially digested λ-EcoR I; 7) SV40-Hind II + Hind III; 8) control, undigested 1.680 DNA; 9) 1.680-Hind II + Hind III; 10) λ-EcoR I; 11) 1.680-Hae III; 12) control 1.680. (b) Calibration of the molecular weights of restricted 1.680 fragments. The calibration curve is constructed from the log M.W. of different standard DNA fragments and their relative distances of migration on the gel shown in Fig. 3a. The log M.W. and M.W. of the restricted 1.680 fragments were found from the calibration curve. x - λcI857-EcoR I Fragments. ▲ - SV40 Hind II + Hind III Fragments.

Some apparent inconsistencies of the M.W. with the intensities could be found in some cases. The three 1.680-Hae III fragments have M.W. 6.31×10^6, 3.95×10^6, and 2.14×10^6, daltons respectively. The relative molar ratio is 1.1:2.5:5.6 as determined from the microdensitometric tracings. In other words, although their M.W. add up approximately to that of the intact mitochondrial DNA (8), they were not generated in 1:1:1 ratios. Similarly the 1.680-EcoR II fragment number 1 (M.W. = 9.1×10^6 daltons) has a

Enzymes Used \ Fragment Number	1	2	3	4	5	6	7	8	9	10	11	Sum of the M.W. of the Fragments
Hae III	6.31	3.95	2.14									12.40
Hind II + Hind III	4.42	3.40	1.77	0.94	0.51							11.04
Hinf	4.84	1.84	0.99	0.72	0.63	0.39	0.36	0.30	0.21	0.21	0.20	10.69
Hpa II	6.10	2.10	1.84	1.28								11.32
EcoR I	7.62	3.35	1.08	0.55								12.60
EcoR II	9.12	4.84	4.84	0.82	0.53	0.36						20.51

Table I. Molecular weights of restricted 1.680 fragments. Molecular weights of each 1.680 fragment generated by different restriction enzymes are listed here as M.W. x 10^6. The fragment numbers are assigned in the order of decreasing molecular weight. The two bands with M.W. 1.17 x 10^6 and 1.35 x 10^6 daltons, respectively, in the middle of the 1.680-EcoR II column in Figure 3 are not listed here because they become very faint when more EcoR II enzyme is used to digest 1.680.

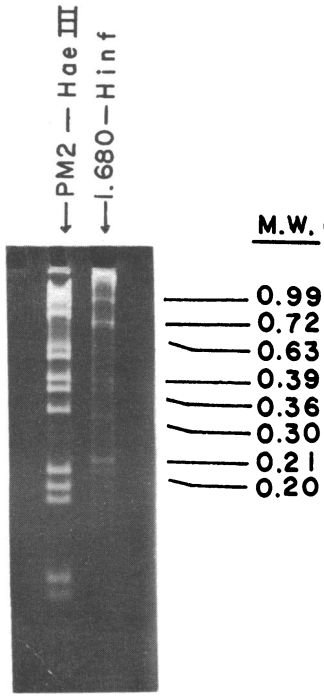


Figure 4. 4% polyacrylamide gel electrophoresis of 1.680-Hinf. PM2-Hae III fragments were co-electrophoresed as markers (left column). The M.W. of the 1.680-Hinf fragments have been determined from a calibration curve constructed from the M.W. and relative mobility of the PM2-Hae III fragments.

much smaller intensity in comparison to that of the doublet number 2 and number 3 which have M.W. 4.84 x 10^6 daltons.

Restriction of the 1.705 Satellite. The purified 1.705 satellite (Fig. 2) was not cut by any of the seven enzymes tested. The digestion products of the 1.705 by all seven enzymes migrated with the same speed as that of the control 1.705 (results not shown). It is thus concluded that the 1.705 DNA isolated here does not contain any of the restriction sequences of the enzymes used.

Restriction of the 1.688 Satellite DNA. As shown in Fig. 5, when the 1.688 satellite DNA was partially digested with Hae III or Hinf, a series of bands with M.W. approximately 0.23 x 10^6 daltons, 0.46 x 10^6, 0.69 x 10^6, 0.92 x 10^6, etc., appeared on a 1% agarose gel. When the reactions of 1.688 with Hae III or Hinf were allowed to go to completion (Fig. 6) and the band patterns were compared to those of 1.680-Hae III and 1.680-Hinf (Fig. 3a and 4), it was concluded that three bands (M.W. $\cong$ 0.23 x 10^6, 0.46 x 10^6, 0.69 x 10^6) were derived from the 1.688 satellite in both the cases of the Hae III and the Hinf digestions. Further-

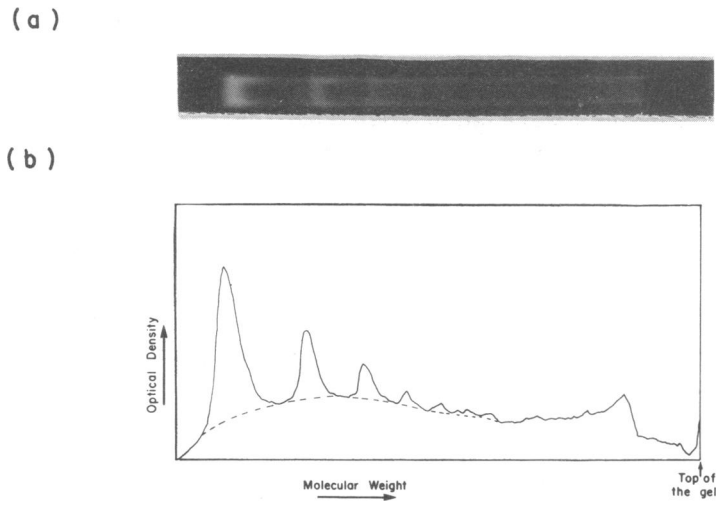


Figure 5 (a). Partial digestion of the 1.688 satellite by Hae III. λ-EcoR I and SV40-Hind II + Hind III were used as markers to determine the M.W. (b) Microdensitometric tracing of the partial 1.688-Hae III fragments (1.688-Hinf gave qualitatively similar patterns).

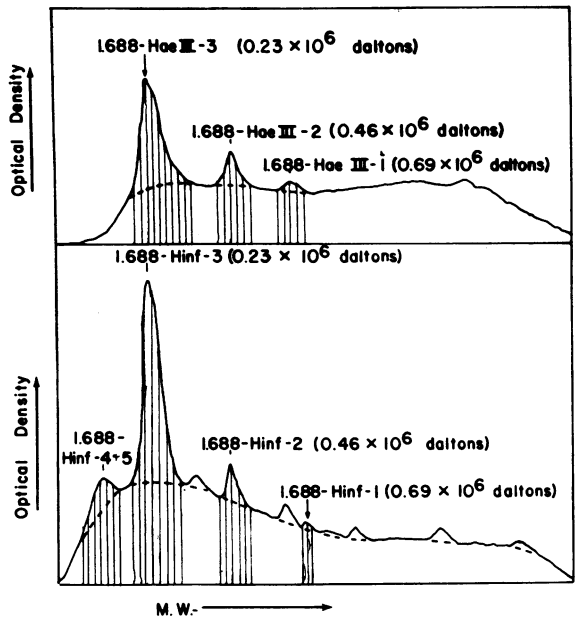


Figure 6. Microdensitometric tracings of 1.688-Hae III and 1.688-Hinf. Three peaks of M.W. 0.23 x 10^6, 0.46 x 10^6 and 0.69 x 10^6 are seen in both 1.688-Hae III and 1.688-Hinf. The vertical lines are drawn here for the calibration purpose and have been described in Materials and Methods.

more, another two fragments were seen when the 1.688-Hinf products were analyzed in a 4% polyacrylamide gel electrophoresis (results not shown). They have molecular weights of 0.12 x 10^6 daltons (1.688-Hinf-4) and 0.093 x 10^6 daltons (1.688-Hinf-5), respectively. They also are not derived from 1.680.

The relative amounts of these 1.688-Hae III and 1.688-Hinf fragments were determined as described in the Materials and Methods. A calibration curve for the gel tracing is shown in Fig. 7. We can see that the curve is linear only over a narrow range of DNA concentrations. The relative amounts are 1.688-Hae III-3 : 1.688-Hae III-2 : 1.688-Hae III-1 = 63.6 : 6.30 : 1 and 1.688-Hinf-(4 + 5) : 1.688-Hinf-3 : 1.688-Hinf-2 : 1.688-Hinf-1 = 72.2 : 403 : 22.0 : 1. Because of the M.W. similarity of 1.688-Hae 1,2,3 and 1.688-Hinf-1,2,3, we suspected that they might originate from the same DNA sequences. One possibility would be that some percent of the 1.688 preparation (estimated to be 13% from either tracing in Fig. 6)

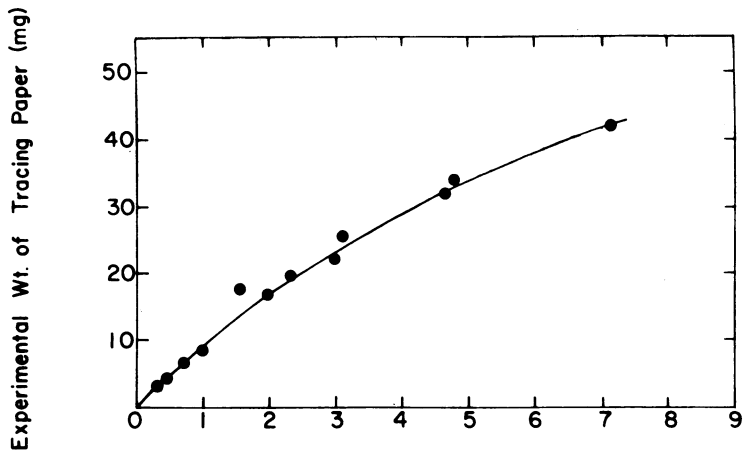


Figure 7. Calibration curve for the relative amounts of sample fragments. Different amounts of λDNA were digested with EcoR I and electrophoresed in 1% agarose gel. The gel was photographed and traced. The peaks corresponding to all the λ-EcoR I fragments except the two containing the cohesive ends were weighed (experimental wt. of tracing paper). The relative proportions with respect to one of the λ-EcoR I fragments were calculated by using the amounts of DNA loaded and their molecular weights. This curve was reproduced in three independent experiments within a range of ± 0.1 unit of the relative proportion. An experimental weight of tracing paper of 10 mg corresponds to 0.025 μg of DNA.

is composed of an array of tandemly repeated sequences 350 base pairs long. Each repeating unit contains one Hae III recognition site and one Hinf recognition site. Some of these sites have been eliminated by some unknown mechanisms (see Discussion section). Thus monomers (0.24×10^6 daltons) as well as dimers (0.46×10^6 daltons) and trimers (0.69×10^6 daltons) are generated after restriction by Hae III or Hinf. To test this hypothesis, the 1.688 DNA was subjected to restriction by a mixture of Hae III and Hinf and the products were analyzed on a 4% polyacrylamide gel (Fig. 8). Three bands were seen with M.W. equal to 0.22×10^6 daltons (fragment M), 0.089×10^6 daltons (fragment B) and 0.13×10^6 daltons (fragment A), respectively. The relative mole ratio is about 0.22 : 1 : 0.95. The sum of the M.W. of fragment

A and B is approximately the same as that of the monomer fragment in 1.688-Hae III or 1.688-Hinf product. This result reinforces the feasibility of the tandem repeat model mentioned above.

After the 1.688 satellite DNA was digested with Hind II + Hind III, Hpa II, EcoR I or EcoR II, 15% to 20% of the loaded DNA migrated to the same position as that of the control (unrestricted 1.688), the exact percentage depending on the enzyme used. The remaining 80% - 85% was cut into heterogeneous patterns. The only bands seen on the 1% agarose gel were faint ones that appear to originate from 1.680 DNA contamination.

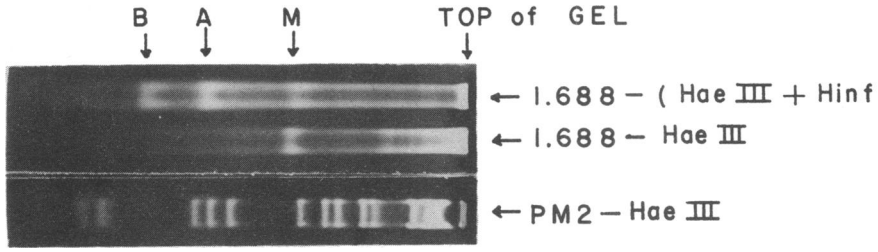


Figure 8. 4% polyacrylamide gel electrophoresis of the digestion product of 1.688 by Hae III and Hinf. Electrophoresis was carried out according to Materials and Methods, SV40-Hind II + Hind III and PM2-Hae III were used as markers. M.W. is 0.22×10^6 daltons for M, 0.089×10^6 and 0.13×10^6 for B and A.

Restriction of 1.672 and 1.686 Satellites. Isolated 1.672 and 1.686 satellites have been tested with the seven enzymes. For Hind II + Hind III, Hinf, Hpa II, EcoR I and EcoR II reactions, the products migrated to the same positions as the control when they were analyzed on a 1% agarose gel. Hae III, however, cut both 1.672 and 1.686 into heterogeneous patterns (Fig. 9) with a M.W. range from as large as 10×10^6 to less than 0.3×10^6 daltons. This result has been reproduced carefully many times. In an attempt to obtain higher resolution in the low M.W. range, the 1.672-Hae III and 1.686-Hae III were also analyzed in a 2.5% polyacrylamide - 0.5% agarose gel and no fine structure was found. A mixture of 1.672 and 1.686 isolated from Act D-CsCl gradient (Fig. 1b and 2) also gave a heterogeneous pattern after restriction by Hae III with less than 10% of the digestion product migrating to the same position as control.

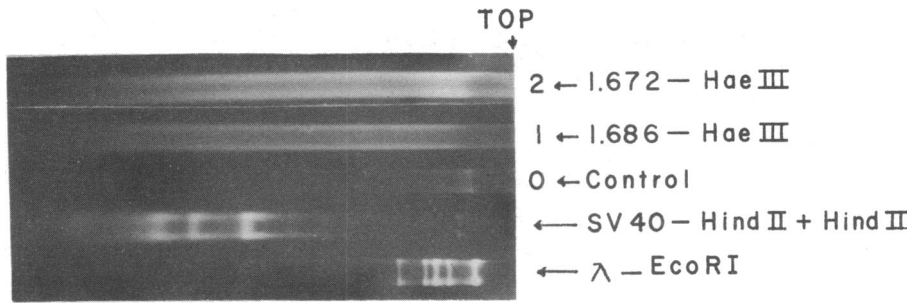


Figure 9. 1% agarose gel electrophoresis of 1.672 and 1.686 restricted by Hae III. 1 µg of 1.672-Hae III was loaded into well #2 and 0.5 µg of 1.686-Hae III was loaded into well #1. 0 is undigested DNA.

DISCUSSION

The studies on the satellite DNAs of many species suggest that satellite DNAs are most likely to have been generated by multiplication of short nucleotide sequences followed by random sequence divergence. Another amplification of large sections of satellite DNAs might occur during the course of evolution. This would not only increase the amount of satellite DNA but would also produce a regular pattern of some sequence modifications previously introduced into the original tandemly repeated array by mutation. This kind of arrangement of the higher order repeating blocks in satellite DNA is difficult to detect by sequencing methods (11,12,13,25). However, it has been found to exist in bovine satellite I (16), guinea pig satellite III (26), and mouse satellite (26,27) by using restriction enzymes.

The EcoR II and Hind III restriction maps of mitochondrial DNA of three mammalian cell lines have been established by Brown and Vinograd (28). The 1.680 DNA data in this report only serve as preliminary evidence on the restriction of Drosophila melanogaster mitochondrial DNA. Abnormalities in the relative amounts of these fragments have been observed in two cases, suggesting that they were generated by restriction of a heterogeneous population of mitochondrial DNA or by the elimination of restriction sites by base changes or other cellular processes.

The 1.672 and 1.686 satellites were both cut into heterogeneous patterns by Hae III. These two satellites have been partially

sequenced (11,12,13). 1.686 contains a major repeating unit 5'-AATAACATAG-3' and 1.672 contains predominantly 5'-AT-3' and 5'-AAT-3' 3'-TTATTGTATC-5' 3'-TA-5' 3'-TTA-5'. The base compositions are 7% GC for 1.672 and 22% GC for 1.686 (upper limit) (4). If these two satellites were generated by slow multiplication and saltatory replication of the above basic repeating units with base mutations randomly introduced, the frequency of appearance for the sequence 5'-GGCC-3' (the restriction sequence 3'-CCGG-5' of Hae III) is at most $(0.035)^4$, i.e., once per 6.7×10^5 base pairs, assuming each strand of 1.672 contains an equal amount of G and C residues. This is certainly not the case since the size range of the 1.672-Hae III fragments is between 2×10^4 base pairs and 500 base pairs (the lower limit may be even smaller), indicating a much larger frequency of -GGCC- than that expected on the basis of random -CCGG- mutation. The frequency for 1.686 is also too large to be accounted for by the simple satellite evolution model. We do not know whether these sequences -GGCC- were introduced into 1.672 and 1.686 by mutations -CCGG- of the original basic repeating unit or by other cellular processes. There may be a selection demanding the existence of such GC clustering in these two satellites. The sequence -GGCC- itself may be important, since another enzyme, Hpa II, whose recognition site is -CCGG- 5'-CCGG-3' does not cut the 1.672 satellite or most of the 1.686 3'-GGCC-5' satellite. The heterogeneous patterns of the 1.672-Hae III and 1.686-Hae III may come from the contamination of some non-specific endonuclease activity in the Hae III enzyme we used. However, the following two facts indicate that it is quite unlikely. First, the 1.705 satellite was not cut by the Hae III enzyme under the conditions that 1.672 and 1.686 were cut. Secondly, we have tested the non-specific endonuclease activity by using PM2 phage DNA as the substrate. When different amounts (5- fold difference) of PM2 DNA were digested to completion using the same amount of enzyme and time of incubation, the same specific band patterns were obtained with less than 5% heterogeneous background in both cases. Furthermore, longer time of incubation or addition of more enzyme did not

increase the background.

The periodicity of the occurrence of the Hae III sites and Hinf sites in the 1.688 satellite suggests that a tandem array of a repeating sequence 350 base pairs long exists in D. melanogaster genome (Fig. 10). This is an intermediate value when compared to that of the bovine satellite I (16), the guinea pig satellite III (26), and the mouse satellite (27). By assuming that random mutations eliminated the EcoR II restriction sites in mouse satellite DNA, Southern (27) calculated the probability of base changes per pair to be 0.030 - 0.032. In fact, the same calculations apply to any model as long as the base change mechanism is assumed to have been random. We have done similar calculations (Appendix) on the 1.688 satellite. If the relative amounts of the 1.688-Hae III-1,2 and 3 are considered, a value of 0.026 - 0.030 is obtained for the probability of base change per base pair. However, the probability of base change is 0.012 - 0.014 per base pair if the relative amounts of 1.688-Hinf-1,2 and 3 are used in the calculation. So the elimination of the two restriction sites are not due to the same random base changes. Knowing that the restriction sequence for Hae III is 5'-GGCC-3'
3'-CCGG-5'
while that for Hinf is 5'-GANTC-3' (B. G. Barrell and C. Hutchinson,
3'-CTNAG-5'

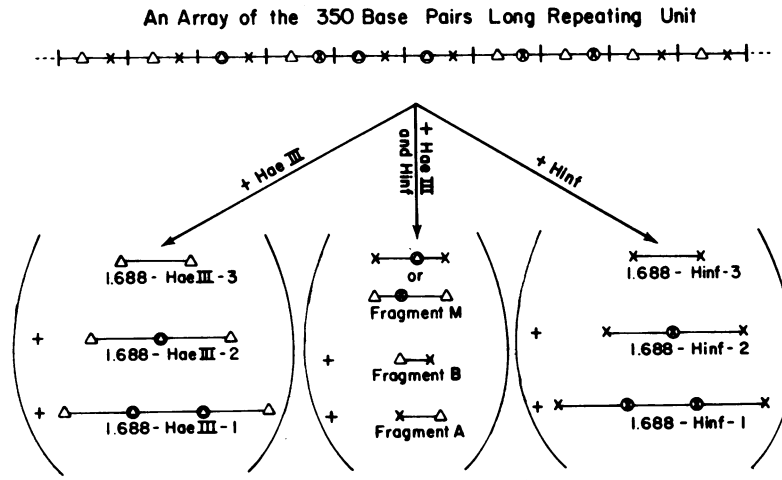


Figure 10. A simple model for the sequences that gave the 1.688-Hae III and 1.688-Hinf fragments. The symbols used are: ⟼ for the 350 b.p. repeating unit; x for Hinf sites; Δ for Hae III sites; ● for eliminated Hinf sites; ○ for eliminated Hae III sites.

personal communication), there are several ways to account for the difference. For instance, the elimination of the restriction sites may be caused by base mutations during evolution but there has been greater selection pressure on the Hinf sequence than on the Hae III sequence. It is also possible that the probability of base mutation of GC base pairs is larger than that of AT base pairs. Alternatively, DNA modifications such as methylation of the bases may also be the cause of restriction site elimination and the difference we obtain from the 1.688-Hae III and 1.688-Hinf data may result from base specific modification mechanisms.

Hamer and Thomas (29) and Manteuil et al. (30) have hydrolyzed D. melanogaster DNA with Hae III, Hind and ECoRI enzymes. Their results differ from ours in two main respects. After the complete digestion of a pooled fraction from an Act D-CsCl gradient, it was found that 1.688 gave five fragments with M.W. multiples of 0.23×10^6 (365 base pairs) daltons (30). However, we observe only three fragments without the tetramers and pentamers after the complete digestion of 1.688 by Hae III. Secondly, studies of the "spared" fragments ($\geq$20,500 base pairs) in Hae III digests of total D.melanogaster DNA (29) or of pools from Act D-CsCl gradient (30) suggested that the 1.672, 1.686 and 1.705 satellites do not have the Hae III sites over long stretches (M.W. $\geq 15 \times 10^6$ daltons) and the random cut DNA was contaminating main band DNA. It is hard to come to a conclusion about the origin of these differences at this time. The difference in enzyme activity is one possibility although we did not find nonspecific endonuclease activity in our Hae III enzyme. Different fractionation procedures also may influence the purity of the DNA preparations. To the extent that buoyant density in Act D-CsCl and pure CsCl gradients establishes the definition for a satellite DNA the heterogeneous fragmentation of these satellites cannot be accounted for by main band contamination.

APPENDIX

Calculation of the probability of random sequence divergence per base pair for the 1.688 satellite. Assumptions: 1) Random base changes of satellite. 2) Dimers and trimers arise from the elimination of the restriction site. 3) One base pair change in a site is sufficient to cause the elimination of the restriction site for the Hinf or Hae III.

The fraction of the DNA in size class n (n = 1 for monomer, 2 for dimer, 3 for trimer) after restriction reaction is given by

$$F(n) = nP^{(n-1)}(1-P)^2$$

where P is the fraction of eliminated restriction sites. Then

$$\frac{F(n)}{n} = P^{(n-1)}(1-P)^2$$

$$\log \frac{F(n)}{n} = (n-1)\log P + 2\log(1-P)$$

P can be obtained from either the intercept or slope of the plot of log (F(n)/n) vs. (n-1). The intercept will be 2 log (1-P) and the slope log P.

Furthermore, if p is the probability of sequence divergence per base pair, then the probability of any consecutive m base pairs not being changed is $(1-p)^m$. For Hae III enzyme, its restriction site is composed of 4 base pairs, i.e. 5'-GGCC-3'. So $(1-p)^4 = 1-P$

3'-CCGG-5'

for the Hae III recognition site. The values of log (F(n)/n) can be calculated from the relative abundance of the monomer, dimer and trimer. They are -1.90, -1.10, -0.095 for the 1.688-Hae III-1, 1.688- Hae III-2, 1.688-Hae III-3, and -2.65, -1.31, -0.048 for the 1.688-Hinf-1, 1.688-Hinf-2, 1.688-Hinf-3, respectively. A plot of log (F(n)/n) vs. (n-1) is shown in Fig. 11.

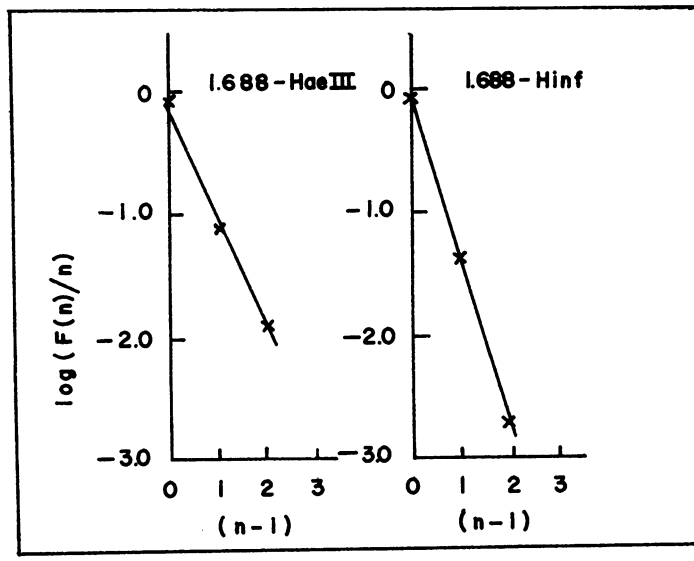


Figure 11. Plots of log (F(n)/n) vs. (n-1) for 1.688-Hae III and 1.688-Hinf.

From Fig. 11, we can calculate the P for Hae III from the intercept and slope. Its value is 0.10 from the intercept and 0.12 from the slope. The probability of base change per base pair is thus 0.026 from the intercept and 0.030 from the slope. For the Hinf sites, P is calculated to be 0.054 and 0.050 and p is 0.014 and 0.013, respectively.

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