A PARTIAL MAP OF THE CIRCULAR MITOCHONDRIAL GENOME OF *DROSOPHILA MELANOGASTER*

Location of *Eco*RI-Sensitive Sites and the Adenine-Thymine-Rich Region

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ABSTRACT

The mitochondrial genome of *Drosphila melanogaster* is a circular DNA molecule of mol wt 12.35 × 10^6 daltons. A single region accounting for approx. 25% of this molecule can be reproducibly differentially denatured presumably because it is rich in adenine and thymine. We have mapped on the circular mitochondrial genome of *D. melanogaster* the relative positions of this adenine-thymine (A-T) rich region and the sites sensitive to cleavage by the restriction endonuclease *Eco*RI, using agarose gel electrophoresis and electron microscopy. Digestion of mitochondrial DNA (mtDNA) molecules to completion with *Eco*RI resulted in the production of four fragments, *A, B, C,* and *D* which represent (±SD) 58.9 ± 1.1%, 27.5 ± 0.8%, 8.9 ± 0.5%, and 4.5 ± 0.3%, of the circular genome length, respectively. Fragments produced by *Eco*RI digestion and circularized by incubation at 2°C also fell into four distinct length groups with means (±SD) of 59.1 ± 0.5%, 27.5 ± 0.5%, 9.2 ± 0.3%, and 4.6 ± 0.2% of the circular genome length. From a consideration of the lengths of fragments resulting from incomplete *Eco*RI digestion, it was determined that the arrangement of the fragments in the circular genome was *A–C–B–D*. By electron microscope examination of partially denatured *Eco*RI fragments, the A-T-rich region was shown to be located in the *A* fragment closer to one end than to the other. By similar partial-denaturation studies of fragments resulting from incomplete *Eco*RI digestion, it was determined that, in the circular genome, of the two *Eco*RI sites which define the limits of the *A* fragment, the site between the *A* and *D* fragment lies nearest to the A-T-rich region.

Mitochondrial DNA (mtDNA) of *Drosophila melanogaster* is in the form of circular molecules with a similar contour length of about 6.2 μm (mol wt = 12.35 × 10^6 daltons) (3, 9, 10, 23, 24, 32). The findings of triphasic (24) and biphasic (3) hyperchromic changes in this DNA upon heating indicated that a portion of it contained a distinctly higher content of adenine-thymine than the remainder. This finding was extended by the demonstration, using the electron microscope denaturation mapping technique of Inman (16, 17), that a single region of each *D. melanogaster* mtDNA molecule, representing approx. 25% of the circular contour length, could be

denatured at a temperature at which no more than 6% of the remainder of the molecule denatured (9, 10, 23). As it is assumed that the DNA in this region is very rich in adenine-thymine (23), it is referred to as the A-T rich region (9, 10).

In agreement with findings for other metazoan mtDNAs (see references 1 and 25), data concerning *D. melanogaster* mtDNA obtained to date, including that presented in the present report, is consistent with the view that the mitochondrial genome contains only a single type of circular molecule.

In the present paper, we report the results of a study designed to map the relative positions on the circular mitochondrial genome of *D. melanogaster*, of the A-T-rich region, and the sites sensitive to cleavage by the restriction endonuclease *Eco*RI (29, 36). This enzyme cleaves double-stranded DNA at the position of the arrows in the specific sequence:

and thus generates fragments with complementary single-stranded ends. It has been shown to cut mtDNA molecules from a number of vertebrates (2, 6, 27) and from yeast (22) into a small number of fragments. The number and size of fragments are uniform for a species but vary between species.

MATERIALS AND METHODS

The *Drosphila melanogaster* strain used in these experiments was Oregon R-Utah (Oakridge, Tennessee) originally obtained by us from Dr. E. W. Hanly, University of Utah, Salt Lake City, Utah. The *D. melanogaster* stock was grown on standard cornmeal medium (30) with live yeast in 6-ounce glass urine specimen jars.

Preparation of mtDNA from Eggs

Approx. 5 days after emergence of the first flies in a group of bottles, about 5,000 flies from the group were placed into each of two, 29.5 × 15 × 16-inch lucite cages, and fed for 4 days on daily changes of live yeast on cornmeal food contained in 5.75 × 2.75 × 1.25-inch lucite trays. Egg collections were made at 24 h intervals from similar food trays. Eggs were washed once in Drosophila Ringer's solution (8), dechorionated by shaking in 3% sodium hypochlorite (4), washed in Ringer's solution (32), and finally suspended in 6 vol of 0.3 M sucrose, 1 mM disodium ethylenediaminetetraacetate (EDTA), and 0.1 M Tris HCl (pH 7.4) at 4°C. Mitochondria were then isolated as described previously (34) except that mitochondria were pelleted by centrifugation at 15,000 *g*. At the end of the procedure, a portion of the final mitochondrial pellet of some preparations was prepared for thin sectioning for examination in the electron microscope, and the remainder was frozen in 0.15 M sodium chloride, 0.1 M EDTA, and 0.05 M sodium phosphate (pH 8.5). Frozen mitochondria resulting from 5 days of egg collection were thawed and pooled, and 20% sodium dodecyl sulfate (SDS) was added to 2%. After 30 min at room temperature (25°C), solid cesium chloride was added to give a refractive index of 1.400. Preparative cesium chloride equilibrium centrifugation was then performed, and the resulting gradients were fractionated. Those fractions expected from refractive index analysis to contain DNA within the buoyant density range 1.660–1.700 g/cm^3 were pooled. The preparative cesium chloride equilibrium centrifugation was then repeated. A portion of the DNA in the pooled fractions resulting from the second centrifugation was subjected to analytical cesium chloride equilibrium centrifugation, a second portion was examined by electron microscopy, and, from the remainder, covalently closed circular molecules and open circular molecules were separated by centrifugation to equilibrium in cesium chloride-ethidium bromide gradients.

Standard DNAs

Bacteriophage fdRF DNA was a gift of Professor Heinz Schalle (Department of Microbiology, University of Heidelberg, West Germany). A preparation of open circular (component II) rat liver mtDNA was a gift of David L. Fouts (Department of Biology, University of Utah). *Eco*RI restriction fragments of bacteriophage lambda DNA, *HindIII* restriction fragments of SV40 virus DNA, and a *HindIII* restriction fragment of PLM-21 plasmid DNA were gifts of Dr. Herbert W. Boyer (Department of Microbiology, University of California, San Francisco, Calif.).

Equilibrium Density Gradient Centrifugations

Details concerning preparative neutral cesium chloride equilibrium centrifugation, cesium chloride-ethidium bromide equilibrium centrifugation, fractionation of the resulting gradients, removal of cesium chloride and ethidium bromide using Dowex, neutral analytic cesium chloride equilibrium centrifugation were described in reference 12.

EcoRI Restriction Endonuclease Digestion

*Eco*RI (activity: 0.01 μl made a single double-stranded cleavage in 50% of 1 μg of SV40 circular DNA molecules contained in 20 μl of reaction buffer at 37°C, in 5 min) was a gift of Drs. Richard Negeher and Herbert W. Boyer (Department of Microbiology, University of California, San Francisco, Calif.). Complete cleavage of *D. melanogaster* mtDNA was accomplished by adding 1 μl of enzyme for each 20 μl of a solution containing

DNA (10–20 μg/ml), 100 mM Tris HCl pH 7.5, 50 mM of sodium chloride, and 5 mM of magnesium chloride and incubating the mixture at 37°C for 30 min (14). When the digests were to be subjected to agarose gel analysis, the reaction was stopped by adding 0.25 vol of a solution containing 5% SDS, 25% glycerol, and 0.025% bromphenol blue. In all other cases, the reaction was stopped by adding 0.1 vol of 200 mM EDTA and heating for 5 min at 63°C. All experiments were controlled by carrying out parallel incubations in which the enzyme was omitted. Incomplete digestion (31) of samples of mtDNA was accomplished as follows: DNA (15 μg/ml in 100 mM Tris HCl pH 7.5 and 30 mM sodium chloride) was cooled to approx. 0°C in an ice bath. 1 μl of enzyme per 20 μl of DNA solution was added, and the mixture was quickly brought to 25°C. 0.1 vol of 50 mM magnesium chloride was added, and the reaction was allowed to continue for 0.5 min, 1 min, and 2 min. The reaction was then terminated by adding 0.1 vol of 200 mM EDTA, followed by heating at 63°C for 5 min. Circularization of fragments of mtDNA produced by *Eco*RI digestion (21) was accomplished by incubating *Eco*RI digests (at DNA concentrations less than 10 μg/ml) at 2°C for 48 h and preparing the DNA for electron microscopy by the aqueous protein monolayer technique in a cold room at 2°C. All solutions were equilibrated at 2°C before use.

Agarose Gel Electrophoresis

Electrophoresis of mtDNA digested with *Eco*RI was carried out (14) using 1.2% agarose (SeaKem, MCI Biomedical Division, Marine Coloids, Inc. Rockland, Maine) slab gels in a buffer containing 0.89 M Tris base, 0.025 M disodium EDTA, and 0.89 M boric acid. The gels were run at room temperature (about 25°C) at 150 V for about 2 h or until the bromphenol blue marker was near the bottom of the gel. The gel was then soaked in electrophoresis buffer containing 0.4 μg/ml of ethidium bromide for 15 min. The DNA bands in the gels were visualized by placing the gels on a short wave UV-transilluminator (Ultra-Violet Products, Inc., San Gabriel, California) and photographed through a Kodak Wratten no. 9 gelatin filter (Eastman Kodak Co., Rochester, N. Y.), using type 55 Polaroid film (Polaroid Corp., Cambridge, Mass.).

Partial Thermal Denaturation

Partial denaturation (16, 17) of open circular molecules and of fragments of mtDNA molecules produced by *Eco*RI digestion was accomplished by heating the DNAs in 0.05 M sodium phosphate (pH 7.8) and 10% formaldehyde at various temperatures for 10 min exactly as described previously (34).

Electron Microscopy

Thin sections of mitochondrial pellets for examination and evaluation in the electron microscope were prepared as described in reference 26. DNAs were prepared for electron microscopy by the aqueous protein monolayer technique of Freifelder and Kleinschmidt (13) and rotary shadowed with platinum and palladium as described previously (33). All grids were examined in a Siemens Elmiskop 101 electron microscope. Shadowed grids were photographed using projector pole piece one at an original magnification of 12,000. Measurements of molecules were made on positive prints using a graphics calculator (Numonics Corporation, North Wales, Pa.). All of the confidence limits given (±) are standard deviations, and the number of comparisons in each case is indicated by n.

Circular, double-stranded, replicative form (RF) DNA molecules of bacteriophage fd were added as internal standards to all DNA samples just before their preparation for electron microscopy. The lengths of molecules and different regions of molecules were converted to percentage lengths of the circular mitochondrial genomes. In order to carry out these conversions, the relative length of uncleaved, native circular *D. melanogaster* mtDNA molecules and fdRF molecules were determined from electron microscope protein monolayer preparations of mixtures of the two DNAs. The molecular weights of uncleaved circular mtDNA molecules and of fragments of mtDNA molecules produced by *Eco*RI digestion were also determined from their lengths relative to the lengths of fdRF DNA molecules, taking the mol wt of the latter to be 4.05×10^6 daltons (32). In order to determine the distribution of lengths of fragments resulting from complete and partial restriction enzyme digests (but not partial denaturation), overlapping photographs were taken of two, approx. 25 μm^2 areas of a grid square. All of the molecules in the areas, except those which ran off the edge, and those which were too tangled (no more than 5% of any sample), were measured. For determinations of the lengths of circularized *Eco*RI fragments, photographs of circular molecules were taken at random. From such a collection, circular molecules were measured until 30 of each apparent size class were obtained.

Determinations of the Relative Mass per Unit Length of Double-Stranded and Single-Stranded DNA in Partially Denatured Molecules

It is known that single-stranded DNA heated in the presence of formaldehyde has a greater mass per unit length than native double-stranded DNA (11, 18, 34). In order, therefore, to be able to compare the lengths of double-stranded and single-stranded regions of molecules heated in the presence of formaldehyde with those of molecules not so treated, we determined the effects of formaldehyde and heat on the mass per unit length (that is, on shrinkage) of double-stranded and single-stranded mtDNA when visualized by the aqueous protein mono-

layer technique. Lengths of mtDNA molecules were determined relative to the lengths of fdRF DNA molecules which in all cases were added no more than 1 min before the DNA protein monolayers were formed.

First, open circular mtDNAs from rat liver and from *D. melanogaster* embryos were prepared in 0.05 M sodium phosphate. 10% formaldehyde was added as for partial denaturation studies, but, instead of heating, the mixtures were left at room temperature (approx. 25°C) for 10 min before preparation for electron microscopy. The lengths of the rat and *D. melanogaster* mtDNAs relative to the length of fdRF DNA calculated from electron micrographs were 2.52 ± 0.06 (n = 30) and 3.04 ± 0.07 (n = 30), respectively. These values are in good agreement with the values of 2.53 ± 0.03 (n = 30) and 3.05 ± 0.05 (n = 30) calculated for these DNAs in the absence of formaldehyde.

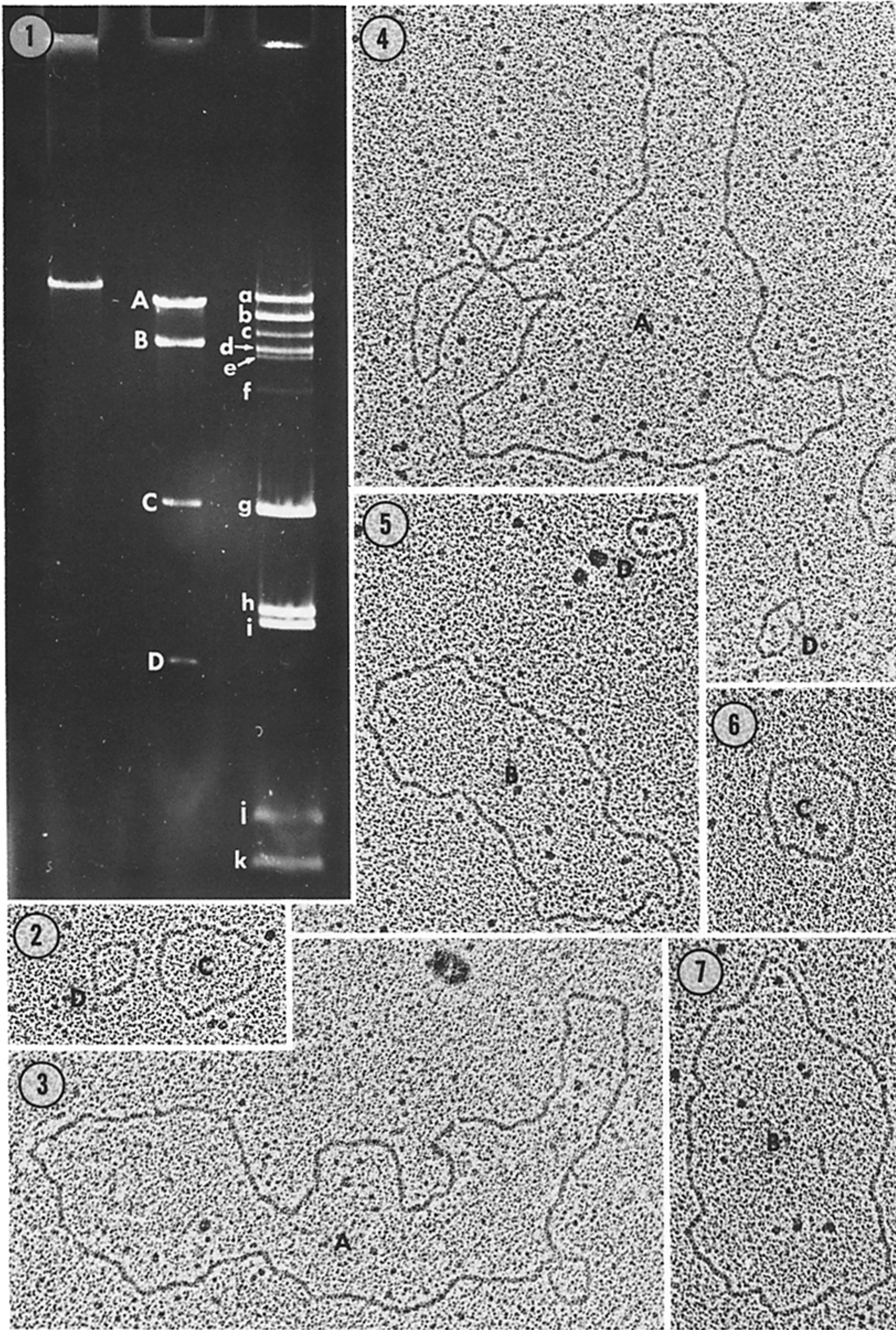
It has been shown (34) that when open circular rat liver mtDNA is exposed to the partial denaturation conditions used in the present study (that is, heated for 10 min in 0.05 M sodium phosphate and 10% formaldehyde) and examined in the electron microscope, regions of denaturation are not apparent until a temperature of at least 48°C is used. In order, therefore, to determine the effects of heating, at temperatures up to 41°C in the presence of formaldehyde, on the mass per unit length of DNA which appears in the electron microscope as double-stranded, we prepared open circular rat liver mtDNA in 0.05 M sodium phosphate and 10% formaldehyde and determined its length in relation to the length of fdRF DNA after the rat liver mtDNA had been left standing at 25°C for 10 min, and after heating at 37°C, 38°C, 39°C, 40°C, and 41°C for 10 min. The relative values obtained were 2.52 ± 0.06 (25°C), 2.52 ± 0.06 (37°C), 2.52 ± 0.05 (38°C), 2.50 ± 0.03 (39°C), 2.51 ± 0.05 (40°C), and 2.50 ± 0.06 (41°C), (n = 20 for each value), indicating that heating this DNA to at least 41°C (at which temperature each molecule appeared to be totally double-stranded) results in a change in the mass per unit length of less than 1%. In view of these results, we did not make any changes in the values obtained by comparison with the lengths of fdRF molecules, for double-stranded regions in mtDNA molecules partially denatured by heating at 40°C in the presence of formaldehyde.

We next performed a similar experiment using single-stranded *D. melanogaster* mtDNA (obtained by heating native open circular mtDNA in 75 mM sodium chloride and 7.5 mM sodium citrate (pH 8.0) at 97°C for 5 min followed by rapid cooling in an ice bath). In this case at 25°C and 40°C, the lengths of the single-stranded circular mtDNA molecules relative to the lengths of fdRF DNA molecules were 2.00 ± 0.07 (n = 30) for DNA left at 25°C for 10 min, and 1.98 ± 0.08 (n = 20) for DNA heated at 40°C for 10 min. These results indicate that the presence of formaldehyde rather than heating is primarily responsible for the greater mass per unit length (shrinkage) of single-stranded DNA. In view of the above findings, the length of single-stranded regions in *D. melanogaster* mtDNA molecules heated to 40°C, calculated by comparison with the lengths of fdRF DNA molecules found on the same grid square, were corrected by multiplying by 1.54 (the length of single-stranded circular *D. melanogaster* mtDNA molecules relative to the length of fdRF DNA molecules divided by the length of single-stranded mtDNA molecules heated at 40°C in the presence of formaldehyde relative to the length of fdRF DNA molecules). Application of this correction factor to partially denatured uncleaved open circular mtDNA molecules (Fig. 14) gave a total length of 98.4% of that expected for native circular molecules.

RESULTS

Examination of thin sections of mitochondrial pellets in the electron microscope confirmed that these preparations consisted mainly of mitochondria, although remnants of other cell organelles were present. Not a single bacterial profile or yeast cell profile was found among 2,000 profiles of mitochondria in each of two preparations. DNA obtained by preparative cesium chloride equilibrium density centrifugation from the mitochondrial pellets formed a single band at ρ = 1.681 ± 0.0007 g/cm^3 (n = 5) when subjected to analytical cesium chloride equilibrium buoyant density centrifugation. Electron microscope examination of protein monolayers of these preparations revealed that between 84% and 94% by length of the DNAs consisted of circular molecules of similar contour length. The remainder of the DNA in each preparation examined was in the form of linear molecules, the lengths of which were less than the contour lengths of the circular molecules. The molecular weight of the circular molecules for one preparation was determined to be 12.35 ± 0.15 × 10^6 daltons (n = 30). These findings are in agreement with the properties of *D. melanogaster* mtDNA reported previously (3, 23, 24, 32).

When these mtDNA preparations were centrifuged to equilibrium in cesium chloride-ethidium bromide gradients, two visible bands were formed. Examination of protein monolayer preparations revealed that at least 95% of the molecules in the lower band were circular, and of these at least 90% had the supercoiled configuration indicating them to be covalently closed (5, 7, 20). The upper band comprised nonsupercoiled (open) circular molecules (circular molecules presumably containing at least one broken phosphodiester bond) and linear molecules. Except where otherwise stated, DNA from the former (covalently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closed) fractions was used in all of the experiments to be described.

Number and Sizes of Fragments Produced by EcoRI Digestion

When mtDNA of *D. melanogaster* was subjected to agarose gel electrophoresis after digestion with the restriction endonuclease *Eco*RI, four bands were detected, *A, B, C,* and *D* (Fig. 1). By comparison with DNA fragments of known molecular weight co-electrophoresed in an adjacent slot, the molecular weights of molecules in the four bands were determined to be between 6.8 and 13.4×10^6 daltons for band *A*, approx. 3.6×10^6 daltons for band *B*, approx. 1.1×10^6 daltons for band *C*, and approx. 0.5×10^6 daltons for band *D*. A single band representing DNA molecules of apparent molecular weight less than 13.4×10^6 was found for mtDNA incubated in the absence of *Eco*RI (Fig. 1).

Electron microscopy of protein monolayer preparations of mtDNA molecules digested with *Eco*RI also revealed four size classes of fragments (Fig. 8*a*) the mean lengths of which represented $58.9 \pm 1.1\%$, $27.5 \pm 0.8\%$, $8.9 \pm 0.5\%$, and $4.5 \pm 0.3\%$ of the circular genome length. Circular mtDNA molecules remained intact when the enzyme was omitted from the incubation mixture. The four fragments resulting from *Eco*RI digestion were found in approximately equal number, and the sum of their mean lengths was equal to 99.8% of the genome length. This suggests that all mtDNA molecules were digested to completion and that these molecules are homogeneous in regard to the number and position of sites sensitive to cleavage by *Eco*RI which they contain.

Fragments of circular DNA molecules resulting from *Eco*RI digestion have complementary single-stranded ends (15, 21). When such fragments are incubated at low temperatures (2–6°C) at a DNA concentration of less than 10 μg/ml, circular molecules resulting primarily from base pairing between the ends of individual fragments are found. The circularity can be maintained for electron microscope visualization by preparing protein monolayers at the temperature of incubation (21). Measurements of the lengths of such molecules provide a means of making very precise determination of the lengths of fragments produced by *Eco*RI digestion, as all linear fragments resulting from breaks not produced by the restriction enzyme are eliminated. Consequently, mtDNA fragments resulting from *Eco*RI digestion were circularized by incubation at 2°C for 48 h, and protein monolayers were prepared for electron microscopy at the same temperature. The lengths of circular molecules measured in these preparations are given in Fig. 8*b* and *c*. Again, four size classes were found (Figs. 2–7 and 8*c*) which were determined (Fig. 8*b*) to have mean lengths equivalent to $59.1 \pm 0.5\%$, $27.5 \pm 0.5\%$, $9.2 \pm 0.3\%$, and $4.6 \pm 0.2\%$ (sum = 100.4%) of the circular genome length. The lengths of the fragments (Fig. 8*b*) *A, B, C,* and *D* are equivalent to molecular weights in daltons of $7.30 \pm 0.6 \times 10^6$, $3.40 \pm 0.06 \times 10^6$, $1.14 \pm 0.04 \times 10^6$, and $0.57 \pm 0.03 \times 10^6$, respectively. Two circular molecules were included in the collections shown in Fig. 8*b* and *c* with contour lengths suggesting that they resulted from circularization of joined *B* + *C* fragments (Fig. 8*b*) and joined *C* + *D* fragments (Fig. 8*c*).

FIGURE 1 Fluoresence photograph showing the distribution of ethidium bromide-stained DNA bands in a 1.2% agarose slab gel after electrophoresis. The center slot contained mtDNA of *D. melanogaster* which had been incubated with *Eco*RI at 37°C for 30 min. Four bands, *A*, *B*, *C*, and *D*, are visible. The slot to the left contained *D. melanogaster* mtDNA which had been similarly incubated but with *Eco*RI omitted. A single band is visible. The slot to the right contained a mixture of bacteriophage lambda DNA fragments (with mol wt in daltons of 13.4×10^6 [*a*], 4.7×10^6 [*c*], 3.5 and 3.7×10^6 which appear as a single band [*d*], 3.1×10^6 [*e*], and 2.0×10^6 [*f*]) produced by *Eco*RI digestion; a fragment of PML-21 plasmid DNA (mol wt = 6.8×10^6 daltons [*b*]) produced by *HindIII* digestion; and SV40 DNA fragments (with mol wt in daltons of 1.1×10^6 [*g*], 0.72×10^6 [*h*], 0.67×10^6 [*i*], 0.34×10^6 [*j*], and 0.27×10^6 [*k*]) produced by *HindIII* digestion. Migration was from top to bottom.

FIGURES 2–7 Electron micrographs of rotary shadowed circularized fragments of *D. melanogaster* mtDNA molecules produced by *Eco*RI digestion for 30 min at 37°C followed by incubation for 48 h at 2°C and protein monolayer preparation at 2°C. Representatives of the four size classes of fragments, *A*, *B*, *C*, and *D* with mean lengths of 59.1%, 27.5%, 9.2%, and 4.6% of the contour length of uncleaved circular mtDNA molecules are shown. All micrographs, × 86,000.

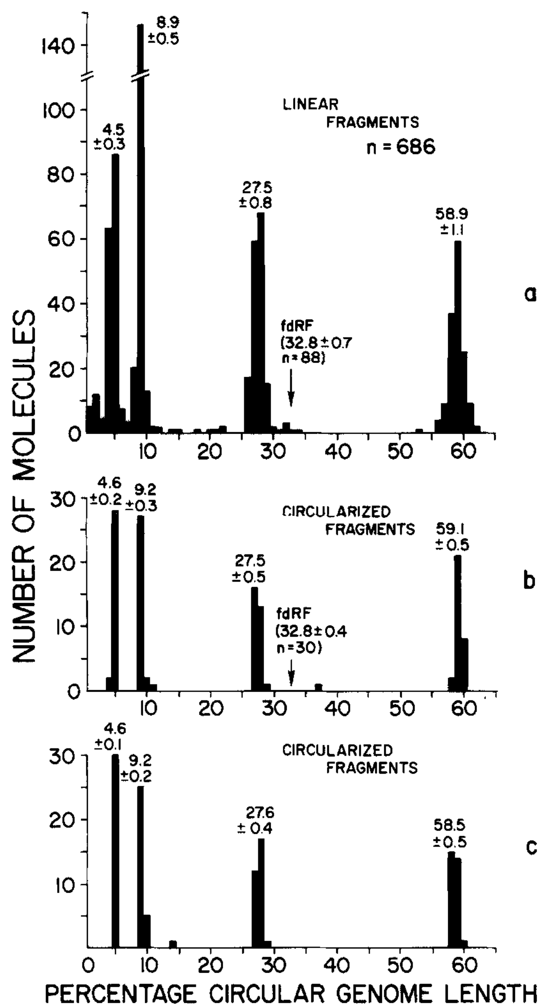


FIGURE 8 Frequency distribution of the lengths of linear and circularized (by incubation for 48 h at 2°C and protein monolayer preparation at 2°C) fragments of *D. melanogaster* mtDNA molecules resulting from digestion for 30 min at 37°C with *Eco*RI. The lengths are expressed as percentage lengths of the uncleaved circular mitochondrial genome. The mean and standard deviation of each size group is given. The lengths of the mtDNA fragments represented in (*a*) and (*b*) were determined by comparison with the lengths of double-stranded circular RF DNA molecules of bacteriophage fd contained as standards in the same preparations. The circularized fragments represented in (*c*) were from a preparation from which fdRF DNA molecules were omitted. The lengths of these molecules were determined by standardizing the mean length of the molecules in the third largest size group with the mean length of the molecules in the third largest size group in (*b*). The linear fragments (and the fdRF circular DNA molecules) in (*a*), represent all of the molecules found within two 25 μm^2 sections of a grid square. The distributions shown in (*b*) and (*c*) were obtained by measuring molecules at random from a collection of photographs of circular molecules until 30 molecules of each of the four size classes were obtained.

Relative Positions of the EcoRI-Sensitive Sites in the Circular Mitochondrial Genome

If genetic polarity is ignored, there are three possible arrangements for the four fragments *A*, *B*, *C*, and *D* in the circular mitochondrial genome; they are *A-B-C-D*, *A-B-D-C*, and *A-C-B-D*. In preparations in which digestion is arrested before all of the *Eco*RI-sensitive sites are cleaved, molecules can be expected which comprise various combinations of two, three, and four fragments, that is, molecules which contain one, two, or three uncleaved *Eco*RI-sensitive sites, respectively. Neither molecules comprising all four fragments nor molecules each comprising three of the four fragments can provide information concerning the arrangement of fragments in the circular genome. In the latter case, regardless of the arrangement of fragments in the genome, the same four classes representing the combined lengths of $A + B + C$, $B + C + D$, $C + D + A$, and $D + A + B$ would be expected. However, in contrast, molecules each comprising two fragments could provide information concerning the arrangement of the fragments in the circular genome because, of the six random, pair-wise combinations for the four fragments, only four are possible as the products of incomplete cleavage for any given fragment arrangement (Table I). In view of this, and the expectation that the sizes of the various possible pair-wise combinations of the four *Eco*RI fragments of *D. melanogaster* mtDNA would be distinct, we studied the products of incomplete *Eco*RI digestion of this DNA.

Mitochondrial DNA was incubated with *Eco*RI under conditions designed to leave various fractions of the enzyme-sensitive sites uncleaved. A digest preparation which, upon preliminary examination, appeared to consist mainly of fragments resulting from complete cleavage but in which some circular molecules were still apparent was chosen for study. The frequency distribution of the lengths of the fragments present in this preparation is shown in Fig. 9. Size groups representing the single fragments *A*, *B*, *C*, and *D* are clearly present. Four other size groups with mean

TABLE I

Determination of the Arrangement of the EcoRI Fragments A, B, C, and D in the D. melanogaster Circular Mitochondrial Genome from a Consideration of Certain Observed and Expected Length Classes of Molecules Which Resulted When mtDNA was Incompletely Digested

Possible pairwise combinations of fragments	Mean length as percentage length of uncleaved circular molecule		Expected presence (+) or absence (−) if order is:		
	Expected	Observed	A-B-C-D	A-B-D-C	A-C-B-D
A-B	86.6	not apparent	+	+	−
A-C	68.3	67.7 ± 1.0	−	+	+
A-D	63.7	63.5 ± 1.0	+	−	+
B-C	36.7	36.8 ± 0.8	+	−	+
B-D	32.1	32.2 ± 0.6	−	+	+
C-D	13.8	not apparent	+	+	−

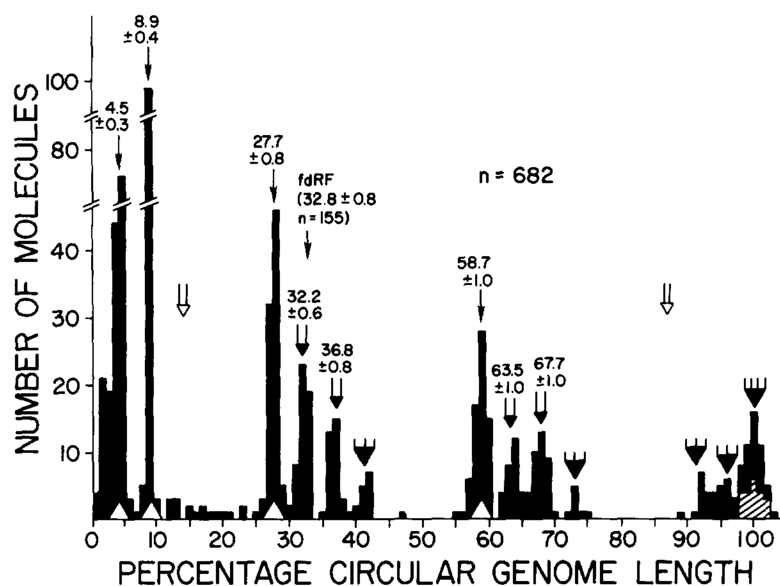
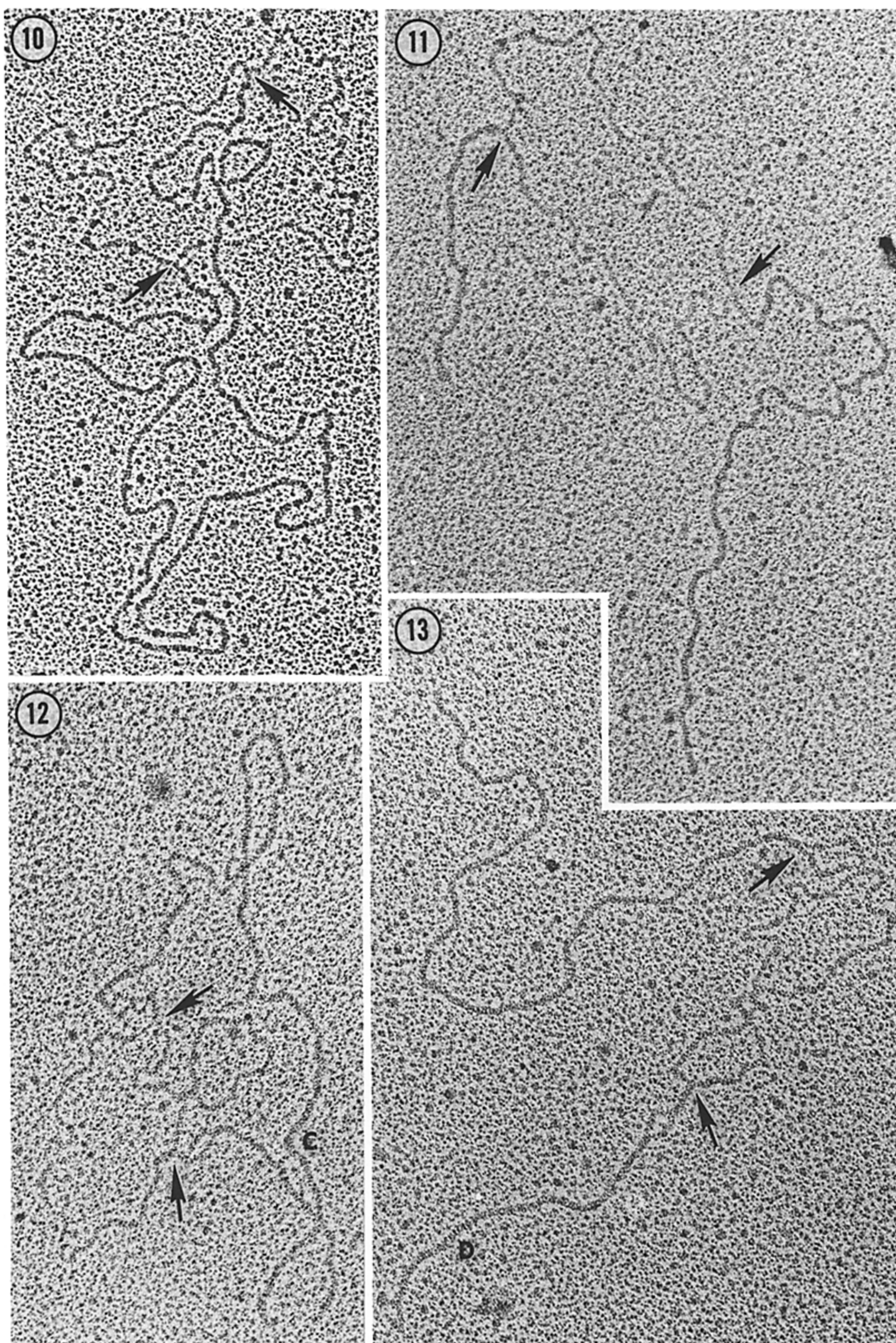


FIGURE 9 Frequency distribution of the lengths of linear fragments (solid blocks) and uncleaved circular molecules (cross-hatched blocks) of *D. melanogaster* mtDNA molecules resulting from incomplete digestion with *Eco*RI. The lengths, which are expressed as percentage lengths of the uncleaved circular mitochondrial genome, were determined by comparison with the lengths of double-stranded circular RF DNA of bacteriophage fd contained as a standard in the same preparation. The linear fragments and uncleaved circular mtDNA molecules (and fdRF circular DNA molecules) represent all of the molecules found within two 25 μm^2 sections of a grid square. The points of the open triangles on the abscissa indicate the mean positions expected for fragments which do not contain uncleaved *Eco*RI sites (that is, for the fragments represented in Fig. 8). The means and standard deviations of the size groups of molecules corresponding to these latter values are indicated by solid arrowheads with a single shaft. The means and standard deviations of size groups of molecules corresponding to the expected values (see Table I) for four of the different classes of molecules containing a single uncleaved *Eco*RI site (that is comprising the fragment combinations *A-C, A-D, B-C,* and *B-D*) are indicated by solid arrowheads with two shafts. The two open arrowheads, each with two shafts, indicate the expected mean positions for molecules comprising the fragment combinations *C-D* and *A-B* (see Table I). The four solid arrowheads, each with three shafts, indicate the mean positions expected for the four possible classes of molecules each containing two uncleaved *Eco*RI sites (that is, combinations of three of the fragments *A, B, C,* and *D*). The solid arrowheads with four shafts indicate the mean position expected for whole circular molecules which have been cleaved at a single site. The mean length and standard deviation of the 21 uncleaved circular molecules (cross-hatched areas) was 99.84 ± 1.36% calculated as were the linear fragments against the internal standard fdRF DNA.


10
11
12
13
C
D

values very similar to the expected lengths for the combinations *A-C*, *A-D*, *B-C*, and *B-D* were also apparent. However, groups of molecules with the mean lengths expected for the combination *A-B* and *C-D* were not apparent. A consideration of the observed length classes, in regard to those expected for the three possible arrangements of the four fragments (Table I), clearly indicates that the arrangements of fragments in the circular molecule is *A-C-B-D*. Four size groups of molecules with mean lengths corresponding approximately to the expected lengths of molecules each comprising three fragments were found (Fig. 9). Fragments were also found with lengths expected for mtDNA molecules in which a single *Eco*RI site had been cleaved (Fig. 9).

Positions of the EcoRI Sites Relative to the A-T-Rich region in the Circular Mitochondrial Genome

Extending a finding reported originally by Peacock et al. (23), we demonstrated by electron microscopy that when *D. melanogaster* mtDNA molecules are heated to 40°C in 0.05 M sodium phosphate and 10% formaldehyde a single region (the A-T-rich region) of each molecule accounting for approx. 25% of the contour length denatures while the rest of the molecule except for various small regions accounting for approx. 6% of the molecular contour length remained undenatured (9, 10). A similar region of denaturation is also apparent when the mtDNA molecules are heated at temperatures of 37°C, 38°C, 39°C, and 41°C. However, at the lower temperatures, small sections of this region remain undenatured, and at 41°C approx. 10% of the remainder of each molecule also denatures.

In order to determine the positions of the *Eco*RI-sensitive sites relative to the A-T-rich region in the *D. melanogaster* mitochondrial genome, we have constructed and compared denaturation maps of fragments resulting from *Eco*RI digestion, and of uncleaved circular molecules. Open circular mtDNA molecules obtained from a cesium chloride-ethidium bromide density gradient, and fragments of mtDNA molecules produced by complete *Eco*RI digestion of covalently closed mtDNA molecules were heated together, but in separate containers, to 40°C in 0.05 M sodium phosphate and 10% formaldehyde for 10 min, and protein monolayers of each sample were prepared for electron microscopy. The first 30 uncleaved circular molecules to be observed on a grid square were photographed. Denaturation maps of these molecules (Fig. 10) were constructed and are shown in Fig. 14. In agreement with our previous findings (9, 10), each circular molecule contained a region of denaturation (the A-T-rich region) with a mean length equal to 24.8 ± 1.6% of the contour length. A similar region bounded on either side by segments of mainly double-stranded DNA was found in some of the restriction fragments (Fig. 11). The two double-

FIGURES 10–13 Electron micrographs of rotary shadowed molecules of *D. melanogaster* mtDNA heated for 10 min at 40°C in 0.05 M sodium phosphate and 10% formaldehyde. The arrows indicate the limits of the large denatured region (the A-T-rich region) in each molecule. All micrographs, × 86,000.

FIGURE 10 An uncleaved circular molecule.

FIGURE 11 An *A* fragment resulting from complete digestion of *D. melanogaster* mtDNA with *Eco*RI. The mainly undenatured segments lying on either side of the A-T-rich region are equivalent in length to approx. 27% and 8% of the contour length of uncleaved circular genomes.

FIGURES 12 and 13 Fragments resulting from incomplete digestion of *D. melanogaster* mtDNA with *Eco*RI.

FIGURE 12 The length of the lower, mainly double-stranded segment of this fragment is within the range of lengths found for the shorter double-stranded segment of the *A* fragment. The length of the upper, mainly double-stranded segment (*C*) is approximately equal to the length expected for the sum of the lengths of the longer double-stranded segment of the *A* fragment and the total length of the *C* fragment.

FIGURE 13 The length of the upper, mainly double-stranded segment of this fragment is within the range of lengths found for the longer double-stranded segment of the *A* fragment. The lower, mainly double-stranded segment (*D*) is approximately equal to the length expected for the sum of the length of the shorter double-stranded segment of the *A* fragment and the total length of the *D* fragment.

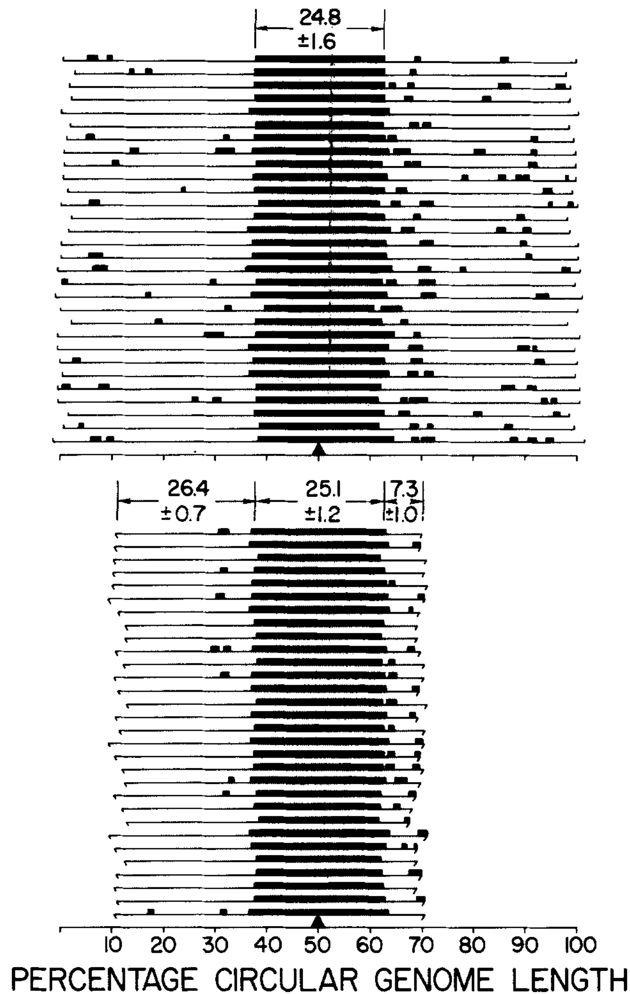


FIGURE 14 Denaturation maps of 30 uncleaved circular molecules (above), and 30 *A* fragments resulting from complete *Eco*RI digestion (below) of mtDNA of *D. melanogaster*, produced by heating at 40°C for 10 min in 0.05 M sodium phosphate and 10% formaldehyde. By comparison with bacteriophage fdRF DNA molecules contained in each preparation, the denatured (thick lines) and undenatured (thin lines) regions of each molecule were converted to percentage lengths of the circular mitochondrial genome. This included correction for the greater mass per unit length of single-stranded DNA under the conditions employed. In both circular molecules and *Eco*RI *A* fragments, the midpoint of the longest denatured region (the A-T-rich region) was taken as the common point by which the molecules were aligned (points of solid triangles on the abscissa). For the circular molecules, direction was defined by placing to the right the segment which contained the region of denaturation lying nearest to the main region of denaturation. For the *Eco*RI *A* fragments, direction was defined by placing the largest mainly undenatured segment to the left. The number above the set of circular molecules is the mean length (as percentage circular genome length) of the A-T-rich region. The numbers above the sets of *Eco*RI *A* fragments are the mean lengths (as percentage circular genome length) of the A-T-rich region and of the mainly double-stranded segments of the molecule on either side of this region.

stranded segments of each such fragment were always unequal in length. The first 30 of these fragments to be observed were photographed and denaturation maps constructed (Fig. 14). They had a mean total length equal to 58.8 ± 1.8% of the circular genome length, indicating them to be the *A* fragment. The length of the A-T-rich region was equal to 25.1 ± 1.2% of the circular genome length, which compares well with the value found for this region in uncleaved circular molecules. The lengths of the double-stranded segments found on either side of the A-T-rich region were equal to 26.4 ± 0.7% and 7.3 ± 1.0% of the circular genome length.

Evidence has been presented above that in the circular genome the *A* fragment lies between the *C* and *D* fragments. We next conducted an experiment to determine the orientation of the *A* fragment in regard to the asymmetrical location of the A-T-rich region, and the *Eco*RI sites defining the limits of the *A* and *C* fragments and the *A* and *D* fragments. An incomplete *Eco*RI digest of *D. melanogaster* mtDNA, similar to the one from which the data in Fig. 9 were obtained, was heated to 40°C in 0.05 M sodium phosphate and 10% formaldehyde for 10 min and prepared for electron microscopy. 60 linear molecules containing the large region of denaturation were photographed and measured. The results are presented in Fig. 15. The mean length of the A-T-rich region (25.9%) was close to the mean length found for the region in previous experiments. The mean lengths of both of the double-stranded segments of 26 of the molecules indicated that they represented the *A* fragment alone (Fig. 15*a*).

The length of one of the double-stranded segments (mean = 27.6%) of each of 14 of the molecules was similar to the length (26.7%) of the longer double-stranded segment of the *A* fragment, but the length (mean = 13.9%) of the other double-stranded segment of each of 13 of these molecules was approximately that expected (13.5%) for the sum of the lengths of the shorter double-stranded segment of the *A* fragment and the whole of the *D* fragment (Figs. 15*b* and 13). The length of the other double-stranded segment (40.3%) of the remaining molecule of this class was approximately that expected (41.1%) for the sum of the lengths of the shorter double-stranded segment of the *A* fragment and the whole of the *D* and *B* fragments.

The length of one of the mainly double-stranded segments (mean = 9.1%) of each of 16 of the molecules was similar to the length (8.9%) of the shorter double-stranded segment of the *A* fragment, but the length (mean = 36.4%) of the other double-stranded segment of each of 14 of these molecules was approximately that expected (35.9%) for the sum of the lengths of the longer double-stranded segment of the *A* fragment and the whole of the *C* fragment (Figs. 15*c* and 12). The length of the other double-stranded segment (mean = 64.3%) of each of the two remaining molecules of this class was approximately that expected for the sum of the lengths (63.4%) of the longer double-stranded segment of the *A* fragment and the whole of the *C* and *B* fragments.

In each of four of the molecules measured, the length (mean = 13.8%) of the shorter double-stranded segment was approximately that expected (13.5%) for the sum of the lengths of the shorter double-stranded segment of the *A* fragment and the whole of the *D* fragment, and the length (mean = 36.3%) of the longer double-stranded segment was approximately that expected (35.9%) for the sum of the lengths of the longer double-stranded segment of the *A* fragment and the whole of the *C* fragment (Fig. 15*d*). These findings indicate that, in the circular genome, of the two *Eco*RI sites which define the limits of the *A* fragment, the site between the *A* and *D* fragments lies nearest to the A-T-rich region.

DISCUSSION

From the data presented, it appears that circular mtDNA molecules of *D. melanogaster*, like circular mtDNA of a number of vertebrates (2, 27), are homogeneous in regard to the number and position of the sites they contain which are sensitive to cleavage by the *Eco*RI restriction enzyme. Each molecule of *D. melanogaster* mtDNA appears to contain four such sites. However, as fragments consisting of less than approx. 200 nucleotides (about 700 Å in length) would not have been detected by the methods employed, it is not ruled out that one or more *Eco*RI-sensitive sites lying very close to those detected are also present in the circular genome. A map of the circular *D. melanogaster* mitochondrial genome showing the relative positions of the *Eco*RI-sensitive sites and the A-T-rich region, derived from the results of this study is presented in Fig. 16.

Brown and Vinograd (2) and Robberson et al. (27) mapped the origin of replication relative to *Eco*RI and *Hin*dIII cleavage sites on human, mon-

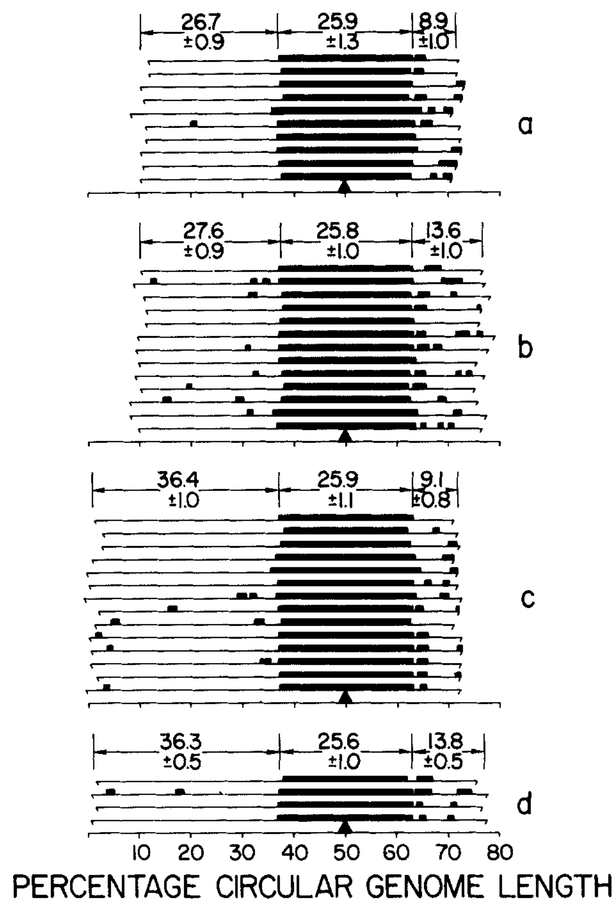


FIGURE 15 Denaturation maps of fragments of mtDNA molecules of *D. melanogaster* resulting from incomplete cleavage by *Eco*RI followed by heating at 40°C for 10 min in 0.05 M sodium phosphate and 10% formaldehyde. All molecules are from the same protein monolayer preparation. By comparison with bacteriophage fdRF DNA molecules contained in the same preparation, the denatured (thick line) and undenatured (thin line) regions of each molecule were converted to percentage lengths of the circular mitochondrial genome. This included correction for the greater mass per unit length of single-stranded DNA under the conditions employed. The molecules have been arranged in length groups which correspond to: the length of the restriction fragment *A* (the 10 molecules shown were chosen at random from the 26 molecules of this size class found in the sample measured) (*a*); the combined lengths of fragments *A* and *D* (*b*); the combined lengths of fragments *C* and *A* (*c*); and the combined lengths of fragments *C*, *A*, and *D* (*d*). For all molecules, the midpoint of the largest denatured region (the A-T-rich region) was taken as the common point by which the molecules were aligned (points of solid triangles on the abscissae), and direction was defined by placing the largest mainly undenatured segment to the left. The numbers above each set of molecules are the means (as percentage circular genome length) of the A-T-rich region and of the mainly double-stranded segments of the molecules on either side of this region.

key, and mouse mitochondrial genomes, from a study of the positions on fragments resulting from digestion with the restriction enzymes of D loops which represent the first step in replication, (19) and of the two forks of expanded D loops which represent various degrees of completion of replication (28, 35). Up to the present time, we have not been able to determine the origin of replication in *D. melanogaster* mtDNA molecules. Employing a variety of techniques including isolation of mtDNA in solutions of ionic strength greater than 0.35 M, glyoxal fixation (2), and formalde-

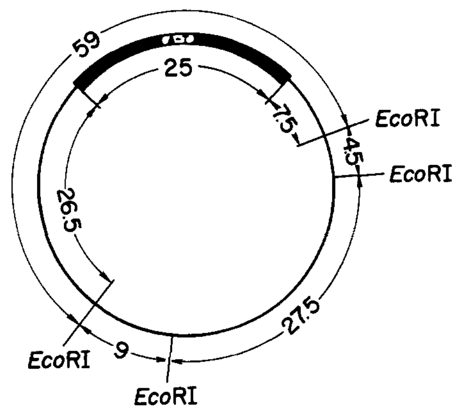


FIGURE 16 A map of the *D. melanogaster* circular mitochondrial genome showing the relative positions of the *Eco*RI-sensitive sites and the A-T-rich region, expressed as percentages of the circular genome length, derived from the results presented. The values for the distances between *Eco*RI sites are taken from Fig. 8*b*. The values for the size of the A-T-rich region and the segments on either side of this region in the *A* fragment are taken from Fig. 14.

hyde fixation, we have failed to detect, with any degree of confidence, D loops in *D. melanogaster* mtDNA molecules. Also, expanded D loops and totally double-stranded replicating forms (35) of a size suitable for determining the position of the origin of replication relative to either *Eco*RI cleavage sites or to the A-T-rich region were present in our *D. melanogaster* mtDNA preparations in too low a frequency to make attempts at such determinations practical (Wolstenholme, D. R. and C. M.-R. Fauron, unpulished observations).

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