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Genetics

Structural heterogeneity of mitochondrial DNA molecules within the genus *Drosophila*

(electron microscopy/molecular weights/denaturation mapping/adenine+thymine-rich DNA)

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ABSTRACT **We have determined by electron microscopy the molecular weights of circular mitochondrial DNA (mtDNA) molecules from 39 species representing 13 groups of five subgenera of the genus *Drosophila*. mtDNA molecules of all species examined, other than members of the melanogaster group, had, with one exception, molecular weights in the rather narrow range 9.90×10^6 to 10.34×10^6. The one exception was *D. robusta*, which had a molecular weight of 10.61×10^6. In contrast, mtDNA molecules from species within the melanogaster group had molecular weights covering the considerably greater range 9.92×10^6 to 12.35×10^6. Applying the electron microscope denaturation mapping technique of Inman to mtDNA molecules of eight species of the melanogaster group, we found each of them to contain a region [presumably rich in adenine and thymine (A+T)] which denatured at a specific temperature (40°) at which most of the remainder of the molecule remained undenatured. The size of the A+T-rich region was constant for mtDNA molecules of a species, but varied from 0.62×10^6 to 3.41×10^6 for mtDNA molecules of different species. It was demonstrated that the differences in molecular weights of the A+T-rich regions can almost completely account for the differences in total molecular weights of the mtDNA molecules from species of the melanogaster group.**

Mitochondrial DNA (mtDNA) of *Drosophila melanogaster* is in the form of circular molecules with contour lengths of about 6.2 μm (molecular weight = 12.35×10^6) (1–4). The findings of triphasic (1, 4) and biphasic (2) hyperchromic changes in this DNA upon heating indicated that a portion of it contained a distinctly higher content of adenine and thymine than the remainder. Using electron microscope denaturation mapping (5, 6), Peacock *et al.* (4) demonstrated that much of this adenine+thymine-rich DNA was confined to a single region of the molecule.

We have examined the mtDNA of a variety of selected species of the genus *Drosophila*, both in regard to total molecular weight and to the presence and size of adenine+thymine-rich regions. Our results are the subject of this report.

MATERIAL AND METHODS

The following *Drosophila* species (stock number and place of origin) were obtained from Mrs. Marietta A. Reveley, Species Stock Collection of the Genetics Foundation of University of Texas at Austin: *D. busckii* (2181.5; Willows Creek, Calif.); *D. duncani* (2311.9; Lake Pierce, Fla.); *D. lebanonensis casteeli* (1865.3; Utah); *D. virilis* (2375.8; Chile); *D. montana* (1218.1D; Cottonwood Canyon, Utah); *D. americana* (2515.8; Neb.); *D. robusta* (2069.3; Neb.); *D. hydei* (1385.11; Vera Cruz, Mexico); *D. neohydei* (H207.26; Carpentaro, Venezuela); *D. funebris* (2093.18; Lebanon); *D. tripuntata* (2003.3; New Orleans, La.); *D. pseudoobscura* (A118.1; Tucson, Ariz.); *D. willistoni* (2268.2; Mexico City); *D. saltans* (H180.4, San Jose, Costa Rica); *D. ananassae*-C (2389.1; Cuba); *D. bipectinata* (3007.3; New Guinea); *D. elegans* (3140.2; Baguio City, Luzon, Philippines); *D. eugracilis* (3056.5E; Palawan, Philippines); *D. ficusphila* (3075.8; Khan-in Long, Taiwan); *D. denticulata* (3021.9; Popendetta, New Guinea); *D. birchii* (3007.1; Cairns, Australia); *D. kikkawai* (H436.64; Colombia); *D. auraria* (3040.15; Nopporo, Japan); *D. takahashii* (3075.4; Wulai, Taiwan); *D. lutea* (3040.12; Sugadaira, Japan); *D. suzukii* (3040.11; Kirishima, Japan); *D. lucipennis* (3068.3; Chi-tou, Taiwan); *D. melanogaster*-H (2370.7; Oahu, Hawaii); *D. simulans*-H (2370-1; Hawaii); *D. simulans*-P (2395.5; Pisco, Peru); *D. yakuba*-IC (2371.6; Ivory Coast). Dr. L. Tsacas, Centre National de la Recherche Scientifique, Gif-sur-Yvette, France, provided *D. yakuba*-Ca (115; Cameroon); *D. teissieri* (128.2; Rhodesia); *D. erecta* (154.1; Ivory Coast); *D. mauritiana* (Mauritius). Dr. Hampton L. Carson, University of Hawaii, provided *D. gymnobasis* (M64G2); *D. grimshawi* (G1); *D. silvarentis* (K18A1); *D. hawaiensis* (J14B8); *D. balioptera* (G34B1). Dr. P. M. M. Rae, Yale University, provided *D. ananassae*-Cr (Cristobal). Dr. W. J. Dickinson, University of Utah, provided *D. mercatorum* [S-1-Im El Salvador (7), a strain maintained parthenogenetically]. Dr. L. H. Throckmorton, University of Chicago, provided *D. yakuba*-C,IC, which originated from the Texas strain listed above. Dr. E. W. Hanly, University of Utah, provided *D. melanogaster*-O (Oregon-R-Utah, Oak Ridge, Tenn.).

All flies used in these experiments (other than the five Hawaiian species which were reared at the University of Hawaii by Drs. K. Y. Kaneshiro and H. L. Carson) were grown on cornmeal medium (8) (with banana and liver modifications for some species) with live yeast in 6 ounce (180 cm^3) glass urine specimen jars.

Mitochondria were prepared from eggs and DNA was prepared from the mitochondria and analyzed by neutral and alkaline CsCl equilibrium density gradient centrifugation and electron microscopy as described elsewhere (9, 10).

Ovaries were hand dissected from anesthetized flies in 0.15 M NaCl, 0.1 M EDTA, 0.05 M Tris·HCl (pH 8.0) and immediately frozen on solid CO_2. Ovaries from approximately 300 flies of a single species were thawed and lysed by addition of sodium dodecyl sulfate to 2%, and the lysate was subjected to preparative CsCl equilibrium density gradient centrifugation (10). The buoyant densities of individual fractions of the resulting gradient were determined from their refractive indices, and the configurations of the DNA molecules each fraction contained were determined by electron microscopy.

DNA samples comprising 50–80% open circular molecules (each containing at least one phosphodiester bond break) used for thermal denaturation studies were obtained as the upper fluorescent band in a CsCl/ethidium bromide equilibrium density gradient (10, 11) of pooled circular-molecule-containing fractions obtained by CsCl centrifugation of ovary lysates.

Abbreviations: mtDNA, mitochondrial DNA; M_r, molecular weight.

Table 1. Data concerning DNA extracted from mitochondrial fractions obtained from embryonated eggs

Species	Buoyant density ±SEM (g/cm³) Neutral CsCl	n^a	Alkaline CsCl	n^a	% Length of DNA as circular molecules	n^b	Mean molecular weight of circular molecules ± SEM (× 10^{-6})	n^c
D. melanogaster	1.681 ± 0.0003	5	1.725 ± 0.0005	3	89.5	580	12.35 ± 0.001	27
			1.731 ± 0.0003	3				
D. simulans	1.680	1	1.725 ± 0.0003	2	94.3	423	12.20 ± 0.017	24
			1.732 ± 0.0003	2				
D. virilis	1.685 ± 0.0004	2	1.728 ± 0.0003	2	84.9	695	10.17 ± 0.022	26
			1.735 ± 0.0002	2				

n^a = number of runs; n^b = number of unit length circular molecule equivalents examined; n^c = number of circular molecules measured.

Partial denaturation (5, 6) of open circular molecules was accomplished by heating the DNAs in 0.05 M sodium phosphate (pH 7.8) and 10% HCHO at various temperatures for 10 min as described in Wolstenholme *et al.* (12).

Thin sectioning of mitochondrial pellets, preparation of DNA for electron microscopy by the aqueous protein monolayer technique, electron microscopy, and measurements of molecules were carried out as described previously (10, 13).

Molecular weights of mtDNAs were determined by electron microscopy using bacteriophage fd replicative form DNA [molecular weight 4.05×10^6 (3)] as an internal standard. Mean molecular weights for mtDNA of each species were obtained from 25 to 30 determinations of the ratio of lengths of one circular mtDNA molecule and the fd DNA molecule lying nearest to it on the grid square.

RESULTS

Examination in the electron microscope of thin sections of mitochondrial pellets obtained from embryonated eggs of *Drosophila melanogaster, D. simulans,* and *D. virilis* confirmed that each of these fractions comprised mainly mitochondria and was free from bacteria. Data concerning DNA extracted from these mitochondrial fractions are given in Table 1. The molecular weight and buoyant density in neutral CsCl of *D. melanogaster* mtDNA were as reported previously (1–4). The two components of the DNA (presumably the complementary strands) found at equilibrium in alkaline CsCl density gradients differed from each other in buoyant density by about the same amount (5–6 mg/cm³) as reported by others (1, 2, 4). However, the values found in the present studies for the two components were considerably lower than those (ρ = 1.740 and 1.735 g/cm³) reported earlier. The buoyant densities and molecular weights of *D. simulans* mtDNA were similar to the corresponding values of *D. melanogaster* mtDNA. However, for *D. virilis* mtDNA the buoyant densities were all higher and the molecular weight about 18% lower than the corresponding values found for *d. melanogaster* mtDNA. In view of this latter finding, we determined the molecular weight of mtDNA molecules of 36 other species of *Drosophila.*

Ovaries from each of 39 *Drosophila* species were lysed with sodium dodecyl sulfate and subjected to preparative CsCl equilibrium centrifugation, and the resulting gradient was fractionated. Fractions of each gradient with buoyant densities in the range 1.670–1.690 g/cm³ were found to be rich in circular molecules. Fractions with buoyant densities in the range 1.691–1.710 g/cm³ contained most of the ovary DNA, which appeared to be exclusively linear and presumably of nuclear origin. The molecular weights of the circular molecules obtained from ovary lysates of each species were determined. The data are presented in Table 2. Only a single length class of circular molecules was observed for each species examined. The molecular weights of circular molecules obtained from lysates of ovaries of *D. melanogaster, D. simulans,* and *D. virilis* were similar to the molecular weights of the circular molecules obtained from mitochondrial fractions of embryonated eggs of each of these species (Table 1). Because of this, the assumption is made that the circular molecules obtained for each species from ovary lysates are the mtDNA molecules of that species.

The 39 species examined represent 13 groups of five subgenera of the genus *Drosophila.* mtDNA molecules of all species examined other than members of the melanogaster group had, with one exception, molecular weights in the rather narrow range of 9.90×10^6 to 10.34×10^6. The one exception was *D. robusta,* which had a molecular weight of 10.61×10^6. In

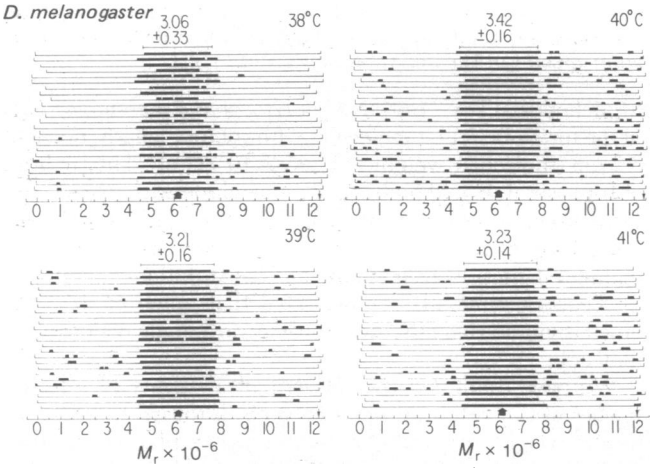

FIG. 1. Denaturation maps of open circular molecules of mtDNA of *D. melanogaster* produced by heating at the temperatures indicated for 10 min in 0.05 M sodium phosphate (pH 7.8) and 10% HCHO. For the purpose of comparison, each circular molecule was converted to a linear rod by opening it at one half the circumference length away from the midpoint of the main region of denaturation. By comparison with the mean lengths of bacteriophage fd replicative form DNA molecules contained in each preparation, the molecular weights (M_r) of the denatured (thick lines) and undenatured (thin lines) regions of each mtDNA molecule were calculated. This included correction for the greater mass per unit length of single-stranded DNA under the conditions employed (9). The midpoint of either the largest denatured region (40°–41°) or the largest series of denatured regions (38° and 39°) was taken as the common point by which the molecules were aligned (large arrows in the center of the abscissae). Direction was defined by placing the segment that contained the region of denaturation lying nearest to the main region of denaturation to the right. The number above each set of molecules is the mean molecular weight (±SD) of the large region of denaturation (A+T-rich region). The fine inverted arrow on the right of each abscissa indicates the mean total molecular weight of the molecules of each set.

Table 2. Molecular weights (M_r) of mtDNA molecules from species of the genus *Drosophila*

	Species	Mean M_r ± SEM ($\times 10^{-6}$)	n
SUBGENUS Scapto-drosophila	*D. lebanonensis*	10.20 ± 0.024	30
SUBGENUS Dorsilopha	*D. busckii*	9.95 ± 0.016	20
SUBGENUS Hirto-drosophila	*D. duncani*	10.11 ± 0.022	27
SUBGENUS Drosophila			
Group Hawaiian picture-wings	*D. gymnobasis*	10.01 ± 0.031	30
	D. grimshawi	10.00 ± 0.076	30
	D. silvarentis	10.01 ± 0.043	30
	D. hawaiensis	10.00 ± 0.027	30
	D. balioptera	9.91 ± 0.024	29
Group virilis	*D. virilis*	10.12 ± 0.047	30
	D. montana	10.32 ± 0.057	30
	D. americana	10.06 ± 0.096	23
Group robusta	*D. robusta*	10.61 ± 0.034	29
Group repleta			
Subgroup hydei	*D. hydei*	10.34 ± 0.086	30
	D. neohydei	9.90 ± 0.043	29
Subgroup mercat-orum	*D. mercatorum*	10.09 ± 0.053	30
Group funebris	*D. funebris*	10.34 ± 0.076	25
Group tripunctata	*D. tripunctata*	10.32 ± 0.034	30
SUBGENUS Sopho-phora			
Group obscura	*D. pseudo-obscura*	10.19 ± 0.070	28
Group willistoni	*D. willistoni*	10.13 ± 0.054	28
Group saltans	*D. saltans*	10.04 ± 0.062	28
Group melano-gaster			
Subgroup ananassae	*D. ananassae*-C	9.94 ± 0.030	30
	D. ananassae-Cr	10.17 ± 0.052	25
	D. bipectinata	10.05 ± 0.050	27
Subgroup elegans	*D. elegans*	10.40 ± 0.020	30
Subgroup eugracilis	*D. eugracilis*	10.31 ± 0.033	26
Subgroup ficusphila	*D. ficusphila*	10.68 ± 0.035	25
Subgroup denticulata	*D. denticulata*	10.44 ± 0.023	30
Subgroup montium	*D. birchii*	11.01 ± 0.048	25
	D. kikkawai	10.68 ± 0.049	25
	D. auraria	10.81 ± 0.039	27
Subgroup takahashii	*D. takahashii*	10.75 ± 0.046	24
	D. lutea	10.67 ± 0.062	24
Subgroup suzukii	*D. suzukii*	10.37 ± 0.044	30
	D. lucipennis	10.96 ± 0.016	30
Subgroup melano-gaster	*D. melanogaster* -O	12.35 ± 0.027	30
	D. melanogaster -H	12.04 ± 0.042	27
	D. simulans-H	11.96 ± 0.054	30
	D. simulans-P	11.94 ± 0.018	30
	D. mauritiana	11.74 ± 0.052	30
	D. yakuba-IC	10.17 ± 0.032	29
	D. yakuba-C,IC	10.28 ± 0.014	30
	D. yakuba-Ca	10.22 ± 0.065	29
	D. teissieri	10.04 ± 0.021	30
	D. erecta	9.92 ± 0.046	29

D. mauritiana and *D. erecta* were classified by Tsacas and David (16) and Tsacas and Lachaise (17), respectively, and other species of the melanogaster group were classified according to Bock and Wheeler (15). All other species were classified according to Throckmorton (20, 21). n = number of mtDNA molecules measured.

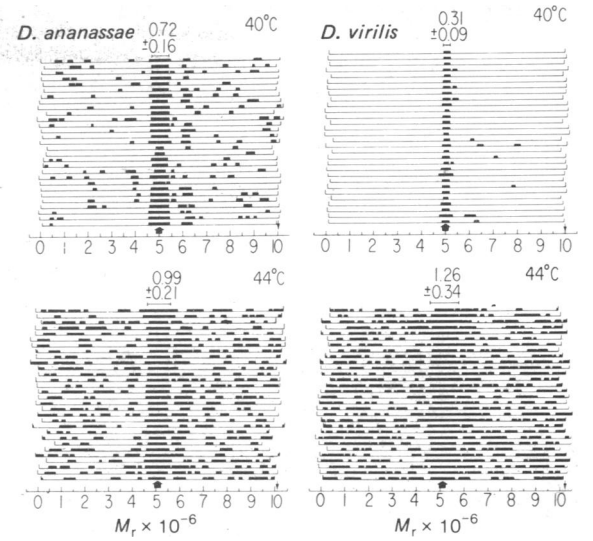

FIG. 2. Denaturation maps of open circular mtDNA molecules of *D. ananassae* and *D. virilis*. Details are as given in Fig. 1.

contrast, mtDNA molecules from the 19 species of the melanogaster group had molecular weights covering the considerably greater range of 9.92×10^6 to 12.35×10^6.

Denaturation of *D. melanogaster* open circular mtDNA molecules was studied using the electron microscope technique of Inman (5, 6) (Fig. 1). In every molecule which had been heated at 40°, a continuous region of denaturation representing, on the average, 3.4×10^6 and accounting for approximately 25% of the molecule was observed. Each molecule also contained a number of small regions of denaturation, but these regions had an average total molecular weight of less than 10% of the molecule. A similar pattern of denaturation was observed for molecules heated at 38°, 39°, and 41° under the same conditions. However, at the two lower temperatures, small segments of the otherwise large region of denaturation remained undenatured.

We made similar denaturation studies (Fig. 2) of mtDNA molecules from *D. ananassae* and *D. virilis*, which have low molecular weights (9.94×10^6 and 10.12×10^6, respectively). The largest regions of denaturation in *D. ananassae* mtDNA molecules after heating at 40° represented only 0.72×10^6 daltons (Fig. 2). In *D. ananassae* mtDNA molecules heated to 44°, at which temperature an average of 44% of each molecule appeared denatured, the largest single region of denaturation had an average molecular weight of only 0.99×10^6. Similar patterns of denaturation were found for *D. virilis* mtDNA (Fig. 2). In this case, in molecules heated at 40°, the largest region of denaturation averaged only 0.31×10^6 daltons.

It is clear from these data that *D. melanogaster* mtDNA molecules are distinguished from *D. ananassae* and *D. virilis* mtDNA molecules by a greater total molecular weight and by the size of the largest single region of the molecule that denatures at a temperature at which most of the remainder of the molecule remains undenatured. It has been shown (14) that, under the conditions of our experiments, as the temperature is increased, the first regions in which strand separation occurs are rich in adenine and thymine. We have, therefore, termed the large region of denaturation found in *D. melanogaster* mtDNA molecules heated at 40° the A+T-rich region. We will also use this term to denote the largest regions of denaturation found in mtDNA molecules heated at 40° from each *Drosophila* species examined.

We next carried out denaturation mapping studies of

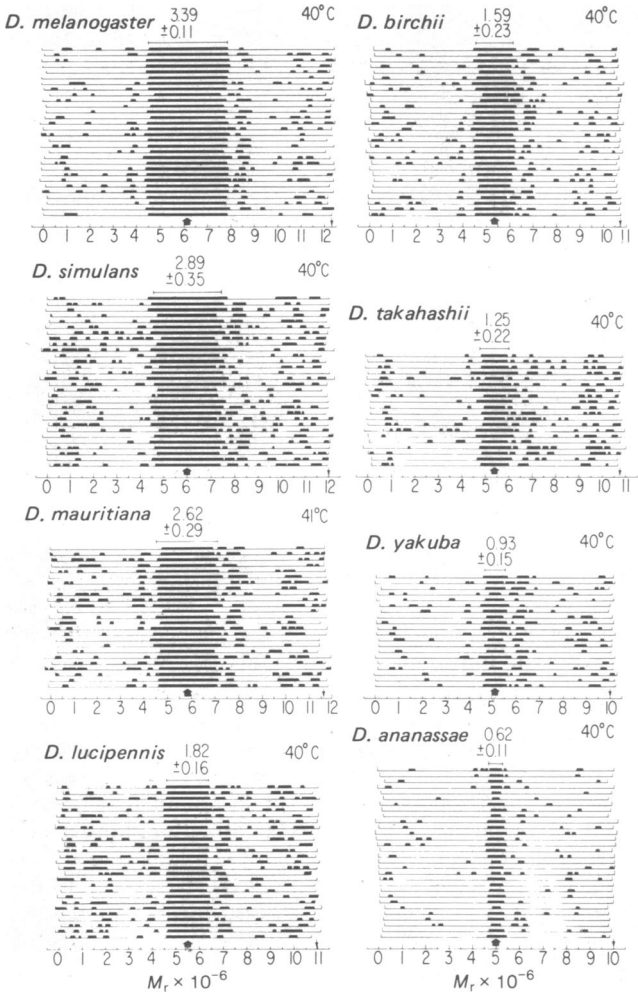

FIG 3. Denaturation maps of open circular mtDNA molecules of the species indicated. Details are as given in Fig. 1. The sets of *D. ananassae, D. yakuba, D. takahashii,* and *D. birchii* molecules are those obtained from the protein monolayer preparations of the various mixtures of these molecules with *D. melanogaster* molecules described in the *text*. The set of *D. melanogaster* molecules is from the protein monolayer preparations of the *D. melanogaster* + *D. birchii* mixture. The set of *D. mauritiana* molecules shown are from a preparation heated at 41°, as the A+T-rich region of these molecules heated at 40° failed to separate completely.

mtDNA molecules from five other species of the melanogaster group which had various total molecular weights (Table 2) between those of mtDNAs from *D. ananassae* and *D. melanogaster*. The species examined (and total mtDNA molecular weight) were *D. yakuba* (10.17×10^6); *D. takahashii* (10.75×10^6); *D. lucipennis* (10.96×10^6), *D. mauritiana* (11.74×10^6), and *D. simulans* (11.96×10^6). mtDNA molecules of each of these species were heated at 40° (41° for *D. mauritiana*, see legend to Fig. 4) and the largest resulting region of denaturation (the A+T-rich region) in each case was measured. The molecular weights ((± SD) of A+T-rich regions obtained in this way were determined to be: *D. yakuba*, $0.87 \pm 0.19 \times 10^6$ ($n = 30$); *D. takahashii*, $1.39 \pm 0.22 \times 10^6$ ($n = 27$); *D. lucipennis*, $1.82 \pm 0.16 \times 10^6$ ($n = 27$); *D. mauritiana*, $2.62 \pm 0.29 \times 10^6$ ($n = 25$); and *D. simulans*, $2.89 \pm 0.35 \times 10^6$ ($n = 30$). These data suggest that mtDNA molecules of each of these species contains a distinct A+T-rich region and the greater the molecular weight of this region, the greater is the total molecular weight of the molecule.

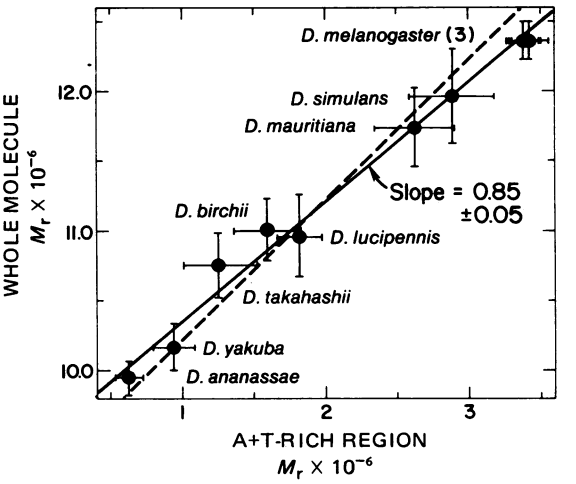

FIG. 4. The relationship of the mean molecular weight of whole circular mtDNA molecules (data from Table 2) to the mean molecular weight of the corresponding A+T-rich region (data from Fig. 3) of each of the eight species of the melanogaster group examined. The data appear to fit a straight line (solid line). The slope of the regression [0.85 ± 0.05 (SEM)] is significantly ($P < 0.01$) less than 1 (broken line drawn for comparison through the mean of the molecular weight values of whole molecules and A+T-rich regions).

In order to determine more accurately the relative molecular weights of the A+T-rich regions of the mtDNAs of some of the melanogaster group species, mtDNA molecules were mixed in the combinations *D. melanogaster + D. ananassae; D. melanogaster + D. birchii;* and *D. melanogaster + D. takahashii + D. yakuba;* and these mixtures (each of which contained fd replicative form DNA as usual) were prepared for electron microscopy after heating at 40°. mtDNA molecules from the individual species were recognizable because of the differences in total length of the molecules. Again, each set of mtDNA molecules was mapped in reference to the A+T-rich region. The molecular weights obtained for the A+T-rich region of molecules in the various combinations were *D. melanogaster*:*D. ananassae*, $3.39 \pm 0.12 \times 10^6$:$0.62 \pm 0.11 \times 10^6$; *D. melanogaster*:*D. birchii*, $3.39 \pm 0.11 \times 10^6$:$1.59 \pm 0.23 \times 10^6$; *D. melanogaster*:*D. takahashii*:*D. yakuba*, $3.41 \pm 0.14 \times 10^6$:$1.25 \pm 0.22 \times 10^6$:$0.93 \pm 0.15 \times 10^6$. These values are in good agreement with the molecular weights of A+T-rich regions of the various mtDNA molecules determined separately.

Denaturation maps of mtDNA molecules of the eight species of the melanogaster group examined are shown in Fig. 3. Fig. 4 is a plot of the molecular weight of the whole mtDNA molecule against the molecular weight of the corresponding A+T-rich region of each of the eight melanogaster group species. The data, which appear to fit a straight line with a slope of 0.85 ± 0.05 (SEM), indicate that most of the variation in total molecular weight of mtDNA molecules of these species can be accounted for by differences in size of the A+T-rich regions. The significant departure of the slope of the line from 1 (Fig. 4) suggests, however, that other factors make some contribution to the observed variation in total molecular weights of these molecules.

The distribution of molecular weights among the shorter A+T-rich regions (Fig. 3) suggested that they represent integral multiples of a 0.31×10^6 dalton unit. Division of the molecular weights of each of the remaining A+T-rich regions by 0.31×10^6 showed that they too were very nearly integral multiples of this molecular weight. The best fit, by the least squares criterion of the hypothesis that all of the A+T-rich regions of the

melanogaster group are integral multiples of a basic unit molecular weight, was obtained with a unit molecular weight of 0.316×10^6.

DISCUSSION

The data presented indicate that circular mtDNA molecules from different species of the melanogaster group have an overall variation of approximately 20% in total mean molecular weight. This is in contrast to the very small variation in total mean molecular weight among circular mtDNA molecules from the other *Drosophila* species examined. Most of the variation in total molecular weight of mtDNA molecules of melanogaster group species appears to be accounted for by variation in size of the A+T-rich region of these molecules.

From our consideration of the sizes of the A+T-rich regions of mtDNA molecules, it seems possible that these regions consist of a basic sequence which is tandemly repeated a different number of times for each species examined. It is interesting to note that the single region that reproducibly melted when *D. virilis* mtDNA molecules were heated at 40° had a mean molecular weight of 0.31×10^6, which is very close to that estimated (0.316×10^6) for the basic unit molecular weight of the A+T-rich region of melanogaster group mtDNA molecules.

The distribution of sizes of A+T-rich regions of mtDNA molecules of the different melanogaster group species does not follow a simple taxonomic pattern (Table 2). Of the six species of the melanogaster subgroup, three (*D. melanogaster, D. simulans,* and *D. mauritiana*) have A+T-rich regions with molecular weights in the range 2.62×10^6 to 3.39×10^6, while at least one (*D. yakuba*) has an A+T-rich region of molecular weight 0.93×10^6. The mtDNA molecules of *D. takahashii, D. birchii,* and *D. lucipennis,* which represent three different subgroups, have A+T-rich regions with molecular weights intermediate to those of *D. yakuba* and the other three melanogaster subgroup species. Assuming that the melanogaster group species have indeed evolved along the lines indicated by the taxonomic classification adopted (Table 2, 15–17), then these data indicate either that increases in size of the A+T-rich region of mtDNA molecules have occurred independently in separate evolutionary paths or that decrease in size of the A+T-rich region has accompanied evolution along at least one path.

An adenine+thymine-rich satellite [7% guanine+cytosine (4)] of nuclear origin (18) has been isolated from *D. melanogaster* (19) and shown to include simple repeat sequences (4). At the present time, little is known about the actual sequence structure of the A+T-rich region of *Drosophila* mtDNA molecules. Data from thermal denaturation studies (2) and from buoyant density analysis of sheared molecules of *D. melanogaster* mttDNA (4) indicate that if guanine and cytosine occur in the A+T-rich region, then they do so in very small amounts. In agreement with this is our finding that a number of bacterial restriction enzymes that cleave specific DNA sequences containing guanine and cytosine failed to cleave the A+T-rich region of the *D. melanogaster* mtDNA molecule (ref. 9; Fauron and Wolstenholme, unpublished).

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