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Origin and direction of replication in mitochondrial DNA molecules from the genus Drosophila

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ABSTRACT

Mitochondrial DNA (mtDNA) obtained from ovaries of Drosophila simulans, D. mauritiana, D. takahashii, D. yakuba and D. virilis was examined by electron microscopy. From a consideration of the structural properties of replicative intermediates, it was concluded that in mtDNA molecules of each species, synthesis on one strand can be up to 97% complete before synthesis on the complementary strand is initiated. MtDNA molecules of each species contain a single A+T-rich region which shows species-specific size variation from 1.0 kb (D. virilis) to 4.8 kb (D. simulans), and maps at the same position in all molecules relative to three common EcoRI sites. The structural properties of complex forms, interpreted as having originated from replicative intermediates, and produced by either partial denaturation or EcoRI digestion, are consistent with the hypothesis that replication is initiated within the A+T-rich region and proceeds unidirectionally around the molecule towards the nearest common EcoRI site. The replication origin is located near the center of the A+T-rich region in D. simulans and D. mauritiana, but lies closer to that end of the A+T-rich region which is distal to the nearest common EcoRI site in D. takahashii, D. yakuba and D. virilis.

INTRODUCTION

Mitochondrial DNA (mtDNA) molecules from metazoan organisms show a remarkable evolutionary conservation of size and shape. In almost all cases, the molecules are circular duplexes containing 14.5 to 16.5 kilobase pairs (kb) (1). However, mtDNA molecules from some species of the melanogaster group of the genus Drosophila, vary in size from 15.7 to 19.5 kb (2), the largest being those of D. melanogaster. A number of studies have shown that mtDNA molecules of D. melanogaster contain a region (~25% of the genome length) which denatures at a lower temperature than the rest of the molecule due to an unusually high content of adenine and thymine (A+T) (2-7). The studies of Fauron and Wolstenholme (2) further demonstrated that mtDNA molecules of other species within the melanogaster group contain a single A+T-rich region, the size of which is constant within a species but varies from 1.0 to 5.4 kb in different species. Moreover, the differences in the size

of the A+T-rich region can completely account for the differences in the size of the mtDNA molecules of the melanogaster group species. Mitochondrial DNA molecules from Drosophila species other than those of the melanogaster group are within the narrow size range of 15.7 to 16.8 kb and contain an A+T-rich region of approximately 1.0 kb (2, 8).

In previous studies of the replication of mtDNA molecules from D. melanogaster (9, 10), we determined by electron microscopy that the majority of molecules are replicated by a highly asymmetric process in which synthesis on one strand can be up to 99-100% complete before synthesis of the other complementary strand is initiated. Replication of other molecules proceeds in a more nearly symmetrical manner. By comparing the relative positions of the replication forks to the A+T-rich region and to EcoRI cleavage sites (6), we further showed that replication of all of these molecules originates near the center of the A+T-rich region and proceeds unidirectionally around the molecule toward the EcoRI site nearest the A+T-rich region. We have now characterized replicative intermediates in mtDNA preparations from four other species within the melanogaster group (D. simulans, D. mauritiana, D. takahashii and D. yakuba) and one other taxonomically more distant species (D. virilis). In addition, we have determined the origin and direction of replication of mtDNA molecules in each of these species with respect to the relative positions of their A+T-rich regions and EcoRI cleavage sites.

MATERIAL AND METHODS

Fly Stocks. The following Drosophila species (stock number and place of origin) were obtained from the Species Stock Collection of the Genetics Foundation of the University of Texas at Austin: D. simulans (3015.8, Nueva, Calif.); D. takahashii (3075.4, Wulai, Taiwan); D. yakuba IC (2371.6, Ivory Coast); and D. virilis (2375.8, Chile). D. mauritiana (Mauritius Island) was provided by Dr. L. Tsacas, Centre National de la Recherche Scientifique, Gif-sur-Yvette, France.

Preparation of mitochondrial DNA. Ovaries were obtained from anesthetized flies which had been maintained for 3-5 days on cornmeal media topped with live yeast in 6 ounce glass urine specimen jars. The ovaries were dissected into 0.15 M NaCl, 0.1 M EDTA, 0.05 M Tris (pH 8.0) and immediately frozen on solid CO_2. Ovaries from approximately 500 flies were thawed and, after the addition of sodium dodecyl sulfate to a final concentration of 2%, were homogenized by hand in a 1 ml micro-homogenizer. The homogenate was subjected to CsCl density equilibrium centrifugation (ρ=1.707 g/cm^3) for 48

hr at 40,000 rpm in a Beckman 50Ti rotor. Fractions with refractive indices between 1.3950 and 1.4000 were pooled and recentrifuged to equilibrium in CsCl which had an initial buoyant density equal to that of D. simulans mtDNA (ρ=1.681 g/cm^3). The refractive indices of fractions were again determined and those expected to contain mtDNA were examined by electron microscopy.

Electron Microscopy. DNA was prepared for electron microscopy using the formamide protein monolayer technique of Davis et al. (11). The concentration of formamide (Matheson, Cole and Bell) in the hyperphase and hypophase was 40% and 10% respectively. Other details of electron microscopy are as described previously (6). Bacteriophage fd single- and/or double-stranded (RF) circular DNA molecules (6408 base pairs (12)) were included as internal standards in all electron microscope preparations.

Formaldehyde Fixation. In order to reduce branch migration (13) in partially replicated molecules, mtDNA from the second CsCl centrifugation was incubated in 10% formaldehyde (Mallinckrodt) for 20 min at 21°C. The formaldehyde was removed before preparation for electron microscopy or before digestion with EcoRI by passage through a small column of G-100 Sephadex (14) equilibrated in either 1 mM EDTA pH 7.5 or in 0.05 M NaCl, 0.1 M Tris pH 7.5, respectively.

Digestion with EcoRI restriction Endonuclease. Formaldehyde-treated mtDNA was digested with EcoRI (New England Biolabs) by adding one µl EcoRI (2500 units/ml, one unit defined as the amount required to degrade 1.0 µg of lambda DNA in 15 min at 37°C in a total assay mixture of 0.05 ml) to 50 µl of a solution containing mtDNA (5-10 µg/ml), 100 mM Tris pH 7.5, 50 mM NaCl, and 5 mM $MgCl_2$ (15) and then incubating for 30 min at 21°C.

Partial Denaturation. Partial denaturation of circular mtDNA molecules was obtained by heating the DNA at 41°C for 11 min in 0.05 M sodium phosphate buffer pH 7.8 and 10% formaldehyde as previously described (16). The formaldehyde was removed before preparation for electron microscopy by passage through a small column of G-100 Sephadex equilibrated in 1 mM EDTA pH 7.5 (14).

RESULTS

Characterization of partially replicated molecules. Mitochondrial DNA (mtDNA) obtained by cesium chloride density equilibrium centrifugation of lysed ovaries of each of the species D. simulans, D. mauritiana, D. takahashii, D. yakuba and D. virilis was examined in the electron microscope. Between 40 and 80 percent of the DNA molecules in the different preparations

were circular. Double-forked, circular molecules with characteristics of size and shape consistent with their being replicative intermediates (17-20) were observed. These molecules accounted for approximately 2% of the circular molecules present in preparations from D. mauritiana, D. takahashii, and D. yakuba, 3% in preparations from D. simulans and 1% in preparations from D. virilis. All of these molecules contained one segment in the replicative region which was totally double-stranded (first daughter segment). The other segment in the replicative region (second daughter segment) was either totally single-stranded, totally double-stranded, or partially double-stranded with single-stranded regions at one or both forks. Data concerning these molecular forms are given in Table 1. In the majority of replicative molecules of each of the five species, the second daughter segment was completely single-stranded and the replicative region ranged from 2 to 97% of the genome length. A total of 11 molecules were found in which the second daughter segment was totally double-stranded. In six of these the second daughter segment measured between 89% and 97% of the genome length. A single-stranded region was present in the second daughter segment at only one fork in 14 molecules, and at both forks in the three others. A distinct class of circular mtDNA molecules with characteristics of D-loop structures (21) was not observed among mtDNAs of any of the five species studied. Non-forked, circular

Table 1. Data concerning the second daughter segment of partially replicated molecules of mtDNA from five species of Drosophila.

	Secondary structure of second daughter segment.					
	Totally single-stranded		Totally double-stranded		Single-stranded only at one or both forks	
Species	n	Range of lengths (% genome)	n	Range of lengths (% genome)	n	Range of lengths (% genome)
D. simulans	15	5 to 72	2	38 to 91	8	17 to 98
D. mauritiana	17	5 to 71	4	50 to 97	3	16 to 96
D. takahashii	16	5 to 97	1	97	4	31 to 65
D. yakuba	22	5 to 83	3	24 to 72	1	98
D. virilis	25	2 to 59	1	96	1	84

n = the number of molecules observed.

molecules containing a single-stranded region within the otherwise double-stranded circle (gap molecules) were also observed in low frequency in each preparation. The size of the single-stranded region varied from 3 to 62% of the genome length. Molecules of this type have been observed in mtDNA from a variety of animals, and evidence has been presented which indicates that they are daughter molecules which are separated before duplex synthesis has been completed (18, 22).

Partial denaturation of circular replicative intermediates. The origin of replication in mtDNA molecules of D. melanogaster is located near the center of the A+T-rich region (9). The sizes of the A+T-rich regions and the locations of these regions relative to EcoRI cleavage sites of the mtDNA molecules of the five species examined in the present study is shown in Fig. 1. In order to determine whether, as in D. melanogaster mtDNA, replication is also initiated within the A+T-rich region of mtDNA molecules of other species, we examined in the electron microscope mtDNA from D. simulans, D. mauritiana, D. takahashii, D. yakuba and D. virilis following partial denaturation. The majority of the circular molecules observed were predominantly double-stranded with one major region of denaturation of a size characteristic of the respective species. In addition, more complex circular molecules were observed in each mtDNA preparation. Examples of these are shown in the electron micrographs in Fig. 2a and 2b. Each of these molecules contained three forks

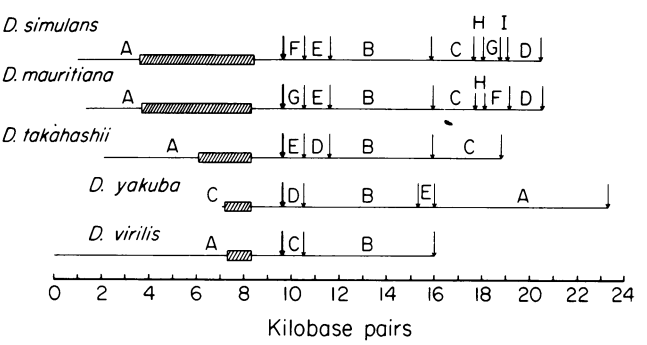

Figure 1. Maps of the mitochondrial genomes of the five Drosophila species indicated, showing the relative positions of the A+T-rich regions (hatched areas) and the EcoRI sites (arrows). The molecules have been aligned by the common EcoRI site nearest to the A+T-rich region (indicated by the heavy arrow) and linearized at the first EcoRI site on the opposite side of the A+T-rich region. The EcoRI fragments of each species are indicated, in order of decreasing size, by the letters A-I. (Modified from the data of Fauron and Wolstenholme (23).)

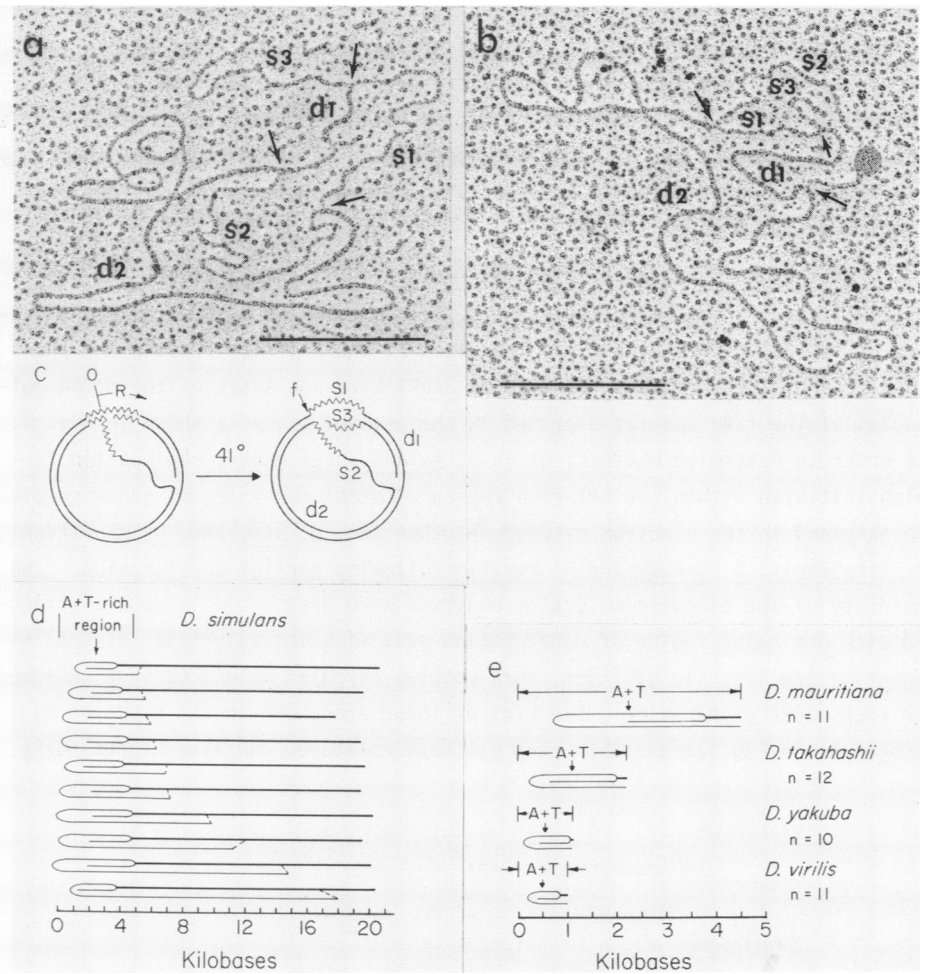

Figure 2. Complex circular structures observed in mtDNA preparations of Drosophila species after partial denaturation. (a) and (b): Representative electron micrographs of molecules from preparations of D. mauritiana and D. yakuba, respectively, which have been heated at 41° in 0.05 M sodium phosphate and 10% formaldehyde. The forks are indicated by arrows; s and d denote single- and double-stranded regions, respectively, and correspond to the numbered s and d segments diagrammed in (c). The short single-strand apparent within the d1 segment most probably results from a small denatured region which was broken at one end. The bars represent 0.5 μm. (c): A diagrammatic interpretation of the molecules shown in (a) and (b). Replication originates at a point (O) within the A+T-rich region (serrated lines) and proceeds in the direction indicated (R). (d): Diagram illustrating the single- and double-stranded segments of ten complex molecules from mtDNA preparations of D. simulans. The molecules were aligned by placing the midpoint of the s1 segment at the center of the A+T-rich region (indicated by

the arrow), linearized by cutting the molecules at the fork (f) in (c) above, and placing the double-stranded end thus generated to the right. Thin and thick lines represent single- and double-stranded regions, respectively; the broken line indicates the difference between the measured length of s2 and the sum of the lengths of s1 plus d1. (e): Diagram illustrating the average lengths of the s1 and s3 segments of complex molecules from D. mauritiana, D. takahashii, D. yakuba and D. virilis. In each case, the structures have been linearized and aligned with respect to the A+T-rich region as described in (d) above. The lengths of the A+T-rich regions are from Fauron and Wolstenholme (2), and as listed in Table 2.

which delimit two double-stranded segments, indicated by d1 and d2 in Fig. 2a-c, two single-stranded segments (s1 and s2) and a single-stranded segment with a free end (s3). As illustrated in the diagram in Fig. 2c, and supported by the data given in Table 2, these structures can be generated by partial denaturation of a molecule in which replication has proceeded unidirectionally from an origin lying within the A+T-rich region. In these molecules, s1 is one of the denatured strands of the A+T-rich region, and s2 is the other denatured strand of the A+T-rich region plus the displaced, unreplicated strand. s3 is the replicated portion of the A+T-rich region, and its length gives the approximate position within the A+T-rich region at which replication was initiated. The length of s2 is equal to the sum of the lengths of

Table 2. Lengths of various segments of partially denatured replicative molecules of Drosophila mtDNAs compared with the sizes expected for the whole genomes and for the A+T-rich regions.

Species	s1 + d1 + d2[a]	Total genome length[c]	s1[a]	A+T-rich region[c]
D. simulans	19.3 ± 0.8[b]	19.3	4.0 ± 0.8[b]	4.8
D. mauritiana	19.0 ± 1.2	18.9	3.1 ± 0.9	4.6
D. takahashii	17.1 ± 1.0	16.7	1.7 ± 0.4	2.2
D. yakuba	16.5 ± 0.4	16.2	0.9 ± 0.2	1.1
D. virilis	16.2 ± 0.8	16.0	0.7 ± 0.2	1.0

a. Segments defined in Figure 2c.

b. Each entry is the mean and standard deviation (kilobases) for 10, 11 or 12 observations.

c. Kilobase pairs, from Fauron and Wolstenholme (2, 23).

s1 and d1 (the replicated strand outside the A+T-rich region), and the sum of the lengths of s1, d1 and d2 (the unreplicated portion of the molecule outside the A+T-rich region) is equal to the total circular genome length. Molecules of this type in mtDNA preparations of D. simulans are represented in Fig. 2d. The average length of s1 in the D. simulans mtDNA molecules was 4.0 ± 0.8 kb compared with a value of 4.8 kb, as listed in Table 2, for the size of the A+T-rich region. The free ended single-stranded segments (s3) of these molecules extended into the A+T-rich region an average of 2.6 kb which is 65% of the length of s1. The average lengths of the s1 and s3 segments of such molecules in mtDNA of the other four species are given in Fig. 2e and Table 2. In molecules of D. mauritiana, D. takahashii, D. yakuba and D. virilis, the length of the s3 segment was 55%, 79%, 65% and 73% of the s1 length, respectively.

The structural form of the molecules from each of the five species rules out any model of replication that requires either bidirectional synthesis from a unique origin or a replication origin lying outside the A+T-rich region. However, the data do not specify the direction of replication around the molecule or indicate whether the direction of replication is the same in all molecules.

EcoRI digestion of partially replicated molecules. Mitochondrial DNA from D. simulans, D. mauritiana, D. takahashii, D. yakuba, and D. virilis was next digested with EcoRI restriction endonuclease. When EcoRI-digested mtDNA was examined in the electron microscope, three classes of double-forked structures were observed. Examples of these from mtDNA preparations of D. takahashii are shown in Fig. 3a-3c. A diagrammatic interpretation of each molecule is shown in Fig. 3a'-3c'.

In molecules of the first class the two forks were contained within the same fragment, and the two DNA segments between the two forks were of the same length. In all cases, at least one of these latter segments was double-stranded (d2); the other was single-stranded (s) in 19 of the molecules and double-stranded in one. The sum of the lengths of the double-stranded segments (d1 + d2 + d3) was 7.5 ± 0.2 kb, indicating that the forks were contained within the A fragment (7.5 kb) of the D. takahashii mtDNA molecule. As illustrated in Fig. 3a', this type of structure would be generated by EcoRI digestion of a molecule in which replication had been initiated at one point in the EcoRI A fragment and then proceeded a short distance to a second point still within this segment. Twenty structures of this kind were observed and are diagrammed in Fig. 3a''. In each, one fork was consistently lo-

cated approximately 3.0 kb from one end. Therefore, in Fig. 3a'', these EcoRI fragments have been aligned by the position of that fork (indicated by the arrow), with the nearest EcoRI-generated end placed to the right. The position of the second fork was variable but was nearer to the right end in all of the aligned fragments. This result is consistent with replication originating at the site of the fork used to align the fragments and proceeding unidirectionally towards the EcoRI site nearest to the origin. From a consideration of the relative positions of the A+T-rich regions and the EcoRI sites in D. takahashii mtDNA, it appears that a point lying 3.0 kb from one end of the EcoRI fragment falls within the A+T-rich region. Thus these results support the data from the partial denaturation experiments indicating that replication proceeds unidirectionally from a point lying within the A+T-rich region. In addition, these results show that the direction of replication around the molecule is the same in each molecule.

Structures of the second class, which are illustrated in Fig. 3b, consist of two double-stranded fragments connected internally by a single-stranded segment (s). As shown in Fig. 3b', such structures could be interpreted as having been derived from a molecule in which replication had originated 3.0 kb from one end of the EcoRI A fragment and proceeded unidirectionally into another fragment: in the example shown in Fig. 3b', through the E and D fragments to a point lying on the B fragment. Four molecules of this kind were observed and are diagrammed in Fig. 3b''.

The third type of structure seen, illustrated in Fig. 3c, was a molecule in which the two forks were again contained within one fragment, but in contrast to the situation in structures of the first class, the single-stranded region between the forks was much larger than the corresponding double-stranded region. As shown in Fig. 3c' this structure could be derived from a molecule in which replication had originated at a point 3.0 kb from one end of the A fragment and then proceeded unidirectionally through the E, D, B and C fragments to a second point on the A fragment. The dimensions of the various segments of this molecule are shown in Fig. 3c''.

Three classes of structures similar to those described for D. takahashii mtDNA were also observed in preparations of EcoRI digested mtDNA from D. simulans, D. mauritiana, D. yakuba and D. virilis. These data are summarized in Figs. 4 and 5. For each species, the dimensions of the different single-stranded and double-stranded segments of the complex structures observed were wholly consistent with the interpretation that replication was initiated in the A+T-rich region of the mtDNA molecule and proceeded toward the nearest

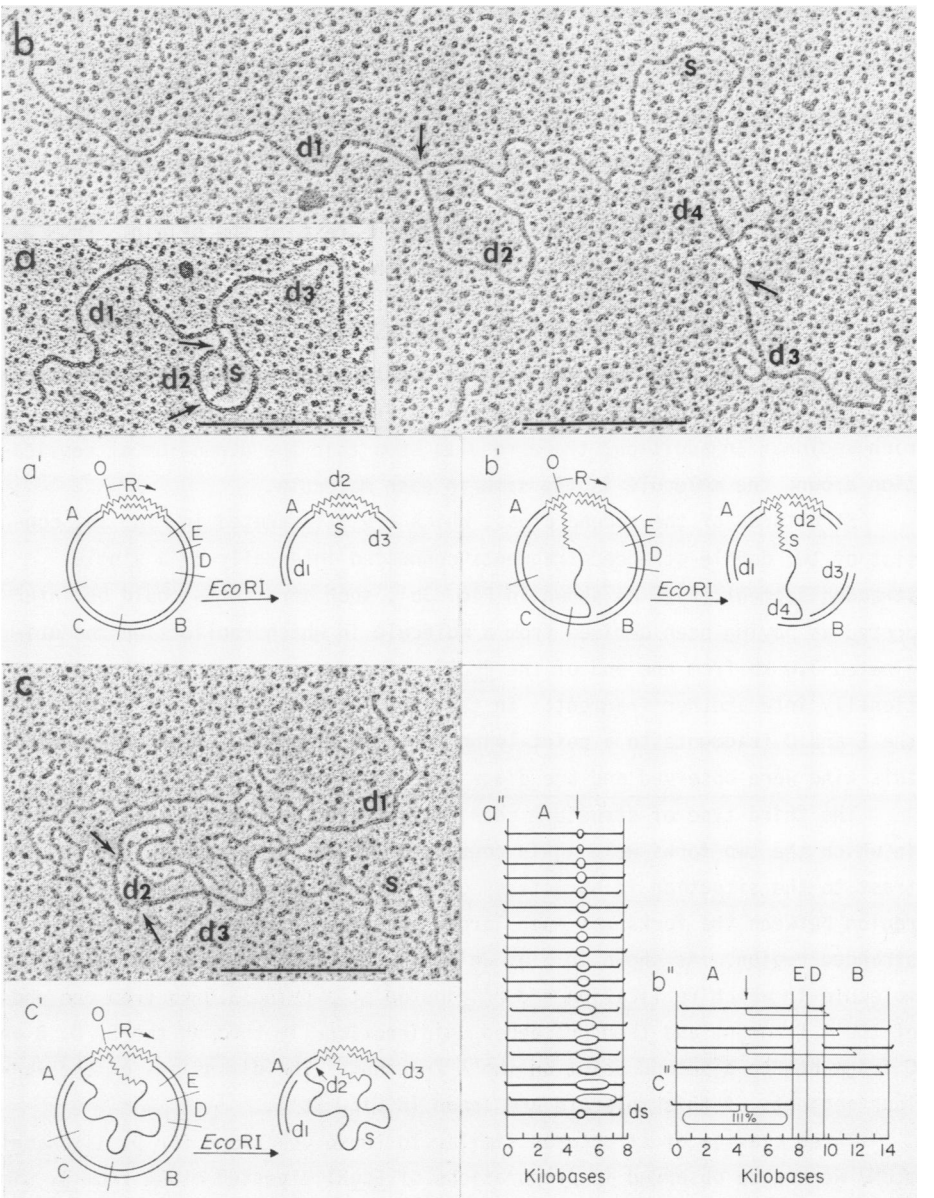

Figure 3. Double-forked structures observed in mtDNA preparations of D. takahashii after digestion with EcoRI. (a), (b) and (c): Electron micrographs illustrating the three classes of structures observed. Forks are indicated by the arrows; s and d represent single- and double-stranded segments, respectively, and correspond to the numbered s and d segments in the diagrams of (a'), (b') and (c'). The bars represent 0.5 μm. (a'), (b'),

and (c'): Diagrammatic interpretations of the structures shown in (a), (b), and (c) respectively. Replication originates at a point (O) within the A+T-rich region (serrated lines) and proceeds in the direction indicated (R). Cleavage of the five EcoRI sites produces five fragments (A-E) as illustrated in Fig. 1. The A+T-rich region is contained within the A fragment. (a''), (b'') and (c''): Diagrams illustrating the relative lengths of the different segments in each of the 25 structures observed. In (a'') are represented those structures of the class shown in (a). The second daughter segment was double-stranded in one molecule (ds) and single-stranded in the other 19. The molecules were aligned by the fork indicated by the arrow, and the longest double-stranded region (dl in (a) and (a')) was placed to the left. Structures of the classes illustrated in (b) and (c), are represented in (b'') and (c'') respectively. The A fragments were aligned by the fork indicated by the arrow, and the segments corresponding to dl (in (b) and (b')) or dl + d2 (in (c) and (c'')) were placed to the left. The corresponding D and B fragments were placed to the right, as indicated. The sum of the lengths of s + d2 in the molecule shown in (c'') was equal to 111% genome length. In two of the 25 structures, a short single-stranded whisker was present at one or both forks. Since these whiskers are assumed to be the product of branch migration (13), the positions of the forks in these structures have been corrected for this effect.

EcoRI site common to the molecules of all five species.

The position of the replication origin in mtDNA molecules of each of the five species examined was determined as the average distance of the fork interpreted as the replication origin in each structure, from the nearest common EcoRI site. These values are plotted in the histogram in Fig. 6. In mtDNA molecules of D. simulans, D. mauritiana, D. takahashii and D. virilis, this distance was 3.8 $\pm$ 0.2 kb, 3.5 $\pm$ 0.2 kb, 2.9 $\pm$ 0.1 kb, 2.2 $\pm$ 0.1 kb, and 2.0 $\pm$ 0.3 kb, respectively. From a consideration of the relative position of the A+T-rich region within the origin-containing EcoRI fragment, it appears that the origin of replication is located near the center of the A+T-rich region in mtDNA molecules of D. simulans and D. mauritiana, but that in mtDNA molecules of D. takahashii, D. yakuba and D. virilis the origin is located closer to that end of the A+T-rich region which is distal to the nearest common EcoRI site. These conclusions agree with those derived from the relative lengths of s3 and s1 in partially denatured replicative molecules from D. mauritiana, D. takahashii, D. yakuba and D. virilis. The difference observed for mtDNA molecules of D. simulans may be due to what appears to be incomplete denaturation of the A+T-rich region in these molecules.

DISCUSSION

Mitochondrial DNA molecules of D. simulans, D. mauritiana, D. takahashii, D. yakuba and D. virilis share certain features with one another and with

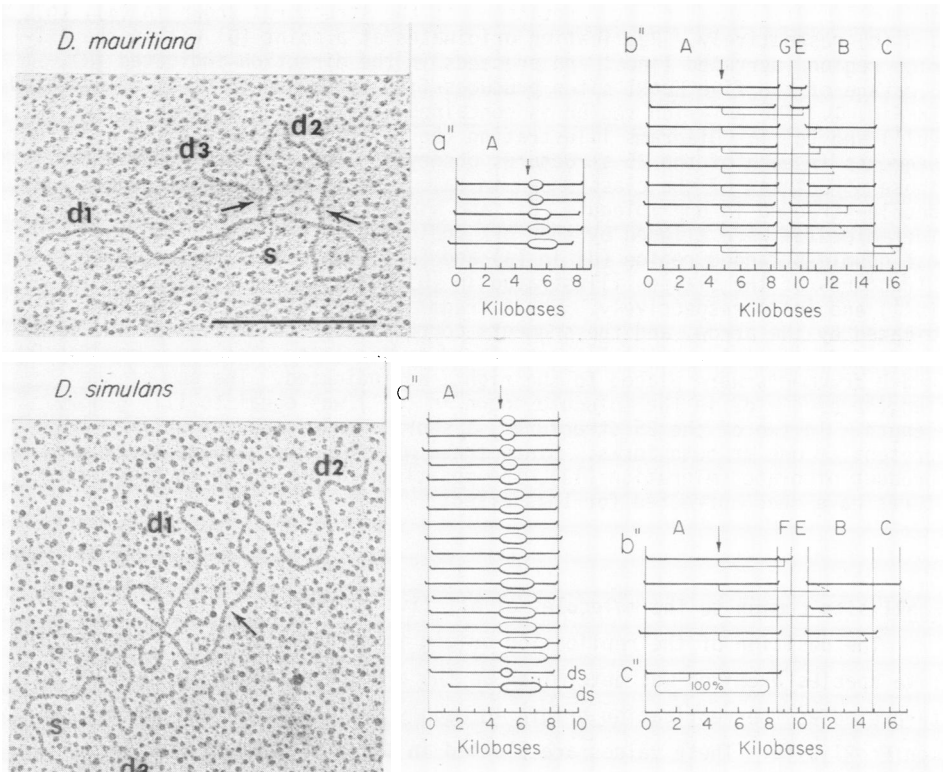

Figure 4. Double-forked structures observed in mtDNA preparations from D. mauritiana and D. simulans after digestion with EcoRI. The upper and lower electron micrographs illustrate structures typical of those observed in mtDNA preparations of D. mauritiana and D. simulans, respectively. The two types of molecules are similar to those illustrated for D. takahashii in Fig. 3a and 3b. The bars represent 0.5 μm. The various segments of individual molecules are illustrated for D. mauritiana and D. simulans in the upper and lower diagrams, respectively. a'', b'' and c'' correspond to the three classes of double-forked structures as described in Fig. 3. The EcoRI digestion of mtDNA preparations from D. mauritiana and D. simulans was not complete. In two of the structures from D. mauritiana, the length of d3 + d4 was longer than the length of the B fragment but was equal to the sum of the lengths of the B + E fragments. Similarly, one structure from D. simulans contained a d3 + d4 segment which was equal in length to the B + C fragments. The sum of the lengths of s + d2 in the molecule shown in c'' (lower diagram) was equal to 100% genome length.

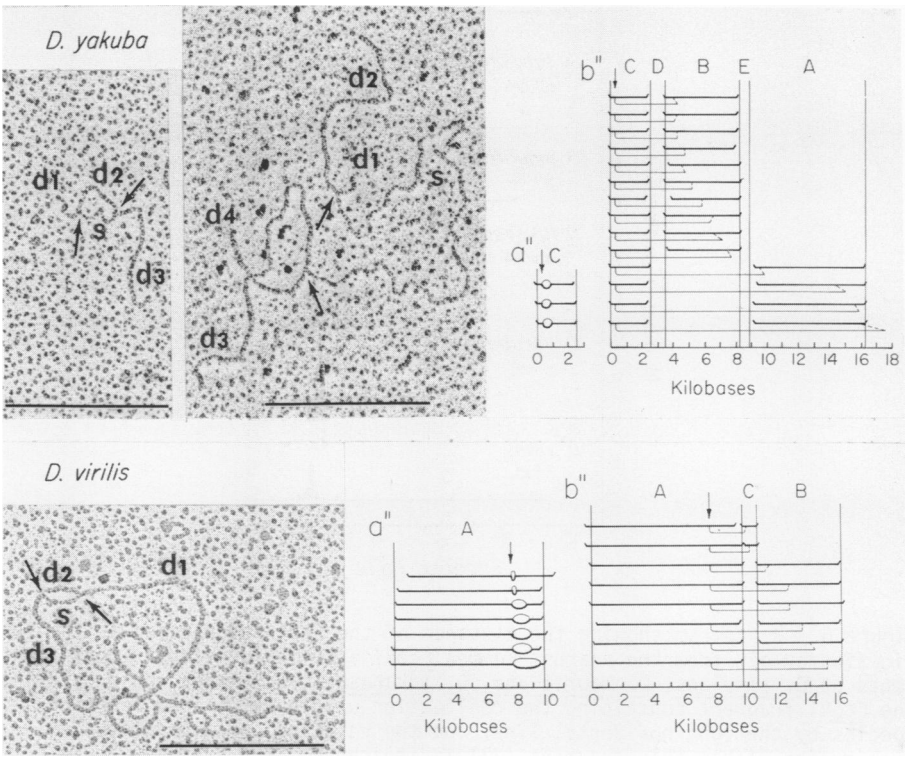

Figure 5. Double-forked structures observed in mtDNA preparations from D. yakuba and D. virilis after digestion with EcoRI. The upper and lower electron micrographs illustrate structures typical of those observed in mtDNA preparations of D. yakuba and D. virilis, respectively. The two types of molecules are similar to those illustrated for D. takahashii in Fig. 3a and 3b. The bars represent 0.5 μm. The various segments in individual molecules are illustrated for D. yakuba and D. virilis in the upper and lower diagrams, respectively. a'' and b'' correspond to the first two classes of double-forked structures as described in Fig. 3.

mtDNA molecules of D. melanogaster. All of these mtDNA molecules have three EcoRI sites in common and an A+T-rich region of specific size, one end of which lies approximately 1.3 kb from the nearest of the common EcoRI sites (23). We have shown previously (9) that in mtDNA molecules of D. melanogaster, replication is initiated at a site near the center of the A+T-rich region and proceeds unidirectionally around the molecule towards the nearest EcoRI site. The data presented here indicate that mtDNA replication in D. simulans, D. mauritiana, D. takahashii, D. yakuba and D. virilis also originates within the A+T-rich region and proceeds unidirectionally around the

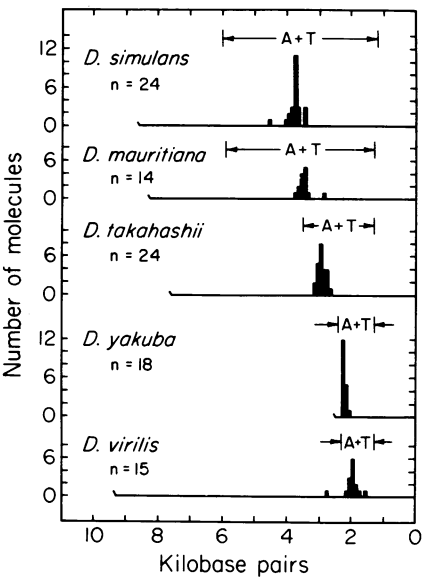

Figure 6. Histogram showing the distance of the fork interpreted as the replication origin from the nearest common EcoRI site in individual EcoRI fragments of D. simulans, D. mauritiana, D. takahashii, D. yakuba and D. virilis. The EcoRI fragment containing the replication origin is defined for each species by the long horizontal line, and the relative position of the A+T-rich region within this fragment (23) is indicated. The average length of the EcoRI fragment of D. yakuba was 6% shorter than the length of the corresponding C fragment indicated in Fig. 1. Therefore, the values shown for D. yakuba molecules were normalized to the length of the C fragment before plotting. The average distance from the replication origin to the nearest common EcoRI site in mtDNA molecules was 3.8 ± 0.2 kb for D. simulans, 3.5 ± 0.2 kb for D. mauritiana, 2.9 ± 0.1 kb for D. takahashii, 2.2 ± 0.1 kb for D. yakuba and 2.0 ± 0.3 kb for D. virilis.

molecule toward this same EcoRI site. In mtDNA molecules of D. simulans and D. mauritiana, the origin lies close to the center of the A+T-rich region, while in molecules of D. takahashii, D. yakuba and D. virilis it is more distal to the nearest common EcoRI site.

The different structural forms of the replicative molecules seen in the mtDNA preparations from D. simulans, D. mauritiana, D. takahashii, D. yakuba and D. virilis are similar to those previously observed in mtDNA preparations from D. melanogaster (9). In 60-90% of the replicative molecules observed, the second daughter segment is completely single-stranded and accounts for as much as 60-97% of the genome length. Thus, replication of the majority of molecules in all Drosophila species examined appears to be

highly asymmetrical. Asymmetric, unidirectional replication has been described in mtDNA preparations from a number of metazoan tissues and cell lines (18-20, 24-26). However, in contrast to mtDNA preparations of other metazoans, no specific class of D-loop molecules (21) has been detected in mtDNA preparations of either D. melanogaster (6, 9, 27, 28) or the five Drosophila species examined here. This may reflect a difference between mtDNA replication in Drosophila and other metazoan organisms or may simply be due to the greater susceptibility of the D-loop structure within the A+T-rich region to loss by branch migration (13) during preparation of the DNA.

The finding of the replication origin within the A+T-rich region is unusual. Studies of partial denaturation and buoyant density indicate that the A+T-rich region of D. melanogaster contains less than 5% guanine and cytosine (GC) (3-5, 7, 23). In HeLa cell mtDNA, there are 13 consecutive adenine-thymine base pairs (bp) beginning 18 bp upstream from the origin of replication. However, the GC content of the 75 bp surrounding the replication origin is 30% (29). Similarly, a 160 bp region surrounding the rat mtDNA replication origin includes a run of 14 adenine-thymine bp but has a GC content of 41% (30). In comparison, the GC content of the 422 bp fragment containing the replication origin of E. coli DNA is 42% (31, 32).

Evidence has recently been obtained from heteroduplex studies which indicates that while sequences outside the A+T-rich regions of mtDNA molecules of D. melanogaster, D. simulans, D. mauritiana, D. takahashii, D. yakuba and D. virilis have retained considerable homology, sequences within the A+T-rich regions of these species have undergone extensive divergence (23). Information concerning the degree of homology at the replication origin of these mtDNAs, however, is lacking. In this regard it is interesting to note that regions with a high degree of sequence divergence have been found at both ends of the D-loop in mtDNA molecules from sheep and goat (33).

In mtDNA molecules of D. melanogaster, the small and large mitochondrial ribosomal RNA (mt-rRNA) genes are located respectively in tandem next to one side of the A+T-rich region (27), and replication proceeds toward these sequences (10). The extensive pairing observed between sequences adjacent to the A+T-rich region in heteroduplexes of mtDNA molecules of D. melanogaster, D. simulans, D. mauritiana, D. takahashii, D. yakuba and D. virilis (23) suggests that the mt-rRNA genes occupy homologous positions in the mtDNA molecules of each species, and furthermore, that the direction of replication is toward these sequences in each case. It has been shown that the two mt-rRNA genes of rat, mouse, human and frog have a similar arrangement,

relative to the origin of replication, to that found in Drosophila mtDNA molecules. However, in contrast to what is found in Drosophila mtDNAs, in each vertebrate mtDNA studied, the direction of replication is away from the mt-rRNA genes (34-37).

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