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Origin and direction of replication in mitochondrial DNA molecules from *Drosophila melanogaster*

(electron microscopy/restriction enzymes/denaturation mapping/adenine + thymine-rich DNA)

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ABSTRACT **From a consideration of the various structural forms of partially replicated mitochondrial DNA (mtDNA) molecules from *Drosophila melanogaster* embryos observed in the electron microscope, it appears that the majority of molecules are replicated by a highly asymmetrical mode in which synthesis on one strand is up to 99% complete before synthesis on the second strand is initiated. Replication of the minority of molecules involves a more nearly symmetrical synthesis of the two complementary strands. The *D. melanogaster* mtDNA molecules have physical features with respect to which the origin and direction of replication could be mapped. These features are (*i*) a single region accounting for approximately 25% of the circular contour length and rich in adenine + thymine, and (*ii*) four *Eco*RI sites, all of which lie outside of this region. Molecules of this mtDNA were subjected to partial denaturation, *Eco*RI digestion, or partial denaturation after *Eco*RI digestion and the products were examined in the electron microscope. Complex forms interpretable as originating from replicative intermediates were observed. The size and structure of the components of these complex forms were wholly consistent with the interpretation that, in all of these mtDNA molecules, replication originates at, or close to, the center of the adenine + thymine-rich region and proceeds unidirectionally around the molecule toward the *Eco*RI site lying closest to the adenine + thymine-rich region.**

Circular mitochondrial DNA (mtDNA) molecules (molecular weight, 12.35×10^6) of *Drosophila melanogaster* are distinguished by a region, accounting for 25% of the circular contour (genome) length, that denatures at a lower temperature than does the rest of the molecule due to a high content of adenine + thymine (A+T) (1–8). By electron microscopy we have studied replicative intermediates present in *D. melanogaster* mtDNA and, using the relative location of the A+T-rich region and sites sensitive to cleavage by the restriction enzyme *Eco*RI (5) we have located the origin and determined the direction of replication in these molecules.

MATERIALS AND METHODS

The *D. melanogaster* strain used in these experiments was Oregon R-Utah (Oak Ridge, TN) (5). Growth of flies, collection of eggs, and preparation of mtDNA from embryonated eggs by use of preparative cesium chloride centrifugation were as described (5).

mtDNA was centrifuged to equilibrium in cesium chloride/ethidium bromide (9). The regions of the gradient containing two distinct fluorescent bands and a third, more diffuse, intermediately located band were collected separately and the ethidium bromide was removed as described (10). The relative concentrations of the DNA in the three regions were determined by measuring absorbance at 260 nm.

Digestion of mtDNA molecules with restriction endonuclease *Eco*RI was accomplished as described (5), except that incubations were carried out at 25° in order to minimize branch migration (11). Partial denaturation of circular mtDNA molecules and *Eco*RI-produced fragments of mtDNA molecules was also accomplished as described (12, 5), except that formaldehyde was removed before preparation for electron microscopy by passing the solution through a 1- to 2-ml column of Sephadex G-100 equilibrated with 1 mM EDTA (pH 7.5) (13).

DNA was prepared for electron microscopy by using the formamide/protein monolayer technique of Davis *et al.* (14); formamide concentrations were 40 and 10% (vol/vol) in the hyperphase and hypophase, respectively. Details of electron microscope visualization of molecules and measurements of molecules were as described (5).

Bacteriophage fd replicative form, circular, double-stranded DNA molecules [molecular weight, 4.05×10^6 (3)] and fd viral, circular, single-stranded DNA molecules or single-stranded, circular *D. melanogaster* mtDNA molecules [obtained as described (5)] were used as internal standards in all electron microscope preparations.

All confidence limits given (±) are standard deviations.

RESULTS

At equilibrium in a cesium chloride/ethidium bromide gradient, 80% (wt/wt) of the *D. melanogaster* embryonated egg mtDNA was found in the upper band, 14% in the intermediate band, and 6% in the lower band. By electron microscopy it was determined that >90% of the DNA in the upper band and >95% of the DNA in the lower and intermediate bands were in the form of circular molecules. Approximately 1% of the circular molecules in the upper and intermediate bands contained two forks and had characteristics of size and structure that indicated them to be partially replicated molecules (15–17). All of these molecules ($n = 59$) had a totally double-stranded segment (here defined as the first daughter) in the replicated region. The molecules differed in the structure of the other segment (second daughter). In 49 of them (with the replicated region ranging from 2 to 99% of the genome length), the second daughter segment was totally single-stranded. A distinct excess of replicative intermediates defined by the presence of a totally single-stranded daughter segment measuring approximately 3.5% of the genome length [a D loop (18)] was not observed. This was also the case for molecules from the lower band of the cesium chloride/ethidium bromide gradient. In six of the replicative intermediates observed, the second daughter segment was also double-stranded, and the replicated region was 90–96% of the genome length. In four of the replicative intermediates, the second daughter segment (10–98% genome length) was single-stranded at both forks but double-stranded in a central region.

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Abbreviations: mtDNA, mitochondrial DNA; A+T, adenine plus thymine.

A separate sample of *D. melanogaster* embryo mtDNA, obtained by cesium chloride centrifugation only, was prepared for electron microscopy by the aqueous protein monolayer technique (5) and scored only for replicative intermediates in which the second daughter segment was either totally or partially double-stranded. Of 156 such molecules observed, 120 were totally double-stranded. Although daughter segment lengths of molecules of this latter group ranged from 5 to 97% of the genome length, in 99 (83%) of the molecules the daughter segment lengths fell within the narrow range of 87 to 97% of the genome length. In 34 of the remaining molecules of this sample the second daughter segment was single-stranded at one fork only, and in 2 others it was single-stranded at both forks.

In formamide and aqueous preparations of mtDNA, simple (nonforked) circular molecules were observed ($n = 77$) in which a single region measuring from 2 to 77% of the genome length appeared to be single-stranded. Such molecules [gapped molecules (15)] have been observed in other metazoan mtDNAs (15–17) and appear to be the products of daughter molecule separation before DNA synthesis is complete in one or both strands (15, 19).

mtDNA from the upper and intermediate bands of the cesium chloride/ethidium bromide gradient was used in the following experiments. First, mtDNA was heated at 40° for 10 min in 0.05 M sodium phosphate/10% formaldehyde and examined in the electron microscope. Approximately 99% of the circular molecules observed exhibited the partial denaturation pattern previously described (5, 6). Each molecule contained a region of denaturation (the A+T-rich region) equal to approximately 25% of the genome length, whereas no more than 6% of the remainder of each molecule appeared denatured. Ten molecules were observed (Fig. 1) that had a more complex structure. All but one of these molecules had three forks, two double-stranded segments and three single-stranded segments; the shortest single-stranded segment had a free end. The remaining complex molecule had these same characteristics with the exception that a free-ended single-stranded segment was not apparent. The second longest single-stranded segment of each of the 10 molecules had a length (mean = 24.1 ± 1.6% genome length) consistent with it being one strand of the A+T-rich region, and the sum of the lengths of this segment and the two double-stranded segments was within the range of lengths of circular molecules in the preparation. The lengths of the free-ended single-stranded segments ranged from 5% to 15% (mean = 11.6 ± 3.0%) of the genome length. The lengths of the longest single-stranded segment varied from 26% to 71% of the genome length. These properties are what would be expected for partially denatured mtDNA molecules in which replication had proceeded unidirectionally, from a unique origin lying approximately in the center of the A+T-rich region, along one strand for 13–58% of the genome length. Whether or not the direction is the same for individual molecules is not indicated by the data. The data are inconsistent with a mode involving (*i*) unidirectional replication from a unique origin lying outside the A+T-rich region and (*ii*) replication in both directions around the molecule (bidirectional) at equal rates, originating at any unique point on the molecule.

The range of lengths of the daughter segments in the 10 partially denatured replicative intermediates observed is consistent with the expectation that denaturation of the A+T-rich region would result in loss of any strands produced by replication of less than 12.5% of the molecule and dissociation of any daughter molecules resulting from greater than 87.5% replication. The latter, as noted above, would include the majority of totally double-stranded replicative intermediates.

Next, mtDNA was digested with the restriction enzyme

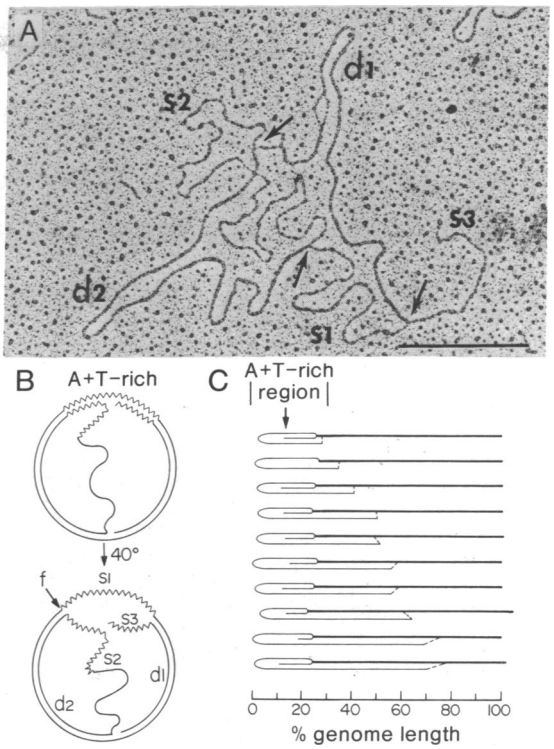

FIG. 1. Complex *D. melanogaster* mtDNA molecules from a preparation heated at 40° for 10 min in 0.05 M sodium phosphate/10% formaldehyde. (*A*) An example electron micrograph. Three forks are indicated by arrows (bar = 0.5 μm). (*B*) Diagrammatic interpretation of the molecule as a replicative intermediate. The interpretation is based on the previously determined size of the A+T-rich region (5) (wavy lines) and the relative lengths of the various segments of the 10 molecules shown in *C*. Corresponding segments of double-stranded DNA (d) and single-stranded DNA (s) in *A* and *B* are indicated by numbers. (*C*) For the purpose of comparing the molecules, each is shown as a linear rod produced by cutting the circular molecule at the fork marked f in *B* and placing this fork to the left. Double-stranded and single-stranded segments are indicated by thick and thin lines respectively. According to the interpretation given in *B* it would be expected that the length of the s2 segment and the sum of the s1 and d1 segments would be equal. The broken line at the fork to the right in each molecule indicates the difference in the lengths observed for s2 and s1 + d1.

*Eco*RI. Agarose gel electrophoretic analysis of the products revealed four fragments, A, B, C, and D, equivalent to 59, 27.5, 9, and 4.5% of the genome length, respectively, as reported (5). In electron microscope preparations, linear fragments containing two forks were located. These fragments were of three structural classes (Fig. 2). The first class (Fig. 2*A–B″*) comprised 23 fragments with a form and size consistent with their being partially replicated A fragments. The replicated region ranged from 4% to 16% of the genome length. In all fragments, one daughter segment was totally double-stranded. The second daughter segment was totally single-stranded in 18 of the fragments, totally double-stranded in 3 of the fragments, and double-stranded at one fork and single-stranded at the other in the remaining 2 fragments. In these 23 A fragments, one fork was found to be located at a constant distance (mean ± SD, 38.2 ± 3.0% genome length) from one end. This strongly suggests that, in each of the molecules from which these fragments were cleaved, replication originated at a unique position and proceeded in the same single direction around the molecule.

The second structural class (Fig. 2 *B–B″*) consisted of three fragments, each comprising a double-stranded segment the length of the A fragment that was joined by a single-stranded segment of variable length to a second double-stranded segment

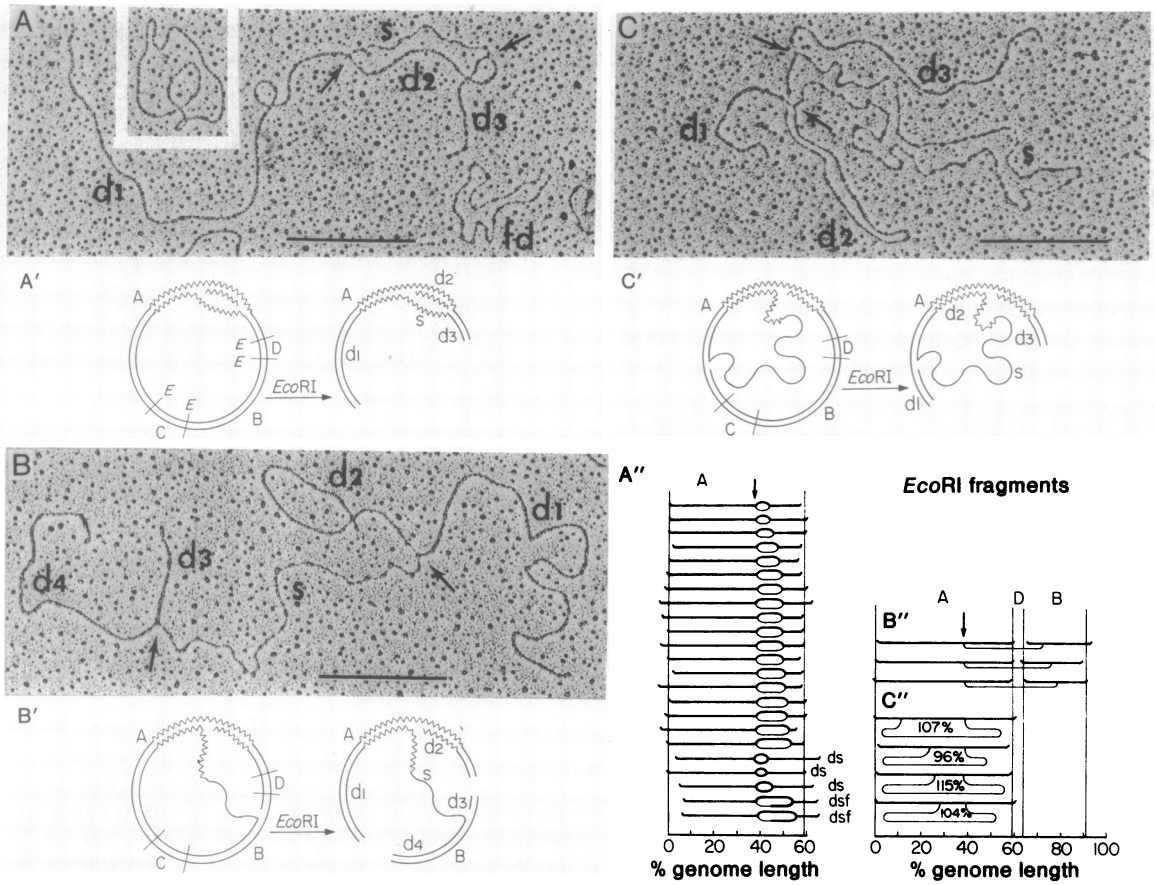

FIG. 2. Fork-containing fragments resulting from *Eco*RI digestion of *D. melanogaster* mtDNA molecules. These fragments were of three structural classes. An example of each is shown in the electron micrographs. A diagrammatic interpretation of each of these fragments as originating from a partially replicated molecule is shown in *A'*, *B'*, and *C'*. The interpretations are based on the previously determined positions of the four *Eco*RI cleavage sites in the *D. melanogaster* mtDNA molecule [*E* in *A'* (5)] and the relative lengths of the various segments of each of the three structural classes of fragments shown in *A''*, *B''*, and *C''*. The four *Eco*RI fragments, A, B, C, and D, are equal to 59, 27.5, 9, and 4.5% of the genome length (5). In *A''* and *B''*, each fragment is aligned (arrow) by the fork associated with the longer double-stranded segment (d1 in *A'* and *B'*) and this double-stranded segment is placed to the left. In *B''* the second fork of each fragment is placed to the right as shown. In *C''* each fragment is aligned (arrow) by the fork associated with the double-stranded segment (d3 in *C'*) which measures approximately 20% of the genome length, and this double-stranded segment is placed to the right. For each fragment in *C''* the combined lengths of the single-stranded loop (s in *C'*) and the double-stranded region (unreplicated portion, d2 in *C'*) which the single-stranded loop bounds are indicated. In *A* the two circular molecules are double-stranded replicative form viral fd DNA (*Inset*) and single-stranded viral fd DNA. Other details are as given in Fig. 1.

In each fragment shown in *A''* one daughter segment was totally double-stranded and the other was either totally double-stranded (ds), double-stranded at one fork and single-stranded at the other fork (dsf), or totally single-stranded (fragments not labeled). The mean length (±SD) of the parental segment left of the fork at which the fragments are aligned is 38.2 ± 3.0% genome length. A short, free-ended, single-stranded segment (a whisker) was present at one or both of the forks of 9 of the 23 fragments shown. Because these whiskers are assumed to have arisen by branch migration (11) the positions of the forks in the appropriate fragment diagrams have been corrected for their presence.

the length of the B fragment (Fig. 2*B''*). One possible arrangement for the different segments of these fragments is consistent with each having been derived from a molecule in which replication began at a point 39% of the genome length from one end of the A fragment and, at the time of *Eco*RI digestion, had proceeded unidirectionally, along one strand only, to different points on the B fragment. Whereas the replicated strand was cleaved at two positions (defining the limits of the A and D, and the D and B fragments), the unreplicated strand remained intact because single-stranded DNA is not cleaved by *Eco*RI under the conditions used (20).

The third structural class (Fig. 2 *C–C''*) consisted of four fragments each comprising a double-stranded segment the length of the A fragment, and a single-stranded loop, the two ends of which joined the A fragment. The lengths of the various segments of these fragments were again consistent with unidirectional replication from a point 39% of the genome length from one end of the A fragment but to variable positions on the A fragment of the parent molecules.

The properties of all of the fragments observed are consistent with replication originating at a unique point on the A fragment and proceeding in a single direction around the molecule. Because one of the two locations for a point 39% of the genome length from one end of the A fragment is the center of the A+T-rich region (5), these observations are in agreement with the conclusions drawn from observations on partially denatured whole mtDNA molecules (Fig. 1), that the origin of replication lies in the center of the A+T-rich region. It follows, therefore, from the data given in Fig. 2, that the direction of replication is toward the *Eco*RI site located closest to the A+T-rich region.

Finally, mtDNA was digested with *Eco*RI and the products were partially denatured. From a distribution of lengths of the linear fragments in the electron microscope preparation, it was determined that all *Eco*RI-sensitive sites of the mtDNA molecules had not been cleaved in this experiment. Among the fragments seen were complex forms containing three forks that delimited single-stranded and double-stranded segments. All of these structures could be aligned by the center of one single-stranded segment the size of the A+T-rich region, and the remaining segments could be arranged into forms expected as the products of incomplete *Eco*RI digestion and partial dena-

turation of replicative intermediates. Structures of one class (Fig. 3 *A–A′*) corresponded to the expected partial denaturation products of *Eco*RI-digestion fragments of the sort illustrated in Fig. 2 *B–B″*. In each of the four molecules from which the members of this class were derived, replication appears to have originated at a common point close to the center of the A+T-rich regions and to have proceeded along one strand of the molecule to different positions in the B fragment.

Structures of a second class (Fig. 3 *B–B″*) corresponded to the expected partial denaturation products of *Eco*RI-digestion fragments of the sort shown in Fig. 2 *C–C″*. Again, members of this class appear to have been derived from molecules in which replication originated close to the center of the A+T-rich region but, prior to digestion, proceeded around the molecule into the A fragment in four cases and into the C fragment in a fifth case.

Two structures found in these preparations contained two free-ended single-stranded segments (Fig. 3 *C–C″*). Each of these fragments could be interpreted (Fig. 3 *C′* and *C″*) as an A (or A+C) fragment derived from a molecule in which partial replication of *both* strands had occurred, having originated in the A+T-rich region and, at the time of *Eco*RI digestion, having proceeded to a point within one of the other fragments. Structures corresponding to these were not located among products of either partial denaturation or *Eco*RI digestion alone (Figs. 1 and 2).

In these preparations, a class of fragments that could be interpreted as originating from molecules less than 20% replicated (and corresponding to the large class of *Eco*RI-produced A fragments shown in Fig. 2 *A–A″*) was not observed. Absence of structures resulting from molecules less than 12.5% (and >85%) replicated was expected for the reasons given above.

DISCUSSION

From a consideration of the frequencies of the various partially replicated molecules in embryonic *D. melanogaster* mtDNA, it appears that, in the majority of molecules, synthesis of a considerable length (up to 99%) of one strand occurs before synthesis on the other strand is initiated. Such an asymmetrical mode of replication seems to be operative in mtDNAs of tissues of various organisms (16, 17, 21) and tissue culture cell lines (15, 20, 22–24). In a minority of *D. melanogaster* mtDNA molecules, as evidenced by observation of molecules in which syn-

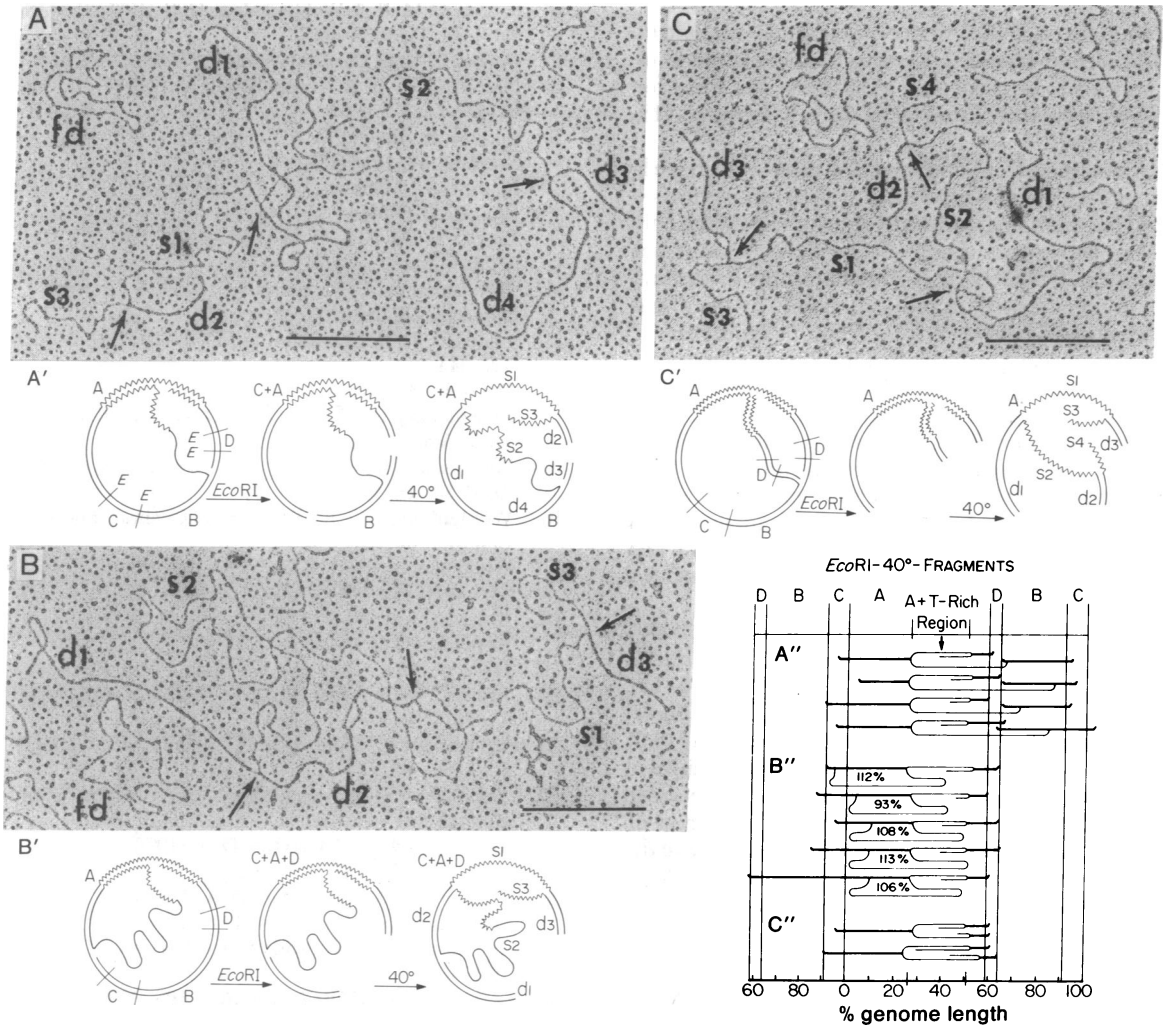

FIG. 3. Complex fragments from preparations of *D. melanogaster* mtDNA molecules digested (incompletely) with *Eco*RI, followed by heating at 40° for 10 min in 0.05 M sodium phosphate/10% formaldehyde. The fragments were of three structural classes. An example of each is shown in the electron micrographs. A diagrammatic interpretation of each of these fragments as originating from a partially replicated molecule is shown in *A′*, *B′*, and *C′*. The interpretations are based on the relative positions of the *Eco*RI sites (*E* in *A′*) and the A+T-rich region (5) and the relative lengths of the various segments in each of the three structural classes of fragments shown in *A″*, *B″*, and *C″*. In *A″–C″* the fragments are aligned (arrow) by the midpoint of the single-stranded segment (s1 in *A′–C′*) of each molecule which has a length consistent with it being one strand of the A+T-rich region, and the free-ended single-stranded segment (s3 in *A′–C′*, as well as s4 in *C′*) is placed to the right. Other details are as given in Figs. 1 and 2.

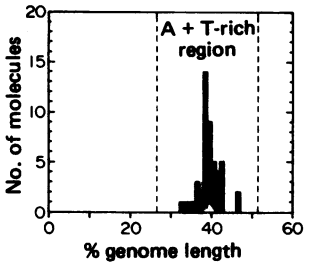

FIG. 4. Location of the replication origin on the *Eco*RI A fragment. The data are a composite of the distance between the fork by which each A fragment is aligned and the left end of the fragments in Fig. 2*A″*, *B″*, and *C″* and the lengths of the free-ended single-stranded segment of each molecule and fragment in Figs. 1*C* and 3*A″* and *B″*, respectively. The latter are plotted as a fraction of the standardized length of the A+T-rich region (25% genome length), in a left-hand direction from the right boundary of the A+T-rich region indicated at 51% in the diagram. The center of the A+T-rich region is 39% of the genome length from the left end (indicated by the open triangle on the abscissa) of the A fragment (5). The mean (± SD) of the values shown is 38.9 ± 2.1% genome length; $n = 48$.

thesis was complete on 5–97% of one strand and complete or partial on the second strand, a more symmetrical mode of replication appears to be used, such as has been described for some mtDNA molecules of tissues taken directly from rats (16, 17) and sea urchins (21).

In mtDNA obtained from embryos and tissue culture cells of *D. melanogaster*, Rubenstein *et al.* (25) observed replicative intermediates that were predominantly duplex, containing only short single-stranded regions near the forks, and concluded that both daughter strands are replicated simultaneously. In contrast, Zakian (26) found evidence for replication only by the asymmetrical mode in mtDNA taken from *D. virilis* embryos.

Our present data clearly indicate that in all molecules the site of initiation (origin of replication) is located at or very close to the center of the A+T-rich region (Fig. 4) and that replication proceeds around the molecule in the direction of the *Eco*RI site nearest to the A+T-rich region. Unidirectional replication from an origin located at a unique site on the molecule has been demonstrated for asymmetrically replicated molecules of various mtDNAs (20, 22–24) and for both asymmetrically and more symmetrically replicated molecules of rat liver mtDNA (27).

D loop-containing molecules have been found in mtDNA from many metazoans, and the D loop has been identified as the first step in replication of mouse L-cell mtDNA (18, 19). Failure to find a distinct excess of D loop-containing *D. melanogaster* mtDNA molecules in this study and previously (5, 7, 25) may indicate a real difference between the mechanisms of replication operative in these organisms and other metazoan mtDNAs studied, as recently suggested by Rubenstein *et al.* (25). However, it is not ruled out that the low resistance to denaturation of the A+T-rich region contributes to loss of D loops by branch migration (11, 28) at some stage in the isolation procedure of this mtDNA.

The function of the A+T-rich region of *D. melanogaster* mtDNA molecules is unknown. A region with similar denaturation properties, but of lesser, variable size, has been demonstrated in mtDNA molecules from a number of *Drosophila* species (6) but not in other metazoan mtDNAs. Data from denaturation studies and buoyant density analysis (1, 2, 4–8) of the A+T-rich region of *D. melanogaster* mtDNA molecules indicate that very little guanine + cytosine can be present. (There is no reason to suspect, however, that the sequence defining the origin of replication lacks these bases completely.) It also appears that there is little or no base bias between the complementary strands of the A+T-rich region (8).

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