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Extensive diversity among Drosophila species with respect to nucleotide sequences within the adenine + thymine-rich region of mitochondrial DNA molecules

Christianne M.-R.Fauron and David R.Wolstenholme

Department of Biology, University of Utah, Salt Lake City, UT 84112, USA

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ABSTRACT

Mitochondrial DNA (mtDNA) molecules from species of the genus Drosophila contain a region exceptionally rich in adenine + thymine (A+T). Using agarose gel electrophoresis and electron microscopy, we determined that in the mtDNA molecules of D. melanogaster, D. simulans, D. mauritiana, D. yakuba, D. takahashii, and D. virilis, the A+T-rich regions, which are 5.1, 4.8, 4.6, 1.1, 2.2, and 1.0 kilobase pairs in size, respectively, are at homologous locations relative to various common EcoRI and HindIII cleavage sites. Under conditions highly permissive for base pairing (35% formamide), heteroduplexes were constructed between EcoRI fragments and whole circular molecules of mtDNAs of the above mentioned six species in a variety of combinations. Complete pairing of molecules outside the A+T-rich region was found in all heteroduplexes examined. However, in contrast, A+T-rich regions of the different species failed to pair in all but those combinations of mtDNAs involving the three most closely related species. In heteroduplexes between D. melanogaster and D. simulans, and between D. melanogaster and D. mauritiana mtDNAs, up to 35% of the A+T-rich regions appeared double-stranded. These data indicate that much more extensive divergence of sequences has occurred in A+T-rich regions than in other regions of Drosophila mtDNA molecules.

INTRODUCTION

Mitochondrial DNA (mtDNA) molecules isolated from species of the genus Drosophila are unique among mtDNA molecules of metazoans studied to date, in that they contain a single region exceptionally rich in adenine and thymine (A+T) (2, 6, 11, 12, 16, 17, 20, 21). This region is constant in size in mtDNA of a single Drosophila species (6, 12, 20). However, A+T-rich regions of mtDNA molecules of different species of the melanogaster group vary from 1.1 kilobase pairs (kb) in D. yakuba to 5.1 kb in D. melanogaster (20, 21). From a consideration of the sizes of whole mtDNA molecules it appears likely that mtDNA molecules of Drosophila species, other than those of the melanogaster group, have an A+T-rich region close to the size of that of D. virilis mtDNA, which is approximately 1 kb (6, 21). Recently it has been shown that the A+T-rich region of mtDNA molecules of a number of Drosophila species contains the molecule's single origin of replication (9, 10).

Using mapping of sites sensitive to cleavage by restriction enzymes and heteroduplex analysis, we have studied relationships between A+T-rich regions found in mtDNA molecules of different species of the melanogaster group, D. melanogaster, D. simulans, D. mauritiana, D. yakuba, and D. takahashii, and in mtDNA molecules of D. virilis, chosen as a taxonomically more distant species. The results of these studies are the subject of this paper.

MATERIALS AND METHODS

Fly stocks. The D. melanogaster strain used in these experiments was Oregon R-Utah (Oakridge TN) (20). The following species (stock number and place of origin) were obtained from the Species Stock Collection of the Genetics Foundation, University of Texas at Austin: D. simulans (3015.8; Nueva, California); D. yakuba IC (2371.6, Ivory Coast); D. virilis (2375.8, Chile); D. takahashii (3075.4, Wulai, Taiwan). The D. mauritiana I strain used was derived from a single fertilized female of a stock (Mauritius Island) provided by Dr. L. Tsacas, Centre National de la Recherche Scientifique, Gif-sur-Yvette, France.

Preparation of mtDNA, ultracentrifugation and partial denaturation. Details of the following techniques were as described previously (8, 9, 20): growth of flies, collection of eggs, preparation of mtDNA from embryonated eggs or from ovaries; preparative cesium chloride and cesium chloride ethidium bromide equilibrium buoyant density centrifugation of mtDNAs (including fractionation of the resulting gradients, removal of ethidium bromide and buoyant density determinations from refractive indices); partial denaturation of circular mtDNA molecules and of restriction enzyme-produced fragments of mtDNA molecules.

Digestion with restriction endonucleases. EcoRI, HindIII and HpaI restriction endonucleases were obtained from New England Biolabs, and Miles Research Laboratories. Digestion conditions were those given by the manufacturer and in reference 20.

Electrophoresis. Electrophoresis of restriction enzyme digestion products was carried out using either horizontal 1%, or 1.8% agarose slab gels (100 V, 3 hr) or vertical composite acrylamide: agarose (3%: 0.5%) slab gels (25 mA, 12 hr) (15) in a buffer containing 40 mM Tris-base, (pH 7.8), 20 mM sodium acetate, 2 mM EDTA, (14) at room temperature. Other details including staining of gels and photography were as described previously (20).

Heteroduplex construction. Heteroduplexes were constructed by first denaturing the DNAs (total concentration <5 μg/ml) by dialysis against a solu-

tion containing 95% formamide and 10 mM EDTA (pH 8.2) for 1 hr, and then renaturing the DNAs by dialysis against a solution containing 35% formamide, 100 mM Tris-HCl (pH 8.2) and 10 mM EDTA for 2 to 2.5 hr (5).

Electron microscopy. DNA was prepared for electron microscopy either by the aqueous protein monolayer technique (8, 20) or by the formamide protein-monolayer technique of Davis et al. (5); formamide concentrations were 35% and 5%, or 50% and 20% (vol/vol) in the hyperphase and hypophase respectively. Other details of electron microscopy were as described previously (8, 20). Estimations of sizes of whole molecules, of restriction fragments, of circularized restriction fragments, and of single- and double-stranded regions of molecules were made using bacteriophage fd single-stranded DNA and double-stranded RF DNA (6,408 bp, [1]) as internal standards. Estimations of sizes of single-stranded regions in partially denatured molecules prepared by the aqueous protein monolayer technique were made using a correction factor as described by Wolstenholme and Fauron (20). All confidence limits given (±) are standard deviations (SD), and the number of observations in each case is given by n.

Mapping of restriction endonuclease cleavage sites and A+T-rich regions. The positions of EcoRI cleavage sites relative to the A+T-rich region, and, separately, of the HindIII cleavage sites relative to the A+T-rich region in mtDNA molecules of D. melanogaster, D. mauritiana, D. simulans, D. takahashii, D. yakuba, and D. virilis were determined by gel electrophoresis, and electron microscopy as described (20). For each species the relative positions of EcoRI sites and HindIII sites on mtDNA molecules were determined by comparing the lengths of fragments produced by sequential digestion of the mtDNA with EcoRI and HindIII, with the two alternative sets of fragment lengths which can be predicted to result from such a digest when the EcoRI and HindIII circular maps are superimposed on one-another, with the A+T-rich regions coinciding, in the two possible relative circular orientations.

The relative positions of EcoRI and HindIII sites which mapped close to each other were ascertained by incubating the fragments produced by sequential EcoRI and HindIII digestion with DNA ligase isolated from T4-infected E. coli (Miles Research Laboratories) (4), and comparing the lengths of circular molecules produced with the lengths of circular molecules predicted for each of the two alternative relative positions of each pair of EcoRI and HindIII sites in question. This test is based on the prediction that the terminal single-stranded segment (5' AATT) produced by EcoRI digestion will not pair with the single-stranded segment (5' AGCT) produced by HindIII digestion and,

therefore, that a circular molecule will only result from a fragment both ends of which are the product of digestion by a single enzyme.

A single HpaI site in D. melanogaster mtDNA and in D. virilis mtDNA was mapped from analyses of electrophorograms of separate and sequential HpaI and EcoRI, and HpaI and HindIII digests on the respective mtDNA.

RESULTS

Restriction maps. The relative locations of EcoRI and HindIII cleavage sites, and of the A+T-rich region in mtDNA molecules of the six Drosophila species studied are shown in Fig. 1. When the parts of the molecules outside the A+T-rich regions are aligned, and oriented so as to maximize the coincidence of restriction sites, it appears that all molecules have three EcoRI sites and two HindIII sites in common. The five species of the melanogaster

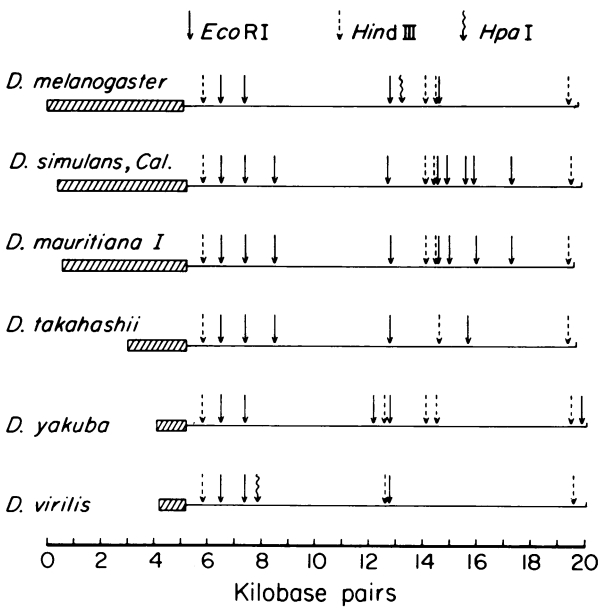

Figure 1. Maps of the mitochondrial genomes of the six Drosophila species indicated, showing the relative positions of the A+T-rich region (hatched areas) and the sites at which the restriction endonucleases EcoRI and HindIII cleave. HpaI-sensitive sites have been mapped only in D. melanogaster and D. virilis mtDNA molecules. The genomes have been oriented so as to maximize the coincidence of enzyme sensitive sites, and then aligned by the common EcoRI site nearest the A+T-rich region. This common site defines the right end of the A+T-rich region in the figure and all molecules are linearized at what is then the left end of the A+T-rich region.

group have one further HindIII site in common. With the exception of the two EcoRI sites which are unique to D. yakuba mtDNA, all EcoRI and HindIII sites are found in mtDNA molecules of more than one species. If these inferences of shared restriction sites and corresponding map homologies are correct, it follows that the A+T-rich region occupies an homologous position in mtDNA molecules of all species studied. It may be further noted from Fig. 1, that the regions outside the A+T-rich region of each of these species vary in size by not more than 0.8 kb.

Heteroduplex analyses. To gain further information on the degree of homology, both outside and within the A+T-rich regions, among the mtDNA molecules of different Drosophila species, we constructed a series of heteroduplexes. First, a sample of D. yakuba mtDNA, shown by electron microscopy to comprise >90% circular molecules (the remainder being linear molecules, heterogeneous in length) of which approximately 80% were nicked, was mixed with a five-fold excess of fragments obtained by EcoRI digestion of covalently closed, circular mtDNA of D. melanogaster. The mixture was dialysed against 95% formamide to denature the DNAs, and a sample removed for electron microscope examination to confirm the completion of denaturation. The denatured DNA mixture was then dialysed against 35% formamide, and samples removed at various times, and prepared for electron microscopy by the formamide protein monolayer technique. The sample in which approximately 50% of the DNA was renatured was used for further analysis. Circular DNA molecules containing double-stranded segments were located and photographed, and analysed in regard to the lengths of the component double-stranded and single-stranded segments.

Some of these selected molecules contained only a single double-stranded segment. In a collection of 29 such molecules the double-stranded segment measured 5.3 $\pm$ 0.2 kb in 7, 1.7 $\pm$ 0.2 kb in 9, and 0.9 $\pm$ 0.1 kb in 9. These lengths are similar to the lengths of the D. melanogaster B, C, and D EcoRI fragments, respectively (Fig. 1). In the remaining four molecules in this sample, the double-stranded segment measured 6.4 kb (n = 2), 7.3 kb and 8.2 kb, similar to the lengths expected for combinations of the C + B, B + D and C + B + D EcoRI fragments of D. melanogaster mtDNAs, respectively. In none of the double-stranded segments of any of these 29 heteroduplex molecules were single-stranded (deletion/insertion) loops, or separated single-strands observed.

Also, among the located circular molecules were ones which contained a single segment in which the strands were separated, and bounded on each side

by apparently totally double-stranded segments (Fig. 2A). The sizes of the separated strands, and of the double-stranded segments (Fig. 3) measured in 17 such molecules were consistent with the interpretation that they were produced by pairing of a circular strand of D. yakuba mtDNA and a strand from that fragment of EcoRI-digested D. melanogaster mtDNA that contains the A+T-rich region (the A fragment). The mean lengths of the two double-stranded

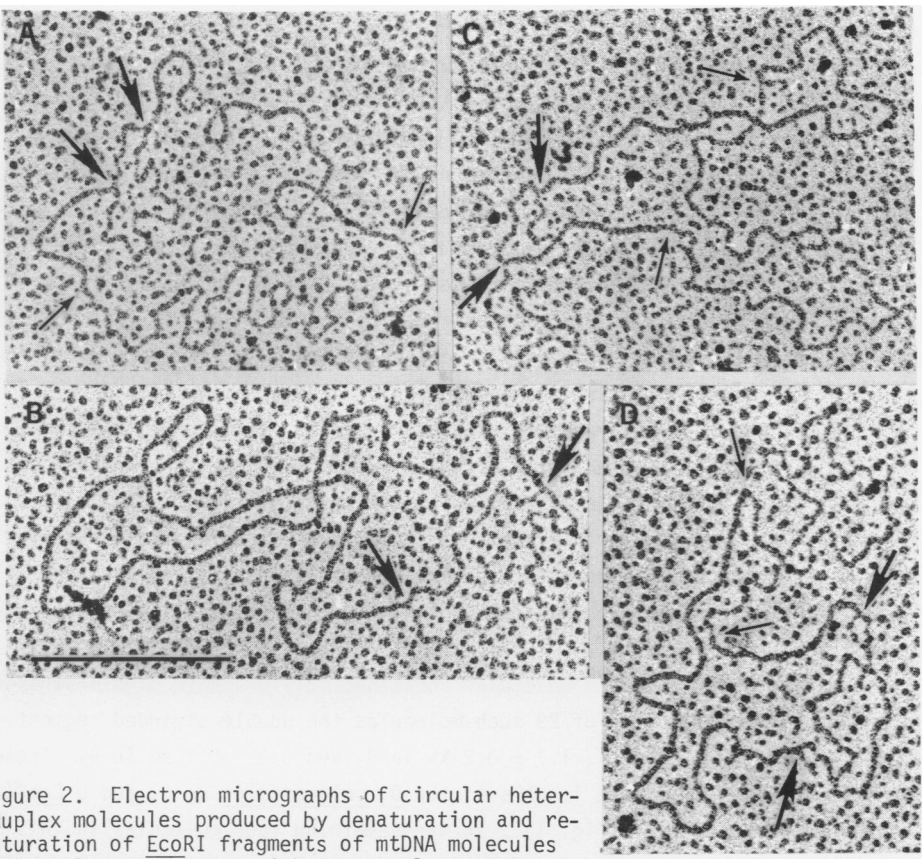

Figure 2. Electron micrographs of circular heteroduplex molecules produced by denaturation and renaturation of EcoRI fragments of mtDNA molecules of D. melanogaster and nicked, circular mtDNA molecules of A, D. yakuba; B, D. takahashii; C, D. virilis; and D, D. simulans. The larger arrows in each micrograph indicate the limits of the regions of strand separation of each molecule. The smaller arrows in A, C, and D indicate the limits of the double-stranded regions which correspond to the regions lying outside the A+T-rich region of the EcoRI fragment of D. melanogaster and the complementary regions of the D. yakuba, D. virilis, and D. simulans mtDNA molecule in A, C, and D respectively. In the molecule shown in B, with the exception of the single unpaired region, the entire molecule is double-stranded and compares in length to the whole mtDNA molecule of D. takahashii lying outside the A+T-rich region. The bar represents 0.5 μm.

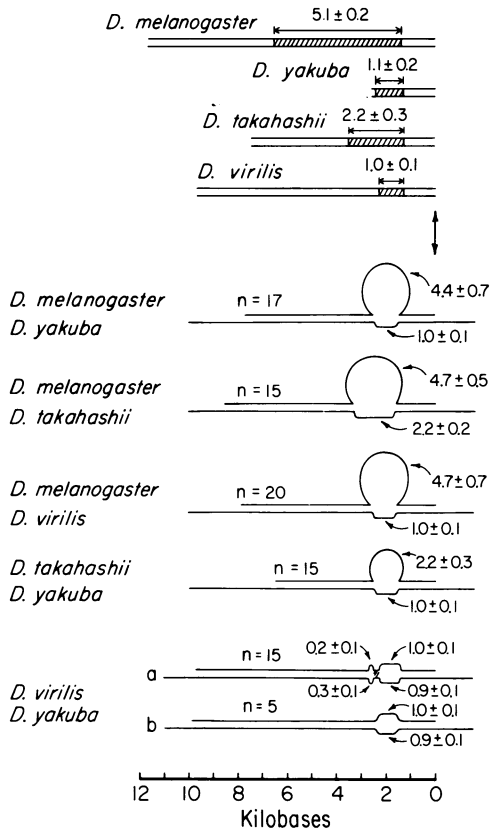

Figure 3. Data concerning heteroduplexes formed between an EcoRI fragment of mtDNA of one Drosophila species and a circular molecule of mtDNA of a different species. The EcoRI fragment containing the A+T-rich region (hatched areas) of each species is shown in the upper part of the diagram. The structures of the heteroduplexes are summarized for different species combinations (indicated on the left) in the lower part of the diagram. In each heteroduplex, parallel lines represent apparently double-stranded segments. The loops represent unpaired segments. The upper line represents the EcoRI fragment drawn to scale, and the lower line represents the single-stranded circular molecule which has been linearized for convenience of illustration, and drawn to scale only within the limits of the complementary EcoRI fragment. The heteroduplexes are shown aligned (double headed arrow) by the end of each EcoRI fragment closest to the single-stranded loop(s) in each case, as this point is taken to represent the EcoRI site which lies at a similar distance from the right end of the A+T-rich region (Fig. 1) in all mtDNAs studied. Heteroduplexes observed following annealing of D. virilis EcoRI fragments and D. yakuba circular molecules, contained either a single unpaired segment (b) or two distinct unpaired segments (a). The sizes (± SD) of the unpaired loops in the lower series of diagrams are given in kilobases.

segments (1.4 ± 0.3 kb, and 5.5 ± 0.5 kb) are similar to the lengths of the two regions of the D. melanogaster EcoRI A fragment outside the A+T-rich region (1.35 ± 0.2 kb, n = 30, and 5.2 ± 0.3 kb, n = 30; Fig. 1). The actual length of each single strand is shorter (4.4 ± 0.7 kb, and 1.0 ± 0.1 kb, Fig. 3) than the sizes determined for the A+T-rich region of D. melanogaster and D. yakuba mtDNA (5.1 ± 0.2 kb, n = 30, 1.1 ± 0.2 kb, n = 30, respectively) in partial denaturation experiments (Figs. 1 and 3). However, in partially denatured D. melanogaster mtDNA, prepared for electron microscopy by the formamide protein-monolayer technique, the mean lengths of the single-stranded A+T-rich regions have been found to vary among preparations (by as much as 16%) far more than the lengths of the double-stranded segments (Fig. 1; Refs 12, 9, Picard & Wolstenholme, unpublished). Therefore, the present findings are consistent with the interpretation that the separated strands in heteroduplexes represent the entire A+T-rich regions of D. melanogaster and D. yakuba mtDNAs.

Next, in experiments similar to those described for D. melanogaster and D. yakuba mtDNA, heteroduplexes were constructed between mtDNAs of D. melanogaster and D. takahashii, D. melanogaster and D. virilis, D. takahashii and D. yakuba, D. virilis and D. yakuba, D. melanogaster and D. simulans, D. melanogaster and D. mauritiana, and D. simulans and D. mauritiana. In each experiment EcoRI fragments of the mtDNA of the first named species were used together with samples containing >75% whole, nicked circular mtDNA molecules of the second named species. For each species combination different heteroduplexes were located which represented the pairing of a strand from each of the EcoRI fragments of one species with a circular strand of the second species. In all heteroduplexes involving EcoRI fragments, other than those containing the A+T-rich region, complete pairing of the strands was observed. In contrast, in all heteroduplexes of D. melanogaster and D. takahashii, D. melanogaster and D. virilis, and D. takahashii and D. yakuba which included an A+T-rich region-containing EcoRI fragment, a single unpaired segment was observed (Fig. 2b and C). Length measurements (Fig. 3) of these heteroduplexes were again consistent with the unpaired segments being the entire A+T-rich regions of the component mtDNA molecules. In all heteroduplexes which included a D. virilis A+T-rich region-containing EcoRI fragment and a D. yakuba circular molecule, an unpaired segment which could be interpreted as containing the entire A+T-rich region of each of the two molecules was also found. However, in the majority of such heteroduplexes (Fig. 3) a second small unpaired segment was observed at the left end of the A+T-rich region.

The resolution of our technique is not sufficient to allow us to determine whether this small unpaired segment and the paired region separating it from the larger unpaired segment lie inside or outside the A+T-rich region.

In each of the renatured preparations from which the data presented above was obtained, we also found totally double-stranded circular molecules the size of the undigested mtDNA, and totally double-stranded linear molecules of each size expected for the EcoRI fragments, including those containing the A+T-rich region, of the digested mtDNA. Since we confirmed denaturation of all DNA in the mixtures prior to annealing, these forms must represent renatured products, and their presence rules out the possibility that the annealing conditions were insufficient to allow renaturation of A+T-rich DNA. Examination of these molecules interpreted as being reannealed, homoduplex products, did not reveal evidence of deletion/insertion loops or of unpaired regions that might be expected if differences in sequence occurred among mtDNA molecules within a single Drosophila species. Further, the preparations containing EcoRI-digested D. melanogaster mtDNA were carefully examined for the presence of linear or circular molecular forms which could have resulted from the pairing of different parts of the A+T-rich region, such as might occur if this region comprised a tandemly repeated sequence. No such forms were found.

The possibility that in each heteroduplex the circularity of one molecule was in some way contributing to failure of pairing of the A+T-rich regions seems unlikely from the results of the following experiments. D. melanogaster and D. yakuba mtDNAs, and separately, D. melanogaster and D. virilis mtDNAs, were mixed in approximately equal amounts and digested with EcoRI. The digestion products were then denatured and annealed as described above and prepared for electron microscopy. Molecules were observed in each preparation which had properties of size and structure consistent with their being heteroduplexes of the A+T-rich region-containing EcoRI fragments of the two respective species, in which the A+T-rich regions were completely unpaired.

The properties of strandedness and size of heteroduplexes of D. melanogaster and D. simulans, and of D. melanogaster and D. mauritiana mtDNAs which included an A+T-rich region-containing EcoRI fragment are summarized in Figs. 4 and 5. As in the species combinations described above, the regions of the restriction fragment outside the A+T-rich region appeared completely paired. However, in contrast to what was found for other species combinations, segments totalling as much as 35% of the D. melanogaster A+T-rich region were

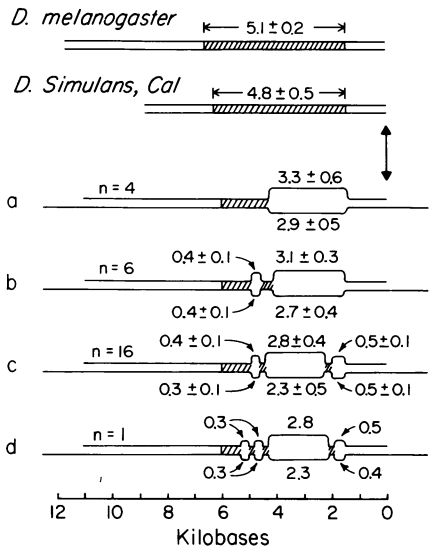

Figure 4. Data concerning heteroduplexes found following annealing of EcoRI fragments of D. melanogaster mtDNA, and circular molecules of D. simulans mtDNA. The EcoRI fragments containing the A+T-rich region of each mtDNA are shown in the upper part of the diagram, and the heteroduplexes, classified (a, b, c and d) in regard to the number of unpaired segments they contained, are shown in the lower part of the diagram. Hatched areas indicate paired segments within the A+T-rich region. The mean sizes (± SD) of unpaired loops only are indicated. Other details are as given for Fig. 3.

found to be paired. Heteroduplexes of D. melanogaster and D. simulans mtDNAs contained 1, 2, 3, or 4 unpaired segments within the A+T-rich region (Fig. 4). In all of these heteroduplexes a 0.5 kb segment at the right end of the A+T-rich region was unpaired, as was a more centrally located segment of approximately 2.8 kb. The left 0.8 kb segment of the A+T-rich region in all heteroduplexes appeared double-stranded.

In all heteroduplexes of D. melanogaster and D. mauritiana mtDNAs, the left 1.7 kb segment of the A+T-rich region was double-stranded (Fig. 5). In two thirds of these structures, a small paired segment of approximately 0.4 kb also occurred towards the right end of the A+T-rich region.

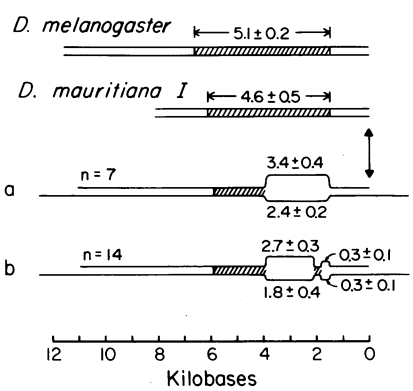

Figure 5. Data concerning heteroduplexes found following annealing of EcoRI fragments of D. melanogaster mtDNA, and uncleaved circular molecules of D. mauritiana mtDNA. The EcoRI fragments containing the A+T-rich region (hatched area) of each mtDNA are shown in the upper part of the diagram, and the heteroduplexes classified (a and b) in regard to whether they contained one or two unpaired segments are shown in the lower part of the diagram. Other details are as given for Figs. 3 and 4.

In heteroduplexes formed between the A+T-rich region-containing EcoRI fragment of D. simulans mtDNA molecules and whole circular D. mauritiana mtDNA molecules, the A+T-rich regions appeared to be completely double-stranded.

DISCUSSION

The data presented establish that the A+T-rich regions of mtDNA molecules of D. melanogaster, D. simulans, D. mauritiana, D. yakuba, D. takahashii, and D. virilis are at homologous locations with respect to shared sequences in parts of the molecules outside the A+T-rich regions.

The observation that all regions of mtDNA molecules outside the A+T-rich region (with one possible exception, Fig. 3) were totally double-stranded in all heteroduplexes examined, indicates that outside the A+T-rich region none of the six mtDNAs studied differ from each other by the presence or absence of a sequence of more than about 50 base pairs, the approximate resolution of the technique employed (5). The same observations argue against gross differences among the Drosophila species in the arrangement of mtDNA sequences outside the A+T-rich region. The observed differences in sensitivity to restriction enzymes of mtDNAs from the different species could result from single base pair differences at specific sites in the molecules. More detailed information concerning sequence relationships in the regions outside the A+T-rich regions of these mtDNA molecules cannot be derived from our data, as the annealing conditions used to produce heteroduplexes were designed to encourage maximum stability of paired regions.

Duplex formation between A+T-rich regions was found only for mtDNA molecules of D. melanogaster, D. simulans, D. mauritiana, three closely related species of the melanogaster subgroup. The degree of pairing between A+T-rich regions of these species follows their relatedness as indicated by taxonomic criteria (3), comparisons of larval salivary gland chromosome bands, and the extent to which they are capable of interbreeding (13).

At variance with our observation that no more than 35% of the A+T-rich regions of mtDNA molecules of D. melanogaster and D. simulans form stable duplexes, Shah and Langley (18) reported complete pairing of these regions under annealing conditions (40% formamide) less permissive than those used by us. However, it is unclear from a consideration of the experimental procedures used by Shah and Langley (18) how they could have distinguished homoduplexes of mtDNA of D. melanogaster and D. simulans, from heteroduplexes of these two mtDNAs.

In the present experiments, complete lack of pairing was observed for A+T-rich regions of D. melanogaster, D. yakuba, D. takahashii, and D. virilis mtDNAs. D. yakuba is the most closely related of these species to D. melanogaster, being a further member of the melanogaster subgroup. D. takahashii is more distantly related, being classified in a different subgroup (takahashii) within the melanogaster group. D. virilis was chosen because it is a member of a different subgenus (Drosophila) than that of the other species (Sophophora) studied. There have been two other recent reports of failure of pairing of the A+T-rich regions in heteroduplexes of D. melanogaster and D. virilis mtDNA. In agreement with our findings, Shah and Langley (19) could not detect any sequence homology between A+T-rich regions of these mtDNAs. Zakour and Bultmann (22), however, interpreted their data as indicating some sequence homology between 0.5 kb of the A+T-rich regions of D. melanogaster and D. virilis mtDNAs.

We previously suggested from a consideration of sizes of the A+T-rich regions of mtDNA molecules of eight Drosophila species (6) that the larger A+T-rich regions of mtDNA molecules of melanogaster group species might each comprise a tandemly repeated sequence. Our present observations on renaturation products of EcoRI fragments of D. melanogaster mtDNA failed to provide evidence in support of this hypothesis. However, in view of the extensive differences of sequences in A+T-rich regions of mtDNAs, even of closely related species, the possibility remains that the A+T-rich regions of D. melanogaster mtDNA and of other melanogaster group mtDNAs did in fact arise as a tandemly repeated sequence.

Neither the results of our restriction enzyme studies nor the results of our renaturation studies provided evidence for sequence differences among mtDNA molecules of flies within any of the individual strains of each species used in these studies. However, restriction cleavage site differences have recently been found between mtDNA molecules from different strains of some Drosophila species (19; Fauron and Wolstenholme, unpublished).

Although the A+T-rich region of the mtDNA of all the species studied contains the origin of replication (9, 10) the function of the remainder of this region is unknown. The guanine + cytosine content of the A+T-rich region of D. melanogaster mtDNA is indicated from the results of thermal melting studies (2, 11, 17) and buoyant density studies (7, Fauron and Wolstenholme, unpublished) to be between 0 and 7.6%. In view of this, the possibility that the A+T-rich region contains sequences coding for proteins seems unlikely. Finally, both the assignment of a function to the A+T-rich region and models

for its evolution (19) must take into account the extensive differences in its sequence (reported here) and size (6, 21) among Drosophila species, and the rigid conservation of its sequence and size within individual strains.

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