INFORMATION TO USERS

This was produced from a copy of a document sent to us for microfilming. While the most advanced technological means to photograph and reproduce this document have been used, the quality is heavily dependent upon the quality of the material submitted.

The following explanation of techniques is provided to help you understand markings or notations which may appear on this reproduction.

1. The sign or "target" for pages apparently lacking from the document photographed is "Missing Page(s)". If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting through an image and duplicating adjacent pages to assure you of complete continuity.

2. When an image on the film is obliterated with a round black mark it is an indication that the film inspector noticed either blurred copy because of movement during exposure, or duplicate copy. Unless we meant to delete copyrighted materials that should not have been filmed, you will find a good image of the page in the adjacent frame.

3. When a map, drawing or chart, etc., is part of the material being photographed the photographer has followed a definite method in "sectioning" the material. It is customary to begin filming at the upper left hand corner of a large sheet and to continue from left to right in equal sections with small overlaps. If necessary, sectioning is continued again—beginning below the first row and continuing on until complete.

4. For any illustrations that cannot be reproduced satisfactorily by xerography, photographic prints can be purchased at additional cost and tipped into your xerographic copy. Requests can be made to our Dissertations Customer Services Department.

5. Some pages in any document may have indistinct print. In all cases we have filmed the best available copy.
ZAKOUR, RICHARD ALLAN
EVOLUTION OF MITOCHONDRIAL DNA IN THE GENUS DROSOPHILA.

RICE UNIVERSITY, PH.D., 1979
RICE UNIVERSITY

EVOLUTION OF MITOCHONDRIAL DNA IN
THE GENUS DROSOPHILA

by

RICHARD ALLAN ZAKOUR

A THESIS SUBMITTED
IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF

Doctor of Philosophy

THESIS DIRECTOR'S SIGNATURE

[Signature]

HOUSTON, TEXAS
JULY, 1978
ACKNOWLEDGEMENTS

I wish to express sincere gratitude to a number of people for their contributions to my research and thesis. To Drs. Charles Stewart and Donald Robberson for valuable advice during the course of my graduate career and research. To Drs. Stephen Subtelny, Roger Storck, and Fredrick Rudolph for critical reading of this thesis. To Drs. James Cregg, Seth Hootman, Virginia Sawin, and Raymond Slomiany for stimulating conversations, scientific and otherwise. To Messrs. Martin Sosland, Bruce Hughes, and Ms. Lynn Richardson for technical and/or moral assistance. To the "Zoo" for much needed diversions. To my family and most especially to Ms. Helen Robinson for all the faith and moral support that one could ever hope to receive.

The role of Dr. Hermann Bultmann during my graduate career has been immense. Without his dedicated guidance, patience, advice, and criticism, this work would not exist. Beyond being a thesis advisor, Dr. Bultmann has greatly influenced my scientific growth and development. As a sagacious abecedarian of recondite omniscience and *jus divinum*, Herr Professor Bultmann has, in his ineffable way, expostulated the inexorable necessitude to eschew obfuscation as I have purported to expound upon multifarious ramifications of arcane edification. For these reasons and much more, I am sincerely grateful to Hermann for a friendship that transcends a teacher-student relationship.
DEDICATION

To the memory of my Father

Charles N. Zakour
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>3</td>
</tr>
<tr>
<td>Sources of DNA</td>
<td>3</td>
</tr>
<tr>
<td>Isolation of mitochondrial DNAs</td>
<td>3</td>
</tr>
<tr>
<td>Bacteriophage DNAs</td>
<td>7</td>
</tr>
<tr>
<td>DNA Reassociation</td>
<td>7</td>
</tr>
<tr>
<td>Electron Microscopy</td>
<td>8</td>
</tr>
<tr>
<td>Calibration of DNA Contour Lengths</td>
<td>9</td>
</tr>
<tr>
<td>Denaturation Maps</td>
<td>15</td>
</tr>
<tr>
<td>RESULTS</td>
<td>16</td>
</tr>
<tr>
<td>Heteroduplex Morphology</td>
<td>16</td>
</tr>
<tr>
<td>Heteroduplex Stability</td>
<td>23</td>
</tr>
<tr>
<td>Denaturation Maps</td>
<td>27</td>
</tr>
<tr>
<td>Differential Stability of C- and V-regions in MtDNA</td>
<td>38</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>45</td>
</tr>
<tr>
<td>The (A+T)-rich Sequences of <em>D. melanogaster</em> MtDNA</td>
<td>45</td>
</tr>
<tr>
<td>Comparison of Sequence Changes in Mitochondrial and Nuclear DNAs</td>
<td>47</td>
</tr>
<tr>
<td>Sequence Organization Among MtDNAs</td>
<td>50</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>57</td>
</tr>
</tbody>
</table>
INTRODUCTION

In mitochondrial DNA (mtDNA) and single-copy nuclear DNA, nucleotide substitutions appear to accumulate at similar rates during the evolution of amphibians (Dawid, 1972; Galau et al., 1976) or mammals (Jakovoic et al., 1975; Laird et al., 1969; Rice and Paul, 1971). Despite changes in nucleotide sequence, some fundamental features of the organization of the mitochondrial genome appear to be conserved among animals. The number of potential functions encoded in mtDNA is restricted by the limited mitochondrial genome size of animals (15-18 kilobases (kb); Borst, 1972), and the types of functions actually specified by mitochondrial genes are similar among widely divergent species. Presumably, all animal mtDNAs specify a small and a large ribosomal RNA, as well as a set of transfer RNAs (Dawid et al., 1976; Attardi et al., 1976). Mitochondrial polyadenylated messenger-like RNAs from species of taxons as distantly related as insects and mammals exhibit similar electrophoretic patterns on polyacrylamide gels (Hirsch et al., 1974). Also, the arrangement of some specific mitochondrial genes in relation to each other is apparently invariant. Thus, in HeLa cells, in Xenopus laevis and Xenopus borealis, or in Drosophila melanogaster, the small and the large ribosomal cistron of mtDNA are adjacent to each other and separated by a small gap, and in all cases transcription proceeds from the small to the large ribosomal RNA. Moreover, the origin of replication is consistently located near the small ribosomal cistron (Attardi et al., 1976; Dawid et al., 1976; Goddard and Wolstenholme, 1977). Most of the sequences specifying the putative transfer RNAs are scattered throughout the mitochondrial
genome, but in both HeLa cells (Angerer et al., 1976) and X. laevis (Dawid et al., 1976), two of the tRNAs are located at either end of the ribosomal DNA (rDNA) locus and one tRNA maps within the gap separating the two ribosomal cistrons.

Based on comparisons of denaturation maps, we have previously shown that the topographic organization of sequences in mtDNA is invariant among different species of the genus Drosophila. Consequently, the large differences in size and buoyant density between mtDNAs from species such as D. melanogaster and D. virilis are entirely attributable to the variable size of a single locus (Bultmann et al., 1976). In D. melanogaster, this locus includes a particularly large region (Fauron and Wolstenholme, 1976) of unusually (A+T)-rich (Polan et al., 1973; Bultmann and Laird, 1973), but not entirely homogeneous sequences (Goldring and Peacock, 1977).

In this thesis, I present a detailed electron microscopic analysis of Drosophila heteroduplex mtDNAs. Examination of circular D. melanogaster : D. virilis hybrid mtDNAs confirms an invariable sequence organization throughout the mitochondrial genome, demonstrating at the same time that Drosophila mtDNAs may accommodate large blocks of species-specific sequences and undergo extensive nucleotide substitutions during evolution.
MATERIALS AND METHODS

Sources of DNA

Isolation of mitochondrial DNA: D. melanogaster (Oregon-R) and D. virilis stocks were obtained from Mrs. Marietta A. Revelry of the University of Texas, Austin, Texas. Mitochondria were isolated by the procedure of Bultmann and Laird (1973) as follows. Ten to thirty grams of adult flies were placed in large population cages, and their eggs harvested on agar plates over periods of 10-15 hours. The eggs were dechorionated in 50% chlorox for 3 min. at room temperature (about 24°C) and rinsed with water. Dechorionated eggs were homogenized in 10 volumes of 0.5 M sucrose, 0.0015 M MgCl₂ 0.01 M Tris-HCl (pH 7.6) at 4°C with motor driven Teflon pestles in glass homogenizers (Tri-R; ten strokes of 0.006 in. clearance and two strokes of 0.004 in. clearance). The homogenate was filtered through glass wool and centrifuged 4 times, each for 4 min, at 3,000 RPM (1,100 x g) in a Sorvall SS-34 rotor to remove nuclei and other large debris. EDTA was added to the final supernatant fraction to a concentration of 0.0015 M and the supernatant centrifuged at 11,500 RPM (16,000 x g) for 20 min in the SS-34 rotor to obtain a crude mitochondrial pellet. This pellet was resuspended in 3 ml of a solution containing 0.5 M sucrose, 0.0005 EDTA, 0.001 M Tris-HCl (pH 7.6) using a Dounce homogenizer with a small clearance pestle (Kontes Glass Company). The resuspended pellet was layered onto

*Abbreviations: EDTA-ethylenediaminetetraacetate (unless otherwise specified, the disodium salt was used); EtBr - ethidium bromide (Sigma Chemical Company); Tris-HCl - tris(hydroxymethyl) aminomethane (adjusted to the desired pH at 25°C with HCl); Tris·HCl - tris (hydroxymethyl) aminomethane hydrochloride; Tris·OH - tris (hydroxymethyl) aminomethane.
a sucrose step gradient (12 ml of 1.1 M and 12 ml of 1.5 M) containing 0.0005 M EDTA, 0.001 M Tris-HCl (pH 7.6) and centrifuged in a Beckman SW 25.1 rotor at 25,000 RPM (90,000 x g) for 90 minutes. The mitochondria, forming a sharp light brown band at the interface of the step gradient, were collected with an ISCO Model D density gradient fractionator. The fractions which contained mitochondria were pooled, diluted to a concentration of 0.5 M sucrose, and centrifuged 20 min at 11,500 RPM (16,000 x g) in a Sorval SS-34 rotor to pellet the purified mitochondria.

The purification procedure was monitored by determining the specific activity of succinate-cytochrome C reductase (Tisdale, 1967). Results similar to those obtained by Bultmann and Laird (1973) were obtained. All preparations were routinely monitored by light microscopy for contamination by nuclei. Electron microscopic examination of sectioned material from purified mitochondrial pellets from both D. melanogaster and D. virilis have confirmed that nuclei were absent (Figure 1).

DNA was obtained from purified mitochondria by the procedure of Bultmann et al. (1976), as follows. Mitochondrial pellets were suspended in 1 ml of a solution containing 0.15 M NaCl, 0.1 M EDTA (pH 8.0) using a Dounce homogenizer with a small clearance pestle. Sodium dodecyl sulfate was added at room temperature to the suspension to a final concentration of 2% to lyse the mitochondria. CsCl was added to a final concentration of 1.0 M, the lysate chilled on ice, and the precipitate of cesium dodecyl sulfate removed by centrifugation. Three or four cleared lysates (approx. 1.2 ml each) were pooled; EtBr and CsCl were added to final concentrations of 0.2 mg/ml and 1.56
Figure 1. Electron micrograph of sectioned *D. viridis* mitochondria purified by isopynic centrifugation

A purified mitochondrial pellet, isolated as described in Materials and Methods, was fixed in 2.5% glutaraldehyde prepared in 0.10 M cacodylate buffer (pH 7.4). After fixation at 4°C for one hour, the pellet was rinsed three times in cold 0.20 M cacodylate buffer (pH 7.4) and postfixed for one hour at 4°C in 1.0% OsO₄ prepared in 0.20 M cacodylate buffer (pH 7.4). The sample was then dehydrated in ethanol and embedded in Epon 812. Thin sections were obtained with a diamond knife on a Porter-Blum Mt-2 ultramicrotome, stained with aqueous uranyl acetate and lead citrate, and observed with an RCA EMU 3F electron microscope. Sections were prepared and photographed by Dr. S.R. Hootman. The line represents 1 μm.
g/ml, respectively. MtDNA was banded in equilibrium density gradients by centrifugation at 32,000 RPM (95,000 x g) for 66 hr at 20°C in a Beckman type 65 Rotor. The mtDNA formed a single fluorescent band and was collected with an ISCO Model D density gradient fractionator by pumping fluorinert (ISCO) into the bottom of the tubes and collecting fractions from the top. Fractions containing the mtDNA were pooled and the EtBr removed by dialysis against 5 grams of Dowex 50 Resin in 50 ml of a solution containing 0.1 M NaCl, 0.05 M Tris-HCl, 0.01 M EDTA (pH 8.5) at room temperature for 24 hours (Kasamatsu et al., 1971). All of the preceding steps involving EtBr were performed in subdued light. Following removal of the EtBr, mtDNA was dialyzed against 500–1,000 volumes of a solution containing 0.01 M Tris-HCl, 0.001 M Na₃ EDTA (pH 8.5) at 4°C and stored at this temperature over a drop of chloroform. MtDNAs isolated by this procedure consisted of a mixture of closed (form I) and open (form II) circular, and linear (form III) molecules with the proportion of circular molecules greater than 90% in all samples.

**Bacteriophage DNAs:** Circular DNA from a Pseudomonas bacteriophage, PM2, was provided by Dr. R. Hewitt, of the University of Texas Cancer System Institute in Houston, Texas. Single-stranded circular DNA from bacteriophage ØX174 was purchased from Miles Laboratories.

**DNA Reassociation**

Heteroduplex mtDNAs were prepared following the procedure of Davis et al. (1971). Approximately equal amounts (20 μg/ml) of *D. melanogaster* and *D. virilis* mtDNAs were mixed and denatured at 37°C for 15 min
after adding 0.25 volumes of 0.5 M NaOH, 0.1 M Na₃EDTA. The solution was readjusted to a pH of about 8.5 (at 24-25°C) by adding 0.1 volumes of 1.8 M Tris-HCl, 0.2 M Tris-OH. After the addition of 99% formamide (Matheson, Coleman & Bell Manufacturing Chemists) to a final concentration of 40%, the DNA was allowed to reassociate at 24-25°C for 4-10 hours. Renaturation was stopped at 4°C and followed by dialysis of the DNA against 1,000 volumes of a solution containing 0.01 M Tris-HCl, 0.001 M Na₃EDTA (pH 8.5) at 4°C. Homoduplex mtDNAs were prepared by the same procedure except that D. melanogaster and D. virilis mtDNAs were reacted separately. The reassociation reactions were monitored by electron microscopy and terminated as soon as multiple nucleation events gave rise to prominent complex aggregates.

**Electron Microscopy**

DNA was spread on protein monolayers in the presence of formamide as described by Davis et al. (1971). The spreading solution was prepared at 4°C and contained 0.5 - 1.0 μg/ml of DNA, 0.1 mg/ml cytochrome C, 0.1 M Tris-HCl, 0.01 M Na₃EDTA (pH 8.5), and various concentrations of formamide. This solution was held at 24-25°C for 15-30 sec before it was applied from a glass ramp onto the hypophase, which contained 0.01 M Tris-HCl and 0.001 M Na₃EDTA (pH 8.5), and which was 30% less than the spreading solution in formamide. After 2 min, the protein film was slightly compressed, usually transferred to self-supporting carbon films on 300 mesh copper grids, and stained with 2-3% phosphotungstic acid in acetone (Lang, 1973). All preparations were made at room temperatures varying between 24 and 25°C. DNA molecules were photographed in an RCA EMU 3F electron microscope and measured as
described previously (Bultmann et al., 1976).

Calibration of DNA Contour Lengths

Based on contour length measurements, the linear base density (i.e., number of bases or number of base-pairs per cm) was consistently higher in single-stranded than in double-stranded DNA. The relative linear base density can be determined by measuring the ratio of double- to single-stranded contour lengths (d/s) for equivalent DNAs, e.g., circular double-stranded and circular single-stranded D. melanogaster mtDNAs. In these experiments, it was necessary to determine d/s for each individual grid, since this ratio varied considerably among different spreads as well as among grids from the same spread. However, d/s was invariant in different areas of a single grid.

For partially denatured molecules, d/s was determined directly, without reference to external standards. For each of nine separate spreads, only molecules from a single grid were scored. As shown in the example of Figure 2, the sum of the double-stranded lengths is plotted as a function of the sum of the single-stranded lengths of each of a set of partially denatured D. melanogaster mtDNA molecules from a single grid. In all nine spreads the individual molecules were denatured to different extents, covering a relatively broad range of about 30% denaturation in each spread, and all the scatter diagrams plotted in this manner defined reliable linear regressions (-0.9 < r < -0.99), the slopes of which corresponded to -d/s. The d/s values determined in this way are listed in Table 1, column 2 for all nine spreads.

These d/s values were checked by measurements of reference mole-
Figure 2. Double- vs. single-stranded DNA lengths in partially denatured *D. melanogaster* mtDNA spread for electron microscopy in 73% formamide.

The sum of the double-stranded DNA lengths \(d\) is plotted as a function of the sum of the single-stranded DNA lengths \(s\) in each of 18 partially denatured *D. melanogaster* mtDNA molecules from the same grid. The linear relationship between these observations is expressed by the least squares fit regression; the \(d/s\) ratio is the negative of its slope.
$d = -1.29s + 6.4$
$(r = -0.95)$
$d/s = 1.29$
**TABLE 1.** Double-stranded/single-stranded (d/s) DNA ratios

<table>
<thead>
<tr>
<th>% Formamide in spreading solution</th>
<th>Denatured molecules</th>
<th>ØX174 DNA and D. melanogaster mtDNA</th>
<th>ØX174 DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>58</td>
<td>1.35</td>
<td>1.30</td>
<td>1.32</td>
</tr>
<tr>
<td>60</td>
<td>1.55</td>
<td>1.30</td>
<td>1.62</td>
</tr>
<tr>
<td>61</td>
<td>1.50</td>
<td>1.30</td>
<td>1.55</td>
</tr>
<tr>
<td>71</td>
<td>1.31</td>
<td>1.30</td>
<td>1.31</td>
</tr>
<tr>
<td>71</td>
<td>1.61</td>
<td>1.30</td>
<td>1.67</td>
</tr>
<tr>
<td>73</td>
<td>1.29</td>
<td>1.33</td>
<td>1.32</td>
</tr>
<tr>
<td>73</td>
<td>1.41</td>
<td>1.33</td>
<td>1.56</td>
</tr>
<tr>
<td>75</td>
<td>1.36</td>
<td>1.37</td>
<td>1.38</td>
</tr>
<tr>
<td>75</td>
<td>1.42</td>
<td>1.37</td>
<td>1.44</td>
</tr>
</tbody>
</table>
cules. Three spreads in which native *D. melanogaster* mtDNA was partially denatured contained undenatured double-stranded form I *D. melanogaster* mtDNA molecules. The average lengths of these molecules were compared with the average lengths of single-stranded ØX174 DNA molecules that were also included in these spreads (Table 1, lines 4, 6, 8). In these three spreads, d/s corresponds to the average length of form I *D. melanogaster* mtDNA divided by 3.6 times the average length of the ØX174 DNA (Table 1, column 2). In two experiments at different formamide concentrations (Table 2), it is shown that single-stranded *D. melanogaster* mtDNA is 3.6 times longer than ØX174 DNA. This is in good agreement with a previous comparison of these two DNAs spread in formaldehyde which yielded a ratio of 3.5 (Bulthman et al., 1976).

All of the grids containing reassociated mtDNA molecules also contained single-stranded ØX174 DNA molecules for reference. Form I *D. melanogaster* mtDNA could not be distinguished from reassOCIATED molecules and therefore was not used as a reference in these spreads. Another available reference molecule, double-stranded PM2 DNA, was not included in these experiments because its length overlaps with the length of single-stranded *D. melanogaster* mtDNA. Despite these limitations, an alternative estimate of d/s was obtained indirectly from length measurements of either single- or double-stranded reference molecules. This was possible because length variations from grid to grid of single- and double-stranded DNAs were correlated. In eleven spreads, including the three in which *D. melanogaster* native mtDNA was partially denatured, the average lengths of double-stranded *D. melanogaster* mtDNA or PM2 DNA were compared to the length of single-stranded
TABLE 2. Comparison of ØX174, and *D. melanogaster* single- and double-stranded mtDNA circular molecules:

<table>
<thead>
<tr>
<th>% Formamide in spreading solution</th>
<th>Circular contour length [µm ± standard deviation (n)]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ØX174 DNA</td>
</tr>
<tr>
<td>61</td>
<td>0.95 ± 0.08 (33)</td>
</tr>
<tr>
<td>75</td>
<td>0.93 ± 0.07 (33)</td>
</tr>
<tr>
<td>$\bar{x}$</td>
<td>0.94†</td>
</tr>
</tbody>
</table>

*D. melanogaster* mtDNAs were incubated at 37°C in 90% formamide prior to being absorbed to protein monolayers along with ØX174 DNA at the indicated formamide concentrations.

$\dfrac{\text{single-stranded } D. melanogaster \text{ mtDNA}}{\text{single-stranded } ØX174 \text{ DNA}} \cdot \dfrac{3.37 \, \mu\text{m}}{0.94 \, \mu\text{m}} = 3.585$
\( \Phi X \)74 DNA. As shown in Figure 3, the correlation of double- and single-stranded length variations is linear. Based on the slope of the regression line, any 14.3% change in the relative length of single-stranded DNA corresponds to a 1% change in double-stranded DNA. From this relationship, as expressed by equation (2) in the legend of Figure 3, \( d/s \) values were derived for the nine spreads listed in Table 1 (column 3) based on the single-stranded \( \Phi X \)74 DNA average length on each grid. For \( \Phi X \)74 DNA, \( s_{\text{max}} \) was 1.29 \( \mu \text{m} \) and \( d_{\text{max}} \) was 1.71 \( \mu \text{m} \); i.e., the maximum double-stranded length of \( D. \text{melanogaster} \) mtDNA, 6.13 \( \mu \text{m} \), divided by 3.6, the ratio of single-stranded \( D. \text{melanogaster} \) mtDNA to single-stranded \( \Phi X \)74 DNA (Table 2). There is good correlation (\( r = 0.93 \)) between this derivation of \( d/s \) values, and the \( d/s \) values obtained from the regression of partially denatured molecules (Table 1, column 1).

**Denaturation Maps**

Mitochondrial DNAs were melted during preparation of protein monolayers according to the isodenaturation procedure of Davis and Hyman (1971) by coordinately varying the formamide concentrations in the spreading solution and the hypophase within an appropriate range. Partially denatured molecules were aligned (in order of increasing denaturation) using the computer program described by Bultmann et al. (1976). The resulting histograms were matched by the same program.
Figure 3. Co-ordinate variations in double- and single-stranded DNA contour lengths

Seven grids contained double-stranded (d) circular *D. melanogaster* mtDNA mixed with single-stranded (s) circular ØX174 DNA (●). Four grids contained double-stranded circular PM2 DNA mixed with single-stranded circular ØX174 DNA (▲). Lengths of single-stranded DNAs are expressed as a fractional length of the largest average value measured for ØX174 DNA ($s_{\text{max}}$, 1.29 μm = 1.0). Lengths of double-stranded DNAs are expressed as a fractional length of the largest average value measured for *D. melanogaster* mtDNA ($d_{\text{max}}$, 6.13 μm = 1.0). *D. melanogaster* mtDNA and PM2 DNA were mixed and spread at formamide concentrations of 61 and 75%. In both spreads, the length ratio of *D. melanogaster* mtDNA to PM2 DNA was 1.91. Based on this value, the lengths of PM2 DNAs were normalized to lengths of *D. melanogaster* mtDNAs. The contour length measurements of twenty-five or more molecules of each type on a single grid were averaged. The linear relationship between these observations is expressed by the least squares fit regression:

$$\frac{d}{d_{\text{max}}} = 0.143 \frac{s}{s_{\text{max}}} + 0.848 \ (r = 0.93) \quad (1)$$

Thus, given equivalent estimates of $d_{\text{max}}$ and $s_{\text{max}}$ (see text), the $d/s$ ratio can be calculated for any observed single- or double-stranded DNA from the equation:

$$d/s = (0.143 \frac{s}{s_{\text{max}}} + 0.848) \frac{d_{\text{max}}}{s} \quad (2)$$
RESULTS

Heteroduplex Morphology

When denatured *D. melanogaster* and *D. virilis* mtDNAs were mixed, renatured, and inspected by electron microscopy after being spread from 40% formamide, I always found molecules that exhibited a highly characteristic, apparently single-stranded, asymmetric bubble. Such a structure was never observed when *D. melanogaster* or *D. virilis* mtDNAs were renatured separately. Thus, the asymmetric bubble specifically identifies heteroduplex molecules (Figure 4). It was seen in complex aggregates of renatured molecules, in linear molecules as long as, or shorter than native mtDNAs, and in circular molecules approximating the length of native mtDNAs. Only the circular heteroduplex molecules have been included in the present analysis. Except for a few of these molecules, which were also excluded because they exhibited obvious single-stranded gaps, every circular heteroduplex appeared to be completely double-stranded outside the diagnostic, asymmetric loop. At formamide concentrations below 55%, exceptional molecules that included a symmetric bubble in addition to the asymmetric loop were found in the first preliminary experiments (Zakour and Bultmann, 1976). In all of my more recent experiments, however, I found additional symmetric bubbles only under denaturation conditions at elevated formamide concentrations. The observed distribution of denaturation bubbles (see below) strongly suggests that the heteroduplex mtDNAs consist of two complete heterologous strands. This conclusion is confirmed by length measurements.

The difference in double-strand equivalent contour lengths between the arms of the asymmetric bubble is 3.3 kb (Table 3, last column).
Figure 4. Electron micrographs of D. melanogaster : D. virilis heteroduplex mtDNAs.

DNA was adsorbed to protein monolayers under non-denaturing conditions, with 40% formamide in the spreading solution (A), or under denaturing conditions in the presence of 60% formamide (B). Arrows point to the diagnostic asymmetric loop in the heteroduplex. The magnification is the same for (A) and (B). The bar represents 0.5 μm.
TABLE 3. Structure of *D. melanogaster* : *D. virilis* mtDNAs*)

<table>
<thead>
<tr>
<th>% Formamide in spreading solution</th>
<th>n</th>
<th>Contour lengths (Kb ± SD)</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Duplex portion†</td>
<td>Asymmetric loop</td>
<td>Long arm</td>
<td>Short arm</td>
<td>Difference</td>
</tr>
<tr>
<td>40</td>
<td>42</td>
<td>13.8 ± 0.8</td>
<td>4.3 ± 0.3</td>
<td>1.0 ± 0.1</td>
<td>3.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>58</td>
<td>34</td>
<td>13.7 ± 0.8</td>
<td>4.4 ± 0.4</td>
<td>1.2 ± 0.2</td>
<td>3.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>30</td>
<td>13.9 ± 0.9</td>
<td>4.8 ± 0.4</td>
<td>1.5 ± 0.2</td>
<td>3.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>61</td>
<td>24</td>
<td>14.2 ± 0.9</td>
<td>4.9 ± 0.4</td>
<td>1.5 ± 0.2</td>
<td>3.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Heteroduplex molecules were adsorbed to protein monolayers at the indicated formamide concentrations.

† The duplex portion includes all of the DNA outside the asymmetric loop. Except in 40% formamide, the duplex portion is actually partially denatured.
This corresponds almost exactly to the size difference between native circular *D. melanogaster* and *D. virilis* mtDNAs, which I estimate to range between 3.2 and 3.3 kb at three different formamide concentrations (cf. also Bultmann *et al.*, 1976; Fauron and Wolstenholme, 1976; Shah and Langley, 1977). Furthermore, heteroduplex molecules do not contain any additional, cryptic regions of strand separation or hidden single-stranded gaps that would cause significant foreshortening of apparent contour lengths. Thus, by adding the duplex portions to either the long or short arms of the asymmetric bubble, I obtain length estimates that closely match the full size of *D. melanogaster* and *D. virilis* mtDNAs, respectively (Table 3).

From previous studies (Bultmann *et al.*, 1976; Fauron and Wolstenholme, 1976), I infer that the long arm of the asymmetric bubble should contain the (A+T)-rich sequences of *D. melanogaster* mtDNA. Thus, the data in Table 3 may be tentatively interpreted as follows. In 40% formamide, the long arm of the asymmetric bubble includes 4.3 kb of the (A+T)-rich sequences of *D. melanogaster* mtDNA; 3.3 kb of these sequences are entirely missing in *D. virilis* mtDNA, whereas 1.0 kb are represented in *D. virilis* mtDNA by sequences included in the short arm of the asymmetric bubble, i.e., by sequences that appear to be unrelated. However, *D. virilis* mtDNA apparently does contain sequences homologous to an additional small portion (0.5 kb) of the (A+T)-rich sequences of *D. melanogaster* mtDNA. As the formamide concentration is increased from 58 to 60%, there seems to be a small, discrete expansion of the asymmetric bubble, increasing the length of its long arm to 4.8 kb. At that point, I consider all of the (A+T)-rich sequences of
D. melanogaster mtDNA to be included in the long arm of the heteroduplex loop.

**Heteroduplex Stability**

Since the asymmetric bubble readily distinguishes heteroduplex molecules from both native and renatured homoduplex mtDNAs, an excellent opportunity exists to assess the relative duplex stability among these three groups of molecules by electron microscopy. Duplex stability was measured by denaturing molecules at increasing concentrations of formamide according to the isodenaturation procedure of Davis and Hyman (1971). For the construction of the melting curves in Figure 5, I analyzed only circular molecules that appeared to include two complete strands. Such molecules were selected as described above for undenatured heteroduplex mtDNA. Circles with at most two small single-stranded tails were also included in the analysis if it was apparent that such tails resulted from a single break within a partially denatured region.

The denaturation of native D. melanogaster mtDNA proceeded as follows. Strand separation was first seen in 68% formamide. Up to concentrations of 70% formamide, a single small bubble was seen in a few molecules. In 71% formamide, the typical big bubble of partially denatured D. melanogaster mtDNA was always present, and some molecules showed additional small bubbles. Most of the melting occurred at formamide concentrations above 71%, with a transition midpoint (50% strand separation) at 74% formamide. The denaturation of native D. virilis mtDNA was very similar to that of native D. melanogaster mtDNA except that the melting transition occurred at 1.5% higher for-
Figure 5. Denaturation of Drosophila mtDNAs in formamide

*D. melanogaster* : *D. virilis* heteroduplex mtDNA (A,B) and homoduplex mtDNAs of *D. melanogaster* (C) and *D. virilis* (D) were melted under isodenaturation conditions (Davis and Hyman, 1971) at the indicated formamide concentrations in the spreading solutions. The extent of strand separation in circular molecules was determined from contour lengths corrected for variations in linear base density as described in Materials and Methods. Curves A and B are alternative representations of the heteroduplex transition, including either the long arm (A) or the short arm (B) of the asymmetric loop in the calculations of strand separation. Homoduplex mtDNAs of *D. melanogaster* and *D. virilis*, which are readily distinguishable by size, were spread together on the same monolayer and consisted of either native (circles) or renatured DNA (triangles). Each point represents a set of molecules derived from a single grid (12 ≤ n ≤ 35).
mamide concentration, with 50% strand separation at a formamide concentration of 75.5%. This difference can be entirely attributed to the interspecies size difference in the early melting (A+T)-rich region. That is, after subtracting 3.3 kb from the big bubble region of D. melanogaster mtDNA molecules, the adjusted melt curve is the same as for D. virilis mtDNA. For both D. melanogaster and D. virilis mtDNAs, denaturation of renatured homoduplex circles closely paralleled the melting transitions of native molecules. Thus, there is no indication for base-pair mismatching in renatured homoduplex molecules. This is consistent with previous optical measurements, indicating that the midpoint of the thermal transition ($T_m$) of renatured mtDNA is at most 10°C lower than that of the native mtDNA (Bultmann and Laird, 1973; Polan et al., 1973).

Compared to native or renatured homoduplex mtDNAs, the heteroduplex melted at much lower formamide concentrations. The diagnostic asymmetric bubble was observed at formamide concentrations as low as 40% and as high as 63%. As already mentioned, at formamide concentrations below 55% heteroduplex molecules appeared to be double-stranded throughout their entire length except within the asymmetric bubble. In 55% and 57% formamide, some heteroduplex molecules first showed a single denaturation bubble in addition to the asymmetric loop. The melting transition of the heteroduplex appears to be as sharp as that of homoduplex molecules. Quantitatively, this can only be shown within the range of 58-61% formamide (Figure 5). Among eight heteroduplex molecules that could unambiguously be identified in 63% formamide, none were denatured less than 65%, and two were almost completely (>95%) denatured. Ap-
apparently, most heteroduplex molecules were completely melted in 63% formamide, 5% below the concentration at which homoduplex molecules began to melt. Including the long arm of the asymmetric bubble (Figure 5, curve A), 50% strand separation occurs at a formamide concentration of 59.5%. Alternatively, including the shorter arm of the asymmetric bubble (Figure 5, curve B), heteroduplex molecules exhibit 50% strand separation at a formamide concentration of 61%. As expected, the 1.5% formamide difference between the two heteroduplex melting curves equals the difference between the melting transitions of homoduplex *D. melanogaster* and *D. virilis* mtDNAs. Consequently, with reference to either the *D. melanogaster* or the *D. virilis* homoduplex, the heteroduplex exhibits 50% strand separation at a 14.5% lower formamide concentration. This corresponds to a depression of the apparent T$_m$ by 10.5°C (McConaughy et al., 1969) and can be attributed to 15.7% base-pair mismatching within heteroduplex molecules. How these mismatched base-pairs are distributed is the next question.

**Denaturation Maps**

If base-pair mismatches were distributed in an entirely uniform fashion, topographic patterns of strand separation in sets of heteroduplex and homoduplex mtDNAs would be indistinguishable, provided that these sets are composed of molecules denatured to the same extent. Alternatively, in comparing such sets of molecules, histograms of melting patterns might exhibit specific regional differences, because base-pair mismatches may be highly clustered in some sections of the heteroduplex, while exceptionally few base-pair mismatches may occur in other sections.
To test which of these alternatives may be realized, histograms were constructed of partially denatured heteroduplex molecules, including the long arm of the asymmetric bubble, and compared to histograms of partially denatured homoduplex *D. melanogaster* mtDNA molecules. Such a comparison takes advantage of the stringent alignment conditions imposed by the long (A+T)-rich sequences of *D. melanogaster* mtDNA (Bultmann *et al.*, 1976) and is therefore preferable to comparisons of maps between heteroduplex molecules and homoduplex *D. virilis* mtDNAs. In Figure 6, denaturation maps of heteroduplex molecules (C,F) and of native (A,D) as well as renatured (B,E) homoduplex molecules are compared. Each of the three groups of mtDNAs has been divided into two denaturation classes, one including molecules denatured between 25 and less than 48% (Figure 6 A,B,C), the other denatured 48-75% (Figure 6D, E,F). From each of the three groups of mtDNA, molecules were selected such that their distribution by percent strand separation was the same within the limits of each denaturation class.

In a general way, the denaturation profiles of heteroduplex molecules are remarkably similar to those obtained from native or renatured homoduplex molecules. This can be expressed quantitatively by measuring the percent area overlap between pairs of histograms (Bultmann *et al.*, 1976). Among the less denatured molecules (25-48%) the histogram areas of heteroduplex (Figure 6C) and renatured homoduplex molecules (Figure 6B) overlap the histogram area of native molecules (Figure 6A) by 81% and 83%, respectively (Table 4). It is not possible to attach any significance even to the 8% difference since Bultmann *et al.* (1976) previously found up to 13% variation in area overlaps be-
Figure 6. Denaturation maps of Drosophila mtDNAs

Partially denatured native (A,D) or renatured (B,E) homoduplex mtDNAs of *D. melanogaster* or partially denatured *D. melanogaster* : *D. virilis* heteroduplex mtDNAs (C,F) were aligned by computer (Bultmann et al., 1976). The resulting two sets of histograms, (A,B,C) and (D,E,F), include molecules denatured between 25 and less than 48% or between 48 and 75%, respectively. The molecules included in each of the three histograms of a set are equally distributed within intervals of 5% strand separation (n ≥ 25 for each histogram). Histograms D, E, and F were aligned to histograms A, B, and C, respectively. Values along the ordinate indicate which fraction of the aligned molecules contained strand separations within each of the 100 segments (each 0.185 kb long) indicated along the abscissa. The principal peaks in histogram profiles fall into eight regions whose boundaries were chosen subjectively as indicated along the upper margin of the figure.
TABLE 4. Comparison of histograms of *D. melanogaster* native and renatured homoduplex mtDNAs and *D. melanogaster : D. virilis* heteroduplex mtDNA*

<table>
<thead>
<tr>
<th>Histogram</th>
<th>% Area overlap with native histogram</th>
<th>% of histogram in regions†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>25-48% denatured</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>native</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>homoduplex</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>heteroduplex</td>
<td></td>
<td>4</td>
</tr>
</tbody>
</table>

| 48-75% denatured  |                                      | 100 | 7 | 13 | 7 | 9 | 41 | 8 | 7 | 8 |
| native            |                                      |  7 | 12 | 5 | 10 | 42 | 9 | 8 | 7 |
| homoduplex        |                                      |  7 | 11 | 5 | 9 | 41 | 3 | 17 | 7 |
| heteroduplex      |                                      |  7 | 11 | 5 | 9 | 41 | 3 | 17 | 7 |

* Histograms compared are those shown in Figure 6.

† Histogram regions are indicated along upper margin of Figure 6.
between different histograms obtained simply by varying the order in which partially denatured molecules of the same set were aligned.

A more detailed comparison of denaturation patterns is based on the examination of the difference maps shown in Figure 7. The map in Figure 7A represents differences in the histogram profiles of heteroduplex molecules (Figure 6F) and the renatured homoduplex mtDNAs (Figure 6E), whereas the map in Figure 7B reflects variations between the histogram profiles of native and renatured molecules (Figure 6D and E). Since the compared sets of molecules are denatured to the same extent, the sum of positive and negative deviations in both maps equals zero. With reference to the (A+T)-rich region in the center of the map, fluctuations around zero values in Figure 7A are distributed in a highly asymmetric fashion. On the left side, fluctuations occur within narrow intervals, whereas immediately to the right of the (A+T)-rich region there is a broad high peak followed by an even broader deep trough. I take the maximal fluctuations (±0.30) in the difference map of Figure 7B (native versus renatured homoduplex) as an estimate of the range of fortuitous variations between any two denaturation profiles; this seems justified, since positive and negative deviations are of the same magnitude. Within the clusters of narrow-interval fluctuations on the left side of Figure 7A, there is only one sharp peak and one narrow trough barely extending beyond the ± 0.30 limits. In contrast, both the broad peak and the wide trough on the right clearly reach beyond these limits. By averaging differences between pairs of histograms over increasingly longer intervals, the magnitude of what is taken to be fortuitous fluctuations in difference maps decreases differentially, leaving the two broad regions to the right of the (A+T)-rich sequences as
Figure 7. Difference maps of Drosophila mtDNAs--1% map intervals

Map (A) was constructed by subtracting within each 1% map interval the values specified along the ordinate of the histogram of heteroduplex molecules (Figure 6F) from the corresponding values in the histogram of renatured homoduplex mtDNAs (Figure 6E). In the same way, the difference map (B) was obtained by subtracting the values in Figure 6D (native mtDNA) from the corresponding values in Figure 6E (renatured homoduplex mtDNA). The dashed lines at ± 0.30 are taken as the extreme limits of fortuitous variations between histogram profiles.
the only two prominent and presumably significant differences between melting profiles of heteroduplex and homoduplex molecules (Figure 8).

Another way to exhibit the two principal differences between denaturation maps of heteroduplex and homoduplex molecules is to compare the fraction of the histogram area included within individual peaks. Boundaries between peaks were subjectively chosen as indicated in Figure 6. The percent area under most peaks is essentially identical for heteroduplex and native or renatured homoduplex molecules (Table 4). However, in the maps of more denatured molecules (Fig. 6D,E,F) the area under heteroduplex peak 6 is one-third and under heteroduplex peak 7 is two times that under the corresponding peaks in the histograms of native and renatured homoduplex mtDNAs. Regions included under peak 6 and peak 7 correspond to the high peak and deep trough, respectively, already identified in the difference maps.

Evidently, there is one contiguous differentially more stable (broad peak) and one contiguous differentially less stable (wide trough) region within heteroduplex molecules. I shall refer to these two segments of Drosophila mtDNA as the C-region (conserved) and the V-region (variable), respectively, since they appear to include differentially fewer (C-region) or more (V-region) base-pair mismatches than the rest of the paired strands of the heteroduplex. The size of the C- and V-regions may be defined by the ± 0.30 limits in the difference map (Figure 7A). Thus, the C-region measures 2 kb extending 0.3 kb into the (A+T)-rich segment (26% of 18.5 kb), while the V-region includes 3 kb and may be separated from the C-region by as much as 0.7 kb (see below, Figure 11). Since outside the C- and V-regions, fluctuations in the
Figure 8. Difference maps of Drosophila mtDNAs--4% map intervals

Map (A) was constructed by subtracting within each 4% map interval the values specified along the ordinate of the histogram of heteroduplex molecules (Figure 6F) from the corresponding values in the histogram of renatured homoduplex mtDNAs (Figure 6E). In the same way, the difference map (B) was obtained by subtracting the values in Figure 6D (native mtDNA) from the corresponding values in Figure 6E (renatured homoduplex mtDNA). The dashed lines at ± 0.20 are taken as the extreme limits of fortuitous variations between histogram profiles.
difference map (Figure 7A) occur within or barely above the ± 0.30
limits of presumably fortuitous variations (Figure 7B), no additional
differentially more stable or unstable regions can be distinguished.
Apparently, through most of the heteroduplex, base-pair mismatches are
distributed in an essentially uniform fashion or in clusters too small
to affect denaturation maps in a readily detectable way.

Differential Stability of C- and V-Regions in MtDNA

Since by this mapping procedure, homologous regions in partially
denatured D. melanogaster mtDNA appear to be aligned with a high degree
of fidelity (Bultmann et al., 1976), it is possible to obtain reasonably
reliable melting curves for each of the eight regions indicated along
the upper margin of Figure 6. In native D. melanogaster mtDNA, immedi-
ately following strand separation in the (A+T)-rich region 5, region 4
is melting, giving rise to the sharp peak in the histogram of Figure
6A. Next, region 2 is melting followed by transitions in the remaining
regions (1,8,7,6,3), all of which are closely clustered around a com-
posite melt curve representing the extent of strand separation within
the entire molecule, excluding only the (A+T)-rich region 5. Figure
9B shows the melting curves of regions 2,6, and 7 in comparison with
the composite melt curve. The sequential pattern of melting transi-
tions in the renatured homoduplex is almost exactly the same as for
native molecules (data not shown).

The non-random distribution of mismatched bases within heteroduplex
molecules, inferred from difference maps, should effect at least some
changes in the sequence in which individual regions denature. Besides
minor shifts in the positions of the four melt curves derived from
Figure 9. Strand separation within individual regions of Drosophila mtDNA

Among circular mtDNAs partially denatured in formamide as described in the legend of Figure 5, eight homologous regions were identified by denaturation mapping (see Figure 6). The extent of strand separation within regions 2 (●), 6 (■), and 7 (▲) at increasing formamide concentrations in the spreading solutions is shown for D. melanogaster: D. virilis mtDNA (A) and for native mtDNA of D. melanogaster (B). Composite melt curves (open circles) reflect the extent of strand separation in the entire molecule excluding only the (A+T)-rich region 5. Each point represents a set of molecules (15 ≤ n ≤ 25) derived from a single grid.
regions 4, 1, 8, and 3, the melting characteristics of three segments in the heteroduplex are strikingly different from those in the homoduplex (Figure 9A). Relative to the composite melt curve, including all of the heteroduplex outside the asymmetric loop, region 7 melts earlier, while region 6 melts much later. In contrast, the three corresponding melt curves of the homoduplex are nearly identical (Figure 9B). The precipitous melting of region 7 in heteroduplex molecules presumably is indicative of a disproportionately larger number of mismatched bases within this region. Region 6, however, must include disproportionately few mismatched base-pairs since strand separation is delayed. Thus, regions 7 and 6 exhibit the expected melting characteristics of the corresponding V- and C-regions identified in difference maps. The third change in melting behavior between heteroduplex and homoduplex molecules is recognized in region 2. In the heteroduplex, strand separation in region 2 closely follows the composite melting transition (Figure 9A), while in the homoduplex the same region melts distinctly earlier (Figure 9B). Apparently, region 2 also includes sequences that are differentially better matched than sequences in some other parts of the heteroduplex. However, these differentially more stable sequences seem to occur in clusters too small to be convincingly resolved in difference maps.

The existence of the C-region can be demonstrated by a comparison of the length of the long arm in the asymmetric loop of heteroduplex mtDNAs with the lengths of separated strands in the (A+T)-rich region in native and renatured homoduplex mtDNAs (Figure 10). As the overall extent of strand separation in homoduplex molecules increases from 25%
Figure 10. Size of the asymmetric loop and the big bubble as a function of increasing denaturation.

The average length of the long arm of the asymmetric loop of the heteroduplex (●) and the average length of separated strands in the big bubble including the (A+T)-rich sequences of the native (■) and renatured (▲) homoduplex are plotted for groups of molecules (n ≥ 5) classified by extent of total strand separation within intervals of 5%. The heteroduplex molecules, denatured in 58-61% formamide, are the same as those included in Figures 6C,F. The native and renatured homoduplex molecules, denatured in 71-75% formamide, are the same as those included in Figures 6A,D and Figures 6B,E, respectively.
to about 50%, the big bubble becomes only slightly larger, but it expands abruptly among molecules that become more than 50% denatured. The proportion of molecules containing an expanded big bubble increases as strand separation proceeds to 75%. The abrupt expansion of the big bubble is apparently due to the collapse of a small double-stranded region separating a denaturation bubble in region 6 from the denatured (A+T)-rich region 5. The denaturation bubble in region 4 on the other side of the block of (A+T)-rich sequences, however, remains separated from the big bubble by a double-stranded bridge except in a few of the most denatured molecules. Even among heteroduplex molecules denatured up to 75%, I have never seen an abrupt expansion of the asymmetric loop into region 6. Consequently, aside from the initial subtle increase of the asymmetric loop noted earlier (Table 3), the length of the single-stranded long arm in the heteroduplex remains essentially constant within the range of 30-75% strand separation (Figure 10). Clearly, the size of the diagnostic loop in heteroduplex molecules is confined by the presence of differentially more stable sequences, in particular by sequences within the adjoining region 6 or C-region.
DISCUSSION

The (A+T)-rich Sequences of D. melanogaster MtDNA

The analysis of denaturation maps confirmed that the long arm of the diagnostic asymmetric loop of heteroduplex molecules includes the (A+T)-rich sequences of D. melanogaster mtDNA. This justifies my earlier suggestion that, based on the comparison with D. viridis mtDNA, three types of sequences may be distinguished within the 4.8 kb long, contiguous, (A+T)-rich region of D. melanogaster mtDNA. I shall refer to these sequences simply by their size (3.3, 1.0, and 0.5 kb). The 3.3 kb sequence is entirely missing in D. viridis mtDNA. The 1.0 kb sequence corresponds to sequences of D. viridis mtDNA that form the short arm of the asymmetric loop in the heteroduplex and thus appear to be unrelated or at least not sufficiently related to form duplex structures. Finally, the 0.5 kb sequences can react with homologous sequences in D. viridis mtDNA to form a heteroduplex at formamide concentrations up to 58%. However, since this short duplex region is unstable in 60% formamide, 8% below the formamide concentration required to melt any part of a homoduplex, the 0.5 kb sequences of D. melanogaster and D. viridis mtDNA cannot be identical. Most likely, the 0.5 kb sequence is located on the same side of the (A+T)-rich region that overlaps the cistron specifying the small ribosomal RNA of D. melanogaster mitochondria (Klukas and Dawid, 1976).

Reconsidering the possibility that at least some limited homologies may exist among the 1.0 kb sequences of D. melanogaster and D. viridis, one would expect that the diagnostic heteroduplex structure I have described should assume alternative configurations. For example, a
heteroduplex might exhibit one simple deletion loop or several such loops clustered within a 1.0 kb region, configurations that could not readily be distinguished from some random overlaps within a DNA molecule. But I have two reasons to believe that such alternative configurations, reflecting homologies within the 1.0 kb sequence, may not be realized. First, I never noticed a disproportionate decrease in the number of heteroduplex molecules bearing the diagnostic asymmetric loop as the formamide concentration in otherwise identical spreading solutions was lowered from 61 to 40%. Second, from the data of Fauron and Wolstenholme (1976), I infer that at least half and possibly all of the 1.0 kb sequences of D. melanogaster have a distinctly different thermal stability than the corresponding sequences in D. virilis mtDNA. The pertinent observation is that under conditions resulting in strand separation of the entire 4.8 kb (A+T)-rich sequence in D. melanogaster mtDNA, the average size of the largest region of strand separation in D. virilis mtDNA is 0.47 kb, merely half the size of the 1.0 kb sequence. The entire 1.0 kb sequence may have different melting characteristics than the corresponding sequences in D. virilis since it seems reasonable to suppose that the earliest melting 0.47 kb region in D. virilis mtDNA may actually correspond to the 0.5 kb sequence at one end of the (A+T)-rich region in D. melanogaster mtDNA.

An interesting implication of the asymmetric loop configuration of the heteroduplex relates to the fact that the origin of replication in D. melanogaster mtDNA is located in the middle of the (A+T)-rich region (Goddard and Wolstenholme, 1977). The origin of replication in D. virilis mtDNA is not known. But, wherever it will turn out to be located, it must be associated with sequences that are substantially
different from those at the replication origin in D. melanogaster mtDNA. Thus, unlike sheep and goat mtDNAs, which share very similar nucleotide sequences at the origin of replication (Upholt and Dawid, 1977), the sequences associated with the origin of replication among the Drosophila mtDNAs are probably not highly conserved.

Comparison of Sequence Changes in Mitochondrial and Nuclear DNAs

For the purpose of comparing sequence changes in mitochondrial and nuclear DNAs, I shall assume throughout the following discussion that a depression of the $T_m$ by 1°C corresponds to 1.5% base-pair mismatching (Laird et al., 1969). My estimate of the $T_m$ of Drosophila mtDNAs by electron microscopy is based on the additional assumption that each 1% increase of the formamide concentration lowers the melting temperature by 0.72°C (McConaughy et al., 1969). From the data in Figure 5, I have calculated 15.7% base-pair mismatching for D. melanogaster : D. virilis heteroduplex mtDNAs. This value is scarcely affected by contributions of the unpaired portion in the heteroduplex to the $T_m$ estimates and essentially reflects sequence changes within the duplex portion alone. Thus, for the two composite melt curves in Figure 9A and B, which exclude the non-homologous portions together with all of the (A+T)-rich sequences, I calculate 15.4% base-pair mismatching. Regionally, estimates of base-pair mismatching vary by less than ± 1% around a value of 15.5%, except for the most conserved, or C-region. This region contains probably about 13% and possibly as few as 10% mismatched base-pairs, depending on how the initial part of the melt curve (Figure 9A) is extrapolated.

Recently, Dr. R.H. Richardson at the University of Texas at Austin
(personal communication) has measured sequence changes between single-copy fractions of nuclear DNAs from D. melanogaster and D. virilis. Trace amounts of $^{125}\text{I}$-labeled fragments of single-copy DNA from D. melanogaster were mixed with an excess of unlabeled fragments of single-copy DNA from D. virilis, denatured and allowed to renature to a $C_{\text{ot}}$ value (Britten and Kohne, 1968) of 1000 M sec$^{-1}$ in 0.12 M phosphate buffer at 60°C. At this point, none of the labeled DNA had a chance to react with itself, while 15-20% of the unlabeled DNA formed double-stranded structures. As determined by thermal elution from hydroxyapatite columns, the $T_m$ of the heterologous duplex was 15.6°C lower than the $T_m$ of the homologous one. This value probably represents an overestimate of base pair substitutions since Richardson found that, in general, the hybridization reactions with iodinated DNA probes lead to estimates of sequence divergence that may be up to 1.5-fold higher than those obtained from alternative hybridization experiments. Accordingly, the nucleotide sequences of mtDNAs have diverged at a similar or possibly slightly lower average rate than single-copy sequences in the nucleus.

Base-pair changes among mitochondrial and nuclear DNAs can be compared in two other cases. First, Jakovic et al. (1975) have estimated about 28% base-pair mismatching among 70% of the sequences in mouse and rat mtDNAs. Estimates of sequence divergence among 40-75% of mouse and rat single-copy nuclear DNAs range from about 23% (Laird et al., 1969; Rice and Paul, 1971) to 33% (Kohne, 1970). As in Drosophila, sequence changes in the two rodents appear to have accumulated in a roughly coordinate fashion in mtDNA and the single-
copy fraction of nuclear DNA. In the second case concerning two frog species, indications are that mtDNA may have changed even more rapidly than most of the single-copy sequences in the nucleus. Of the separated strands of *Xenopus laevis* and *Xenopus borealis* mtDNAs, 70% will cross-hybridize. In contrast to the sharp melting transition obtained by electron microscopy for *D. melanogaster : D. virilis* heteroduplex mtDNA, optical measurements on *X. laevis : X. borealis* hybrid mtDNAs indicate a very broad range of thermal stabilities among various sequences corresponding to base-pair mismatchings between 0-50%, with a mean of about 21% (Dawid, 1972; Brown *et al.*, 1977). According to Galau *et al.* (1976), among single-copy sequences of nuclear DNAs of *X. laevis* and *X. borealis*, at least 10-15% of the base pairs have changed. This estimate probably applies to at least 70% of the single-copy fraction. In general, comparing two closely related species among animals including insects, amphibians, and mammals, nucleotide sequences of mtDNAs do not appear to be conserved more than major fractions of single-copy sequences in the nucleus. Certainly, in all three known cases, rates of nucleotide sequence divergence among portions of mitochondrial and nuclear DNAs overlap extensively.

Based on the assumption that the C-region is equivalent to the rDNA locus in *Drosophila* mtDNA (Klukas and Dawid, 1976), it is possible to compare rates of nucleotide substitutions between mitochondrial and nuclear ribosomal cistrons. Measurements of the thermal stability of heterologous rRNA:DNA hybrids and competition hybridizations indicate about 9% non-homology between sequences specifying 18 and 28S rRNA in *D. melanogaster* and *D. virilis*, while higher estimates (up to 17% non-
homology) are obtained in saturation hybridization experiments (Weber et al., 1976). My estimate of the extent of base-pair mismatching within the C-region of mtDNA hybrids (10-13%) falls within the lower range of the values for 18S28S rDNA. I conclude that mitochondrial and nuclear ribosomal cistrons have changed at a similar rate, which, however, is differentially lower than the rate of fixation of nucleotide substitutions in other portions of the mitochondrial and nuclear genomes.

The assumption that the C-region and the rDNA locus of Drosophila mtDNA are equivalent is justified for two reasons. First, the C-region and the rDNA locus are similar in size and both overlap the block of (A+T)-rich sequences of *D. melanogaster* mtDNA. Second, the comparison of mtDNAs among Xenopus species (Dawid, 1972) has already shown that the ribosomal cistrons represent the evolutionarily most stable, large region of mtDNA. This places the rDNA locus to the right of the schematic denaturation map of *D. melanogaster* oriented as shown in Figure 11, and thereby also orients the denaturation map with respect to cleavage sites of restriction endonucleases (Klükas and Dawid, 1976). The available analysis of partially denatured mtDNA fragments obtained by digestion with EcoRI endonuclease (Wolstenholme and Fauron, 1976) is compatible with the map orientation shown in Figure 11 but does not independently confirm it.

**Sequence Organization Among MtDNAs**

In the mitochondrial genome, rearrangements such as inversions or transpositions do not appear to have occurred since divergence of *D. melanogaster* and *D. virilis* from a common ancestor. Within the
**Figure 11.** Summary diagram

Blocks separated by lines in the middle diagram indicate the positions of the eight major peaks in the denaturation profile of Figure 6D. The peaks fall into the eight regions indicated along the upper margin (as in Figure 6). Region 5 includes the unusually (A+T)-rich sequences comprising 26% of the 18.5 kb long mtDNA of *D. melanogaster*. During denaturation of mtDNA, region 5 melts first, followed by strand separation in regions 2 and 4 and subsequently in the other regions. The analysis of *D. melanogaster : D. virilis* heteroduplex mtDNA draws attention to the three regions marked L, C, and V. Region L corresponds to the long arm of the asymmetric loop of undenatured heteroduplex molecules and includes sequences that are entirely missing (3.3 kb), plus sequences (1.0 kb) that are represented by non-homologous sequences in mtDNA of *D. virilis*. The V-region includes sequences that have changed the most among the homologous sequences of *D. melanogaster* and *D. virilis* mtDNA, while the C-region includes sequences that have changed the least. The rDNA locus of *D. melanogaster* mtDNA specifying the small (left) and the large (right) ribosomal RNAs are proposed to be oriented as shown below the denaturation map.
limits of heteroduplex analysis, this confirms the previous suggestion, based on comparisons of denaturation maps, that the topographic organization of sequences among Drosophila mtDNAs is highly conserved (Bultmann et al., 1976). Specifically, not only single members of the Sophophora and the Drosophila subgenera such as D. melanogaster and D. virilis, respectively, but all members of the two subgenera may share the same archetype sequence organization of mtDNA. Within the subgenus Drosophila, this has been confirmed, at least tentatively, by denaturation mapping of D. hydei mtDNA (Bultmann et al., 1976). Based on the data of Fauron and Wolstenholme (1976), it is possible to test whether within the subgenus Sophophora, the sequence organization of mtDNA is also invariant. Figure 12 shows a histogram constructed from partially denatured mtDNAs of D. ananassae (B) in comparison with a histogram of partially denatured D. melanogaster mtDNAs (A) derived from the histogram in Figure 3A simply by eliminating about 3.6 kb from the (A+T)-rich region 5 to match the contour length of mtDNA of D. ananassae. The two histograms are essentially identical, indicating that no major sequence rearrangements have occurred in the mtDNAs of the two species.

Presumably, there are topographic constraints that specifically limit rearrangements of sequences within mtDNAs in Drosophila and probably in other animals as well (see Introduction), despite the fact that in at least two other ways, the mitochondrial genome is not unusually conserved during evolution. First, and particularly striking in Drosophila, mtDNAs may sometimes accommodate a large block of species-specific sequences with unusual base composition, which
Figure 12. Comparison of denaturation maps of *D. melanogaster* and *D. ananassae*.

Histogram (A) of *D. melanogaster* mtDNA (right scale) is the same as the one in Figure 6D, except that the map intervals 0.44 to 0.63, inclusive, were eliminated to match the shorter contour length of *D. ananassae* (Cuba) mtDNA, which, according to Fauron and Wolstenholme (1976), is 80.5% of the contour length of *D. melanogaster* (Oregon-R). Histogram (B) (left scale) was constructed by realigning the partially denatured mtDNAs of *D. ananassae* (Cuba) depicted in Figure 2 in the article of Fauron and Wolstenholme (1976), according to Bultmann et al. (1976). On the average, the *D. ananassae* molecules are slightly less denatured (47 ± 6%; n = 30) than the *D. melanogaster* molecules (51 ± 10%; after the length adjustment).
apparently, is not transcribed (Chooi and Laird, 1976; Bonner et al., 1978). This phenomenon appears to be analogous to the accommodation of satellite DNAs in the nuclear genome. Second, among mtDNAs, including the mtDNAs of Drosophila, base substitutions may accumulate as rapidly as among large portions of nuclear DNAs. The stability of topographic relationships between sequences in the mitochondrial genophore of Drosophila is certainly conspicuous in contrast to the common occurrence of genetic rearrangements in the evolution of Drosophila chromosomes (cf. Patterson and Stone, 1952).
REFERENCES


