Volume 8 Number 22 1980 Nucleic Acids Research

# Intraspecific diversity of nucleotide sequences within the adenine + thymine-rich region of mitochondrial DNA molecules of Drosophila mauritiana, Drosophila melanogaster and Drosophila simulans

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

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

Received 22 June 1980

## ABSTRACT

Mitochondrial DNA (mtDNA) molecules from Drosophila mauritiana, D. melanogaster, and D. simulans contain a single adenine + thymine (A+T) -rich region, which is similarly located in all molecules, but varies in size among these species. Using agarose gel electrophoresis and electron microscopy, a difference in occurrence of one EcoRI site, and a difference in size (approximately 0.7 kb) of the A+T-rich regions was found between mtDNA molecules of flies of two female lines of D. mauritiana. In heteroduplexes constructed between these two kinds of mtDNA molecules, two or three regions of strand separation, each comprising single strands of unequal length, were apparent near the center of the A+T-rich region. Using the structural differences between D. mauritiana mtDNA molecules it was demonstrated that mtDNA of this species is maternally inherited. Differences in length of A+T-rich regions were also found between mtDNA molecules of two geographically separated strains of D. melanogaster, and between mtDNA molecules of two geographically separated strains of D. simulans. However, in both cases, in heteroduplexes constructed between mtDNA molecules of different strains of one species, the A+T-rich regions appeared completely paired.

## INTRODUCTION

Mitochondrial DNA (mtDNA) molecules isolated from species of the genus Drosophila contain a single region which is exceptionally rich in adenine and thymine (A+T) (3, 8, 13, 14, 17, 19, 22, 23). This region has been reported to be constant in size in mtDNA molecules of a single species (8, 14, 22). However, the A+T-rich regions of mtDNA molecules of different species vary from 1.0 kilobase pair (kb) in D. virilis to 5.1 kb in D. melanogaster (8, 23).

We have recently shown that in mtDNA molecules of D. melanogaster, D. simulans, D. mauritiana, D. yakuba, D. takahashii and D. virilis, the A+T-rich region is at a homologous location (9), and that the single origin of replication of the mtDNA molecules of each species lies within the A+T-rich region (11, 12). However, from the results of heteroduplex analyses it appears that there is extensive diversity of nucleotide sequences within the

A+T-rich region, among the six species mentioned above (9). Under conditions highly permissive for base pairing, the entire A+T-rich regions of D. melanogaster, D. yakuba, D. takahashii and D. virilis failed to pair. In heteroduplexes constructed between D. melanogaster and D. simulans, and between D. melanogaster and D. mauritiana, up to 35% of the A+T-rich region paired. Only in heteroduplexes between D. simulans and D. mauritiana mtDNAs did the A+T-rich region appear fully paired.

The purpose of the present paper is to report the finding of variant mtDNAs among flies of individual species of D. mauritiana, D. melanogaster and D. simulans in regard to the nucleotide sequences within the A+T-rich region.

## MATERIALS AND METHODS

Fly stocks. The Drosophila melanogaster strains (and places of origin) used in these experiments were as follows: 1) Oregon R-Utah (Oakridge, Tennessee) (22); 2) Nagasaki (Japan); 3) L-M (U.S.S.R.). The latter two strains were obtained from Dr. G. Picard, Université de Clermont-Ferrand, Aubière, France. The two D. simulans strains used (stock number and place of origin) were obtained from the Species Stock Collection of the Genetics Foundation, University of Texas at Austin; 1) 3015.8, Nueva, California; 2) 2395.5, Pisco, Peru. The D. mauritiana stock (Mauritius Island) from which lines I and II were derived (see Results) was provided by Dr. L. Tsacas, Centre National de la Recherche Scientifique, Gif-sur-Yvette, France.

Details of all of the techniques used in this work which are not mentioned in the text, have been given previously (4, 9, 10, 11, 22).

## RESULTS

Drosophila mauritiana mtDNAs. A stock of D. mauritiana, originating from more than 10 males and 10 females, obtained from Dr. L. Tsacas in 1978, has been maintained in our laboratory by multiple-pair transfer every 21 days. When mtDNA from ovaries of flies of this stock was subjected to electrophoresis after digestion with EcoRI, 10 bands were detected (Figs. 1 and 8). The sum of the sizes (8.2, 7.4, 4.2, 2.6, 1.8, 1.4, 1.1, 1.0, 0.9, and 0.4 kb) of the fragments in the bands is approximately 29.0 kb, which is 1.6 times the size of D. mauritiana circular mtDNA molecules (approximately 18.6 kb) determined previously from electron microscope data (23). These results suggest that the D. mauritiana mtDNA used contains circular molecules which are heterogeneous in regard to the number and relative location of EcoRI

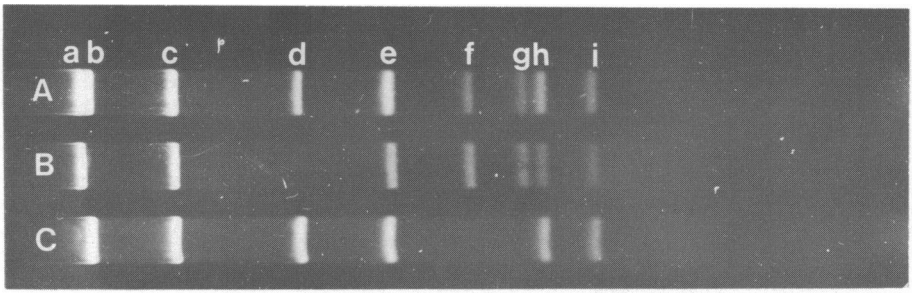



Figure 1. Fluorescence photograph showing the distribution of ethidium bromide-stained bands representing the EcoRI digestion products of D. mauritiana mtDNAs in a 1.0% agarose slab gel after electrophoresis. Migration was from left to right. Lane A contains mtDNA of the original D. mauritiana stock. Nine bands (a-i) are visible. Lanes B and C contain mtDNAs obtained from the progeny of two individual females, respectively. The mtDNA in lane B comprises 7 bands; bands b and d are not visible. The mtDNA in lane C comprises 6 bands; bands a, f and g are not visible. A further band (k) containing fragments of 0.4 kb was visible in some other electrophorograms (see Fig. 8) of each of these mtDNA preparations.

sites they contain.

In order to determine the origin of the different mtDNA molecules predicted, we prepared mtDNA from the ovaries of 5 different groups of female flies, each group being the $F_3$ progeny of a single pregnant female taken from a D. mauritiana stock bottle. A sample of each mtDNA was digested with EcoRI and analysed by agarose gel electrophoresis. Eight bands containing fragments totalling 18.2 kb were apparent for the mtDNAs derived from the progeny of each of three females (Figs. 1 and 8). Seven bands containing fragments totalling 18.4 kb were apparent for the mtDNAs derived from the progeny of each of the remaining two females (Figs. 1 and 8). The flies from which mtDNAs are cleaved with EcoRI into 8, and into 7 fragments have been designated line I and line II respectively.

From a consideration of the data in Fig. 1, it appears that 1) the differences in occurrence of bands d, f and g among line I and II mtDNAs result from the line I mtDNA containing a unique EcoRI site, and 2) the difference in size of the largest EcoRI fragments from line I and line II mtDNAs results from a difference in length of the nucleotide sequence between the two EcoRI sites defining this fragment.

Both line I and line II mtDNAs were cleaved into four fragments with the restriction endonuclease HindIII (Fig. 2). Three of these fragments migrated

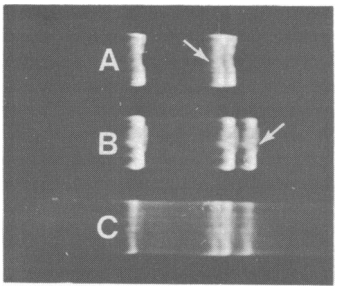



Figure 2. Fluorescence photograph showing the distribution of ethidium bromide-stained bands representing the 3 largest of the 4 fragments produced by HindIII digestion of mtDNA from D. mauritiana in a 1.0% agarose slab gel after electrophoresis. Migration was from left to right. Lane A contains line I mtDNA, lane B contains line II mtDNA, and lane C contains a mixture of line I and line II mtDNAs. The two bands common to line I and line II mtDNAs contain fragments of 8.4 and 4.8 kb. The single band unique to line I mtDNA (arrow, lane A) contains fragments of 5.0 kb and the single band unique to line II mtDNA (arrow, lane B) contains fragments of 4.3 kb.

to similar positions upon electrophoresis of the two mtDNAs. However, a band similar in position to that representing the second largest fragment (Fig. 2, arrow; 5.0 kb) of line I mtDNA was absent in line II mtDNA and was replaced by a band (Fig. 2, arrow) which contained fragments of 4.3 kb.

Both the differences in size of the largest EcoRI fragments and of one of the HindIII fragments of line I and line II mtDNAs, determined by agarose gel electrophoresis, were confirmed by making length measurments of restriction fragments in electron microscope preparations.

We have shown previously (9) that the largest EcoRI fragment, and the second largest HindIII fragment of D. mauritiana line I mtDNA contain the A+T-rich region. Examination in the electron microscope of EcoRI-digested line I and line II mtDNAs which had been exposed to conditions sufficient to denature the A+T-rich region (Fig. 3), indicated that the observed difference in size between line I and line II mtDNA molecules could be completely accounted for by a difference in size of the A+T-rich region in the two molecules.

The relative positions of EcoRI sites and HindIII sites, and of the A+T-rich regions of D. mauritiana line I and line II mtDNAs are shown in Fig. 4.

In order to gain information on the distribution of nucleotide sequences within the A+T-rich region, which differ in line I and line II mtDNAs we constructed heteroduplexes. A sample of line II mtDNA shown by electron microscopy to comprise > 85% circular molecules, of which approximately 90% were nicked, was mixed with a 5-fold excess of fragments obtained by EcoRI digestion of line I mtDNA. The mixture was dialyzed against 95% formamide to denature the DNAs and a sample removed for electron microscope examination to confirm the completeness of denaturation. The denatured DNA mixture was then

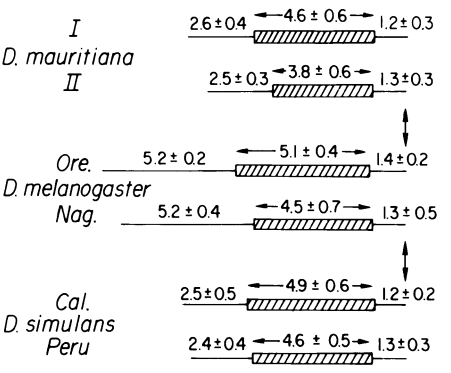



Figure 3. Denaturation maps of the largest EcoRI fragments of mtDNA molecules of each of the lines or strains of each of the Drosophila species indicated, produced by heating at 41°C for 10 min in 0.05 M sodium phosphate (pH 7.8) and 10% formaldehyde (8). Single lines represent double-stranded segments. The single denatured segment (A+T-rich region) of each fragment is indicated by the hatched area. The molecules of each species are shown aligned (double-headed arrows) by the end of each EcoRI fragment closest to the A+T-rich region in each case, as this EcoRI site is common to all Drosophila mtDNAs studied (Fig. 4). All values are given in kilobase pairs (± SD), and means are derived from measurements of 20 EcoRI fragments of mtDNAs of each strain.

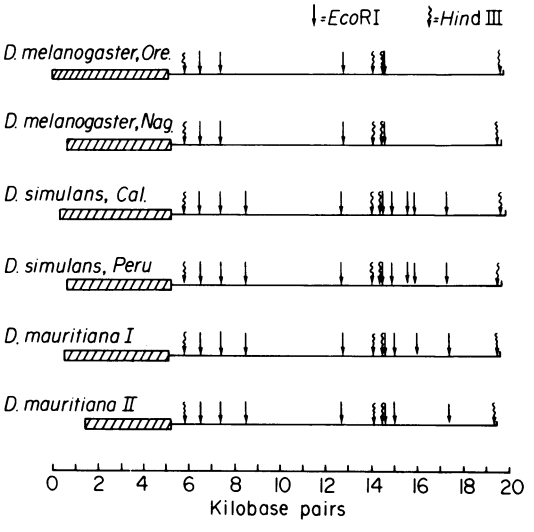
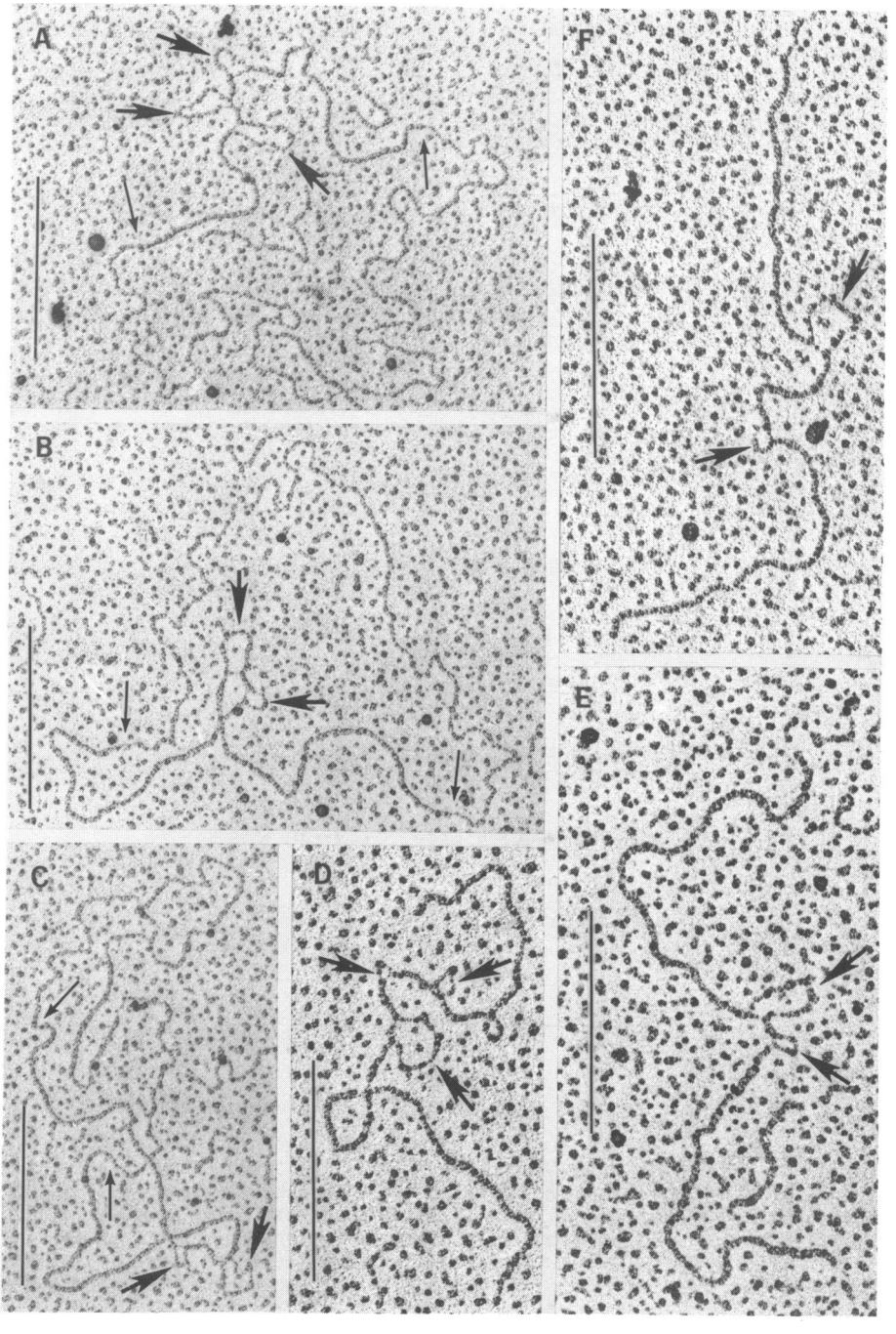



Figure 4. Maps of the mitochondrial genomes of two different lines or strains of each of the three Drosophila species indicated, showing the relative positions of the A+T-rich regions (hatched areas) and the sites at which the restriction endonucleases EcoRI and HindIII cleave. The genomes have been 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.


A
B
C
D
E
F

dialyzed against 35% formamide, and a sample removed at various times, and prepared for electron microscopy by the formamide protein monolayer technique. The samples in which approximately 50% of the DNA was renatured were used for further analysis.

Circular molecules containing a single double-stranded segment were located and photographed, and analysed in regard to the lengths of the component double- and single-stranded segments. In some of the selected molecules, the double-stranded segment was approximately equal in length to either the second largest (4.3 kb) or the third largest (1.8 kb) EcoRI fragment of line I mtDNA (more than 10 of each were measured). Other selected molecules contained a double-stranded segment which varied among molecules from 0.8 to 1.4 kb, lengths expected for the smaller EcoRI fragments of line I mtDNA (Fig. 4). In none of these double-stranded segments of heteroduplexes were single-stranded (deletion/insertion) loops, or separated single strands observed.

Also among the located circular molecules were ones in which the double-stranded segment was of approximately the size expected for the largest EcoRI fragment of line I mtDNA. In all of these molecules (Figs. 5 and 6) two or three segments of strand separation were observed in a central 1.0 kb portion of the double-stranded segment. In each of these segments of strand separation (or loops) the length of one of the single strands was larger than the other, suggesting that they represent nucleotide sequences which are different in length as well as non-homologous. From a consideration of the location of the A+T-rich region within the larger EcoRI fragment (Fig. 4), it is clear that, regardless of whether the smaller loops observed were on the same side of the larger, common loop in different molecules, all observed loops must lie within the A+T-rich region.

Figure 5. Electron micrographs of heteroduplex molecules of D. mauritiana line I and line II mtDNA molecules. The circular heteroduplex molecules (A, B and C) were produced by denaturation and renaturation of EcoRI fragments of line I mtDNA molecules, and nicked, circular line II mtDNA molecules. The smaller arrows in the micrographs A, B, and C indicate the limits of the double-stranded region which correspond to the ends of the A+T-rich region-containing EcoRI fragment of line I mtDNA and the complementary region of the line II circular mtDNA molecule. The large arrows indicate the unpaired regions, which in A, B, and C, correspond to the looped, unpaired regions represented in Fig. 6, c, a, and b respectively. The linear heteroduplex molecules (D, E, and F) were produced by denaturation and renaturation of a mixture of EcoRI fragments of line I and line II mtDNAs. The large arrows again indicate unpaired regions which in D, E, and F, correspond to the looped, unpaired regions represented in Fig. 6, $c_1$, $a_1$, and $b_1$ respectively. In all micrographs the bar represents 0.5 μm.

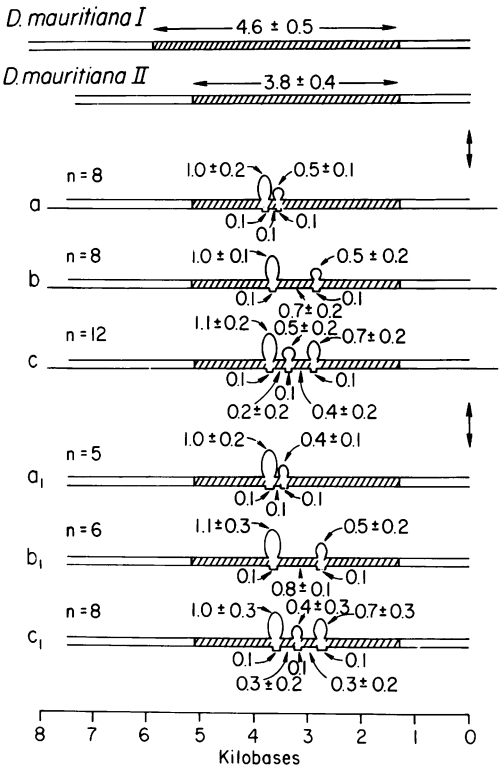



Figure 6. Data concerning heteroduplexes formed between either a strand of an EcoRI fragment of D. mauritiana line I mtDNA and a circular strand of D. mauritiana line II mtDNA (a, b, and c), or between strands of EcoRI fragments of line I and line II mtDNA molecules ($a_1$, $b_1$, and $c_1$). The EcoRI fragment containing the A+T-rich region (hatched area) of the mtDNA molecules of each line is shown in the upper part of the diagram. The two sets of heteroduplexes, each classified into three groups in regard to the number and distribution of the unpaired segments (loops) they contain, are shown in the lower part of the diagram. Orientation of the loops within the EcoRI fragment of circular heteroduplexes was determined from the data given in Fig. 7. In each heteroduplex, parallel lines represent double-stranded segments and hatched areas indicate paired segments within the A+T-rich region. All heteroduplexes are shown aligned (double-headed arrows) by the end of each EcoRI fragment closest to the A+T-rich region (see Fig. 4). In both sets of diagrams representing heteroduplexes, the placement of all loops on the upper strand is arbitrary, as the origin of the individual strands in each unpaired segment could not be determined in these molecules. The mean sizes (± SD) in kilobases, of unpaired segments and of the distances between unpaired segments are indicated. All 0.1 kb values have an SD of approximately ± 0.1 kb.

Heteroduplex molecules were located which contained one of the largest EcoRI fragments, and a second fragment with a length indicating it to be either the second largest (4.3 kb) or third largest (1.8 kb) EcoRI fragment of line I mtDNA. By comparing the locations of the loops in the larger fragment relative to each other, and to the ends of the fragment, with the position in the heteroduplex of the second fragment (Fig. 7) it was determined that the smaller loops were always located between the large, common loop and the EcoRI site which is closest to the A+T-rich region (and common to all Drosophila mtDNAs (9)). Given this information, it was possible to classify all of the heteroduplexes which contain the largest EcoRI fragment into three types as shown in Fig. 6.

In the renatured preparation from which the data presented above were 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 reaction mixture prior to annealing, these forms must represent renaturation products. 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 D. mauritiana line.

A second set of heteroduplexes of D. mauritiana line I and line II mtDNAs were constructed. Approximately equal amounts of circular mtDNAs of

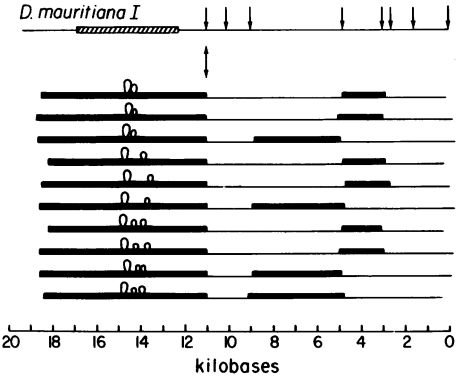



Figure 7. Heteroduplexes constructed using EcoRI fragments of D. mauritiana line I mtDNA, and nicked circular molecules of D. mauritiana line II mtDNA. Each heteroduplex shown was selected as containing two double-stranded segments (thick lines), one the length of the largest EcoRI fragment and a second with a length indicating it to be either the second or third largest EcoRI fragment. The relative locations in each heteroduplex of the two double-stranded segments were then compared with the EcoRI restriction map of D. mauritiana line I mtDNA (top of diagram) and aligned (following linearization for convenience of illustration) to best fit this map at the end of the largest EcoRI fragment closest to the A+T-rich region (hatched segment of upper diagram). The thin lined loops represent segments of strand separation.

the two lines were mixed, digested with EcoRI and then denatured and renatured as described above. In each preparation, among the renaturation products, double-stranded linear molecules were found which were of a size indicating them to be the renaturation products of the largest EcoRI fragments. Some of these molecules were free from strand separations and were presumably homoduplexes, that is the renaturation products of complementary strands of EcoRI fragments of mtDNA from the same line. Others of these molecules contained either two or three segments of strand separation. From a consideration of the sizes and locations of these segments, the molecules could be classified into three types (Fig. 6, $a_1$, $b_1$ and $c_1$), similar to those found for heteroduplexes comprising a strand of an EcoRI fragment of line I mtDNA and a circular strand of line II mtDNA.

In each of the three types of molecules shown in Fig. 6, a-c, and $a_1$-$c_1$, the sum of the differences in length of the two single-strands of each of the two or three segments of strand separation (1.2 kb to 2.0 kb) is greater than the difference in length of the A+T-rich regions of line I and line II mtDNA molecules (Fig. 3).

We have recently shown that the same renaturation conditions as those presently used to construct heteroduplexes between D. mauritiana line I and line II mtDNAs, resulted in complete pairing of the A+T-rich regions in heteroduplexes of D. mauritiana line I and D. simulans Cal. mtDNAs (9). We therefore constructed heteroduplexes between EcoRI fragments of D. mauritiana line II mtDNA, and nicked circular mtDNA molecules of D. simulans Cal. Circular heteroduplexes were located which contained a single double-stranded segment approximately the size of the largest EcoRI fragment of D. mauritiana line II mtDNA. All of these double-stranded segments were interrupted by segments of strand separation (loops) which compared in number, size and position to the loops found in similar heteroduplexes constructed between D. mauritiana line I and II mtDNAs.

In view of the heteroduplex data concerning D. mauritiana and D. simulans mtDNAs, it seemed worthwhile to determine the extent to which D. mauritiana line I and line II flies are capable of interbreeding. Using virgin females, 25 single pair matings of line I and line II flies were set up in 1" X 3.75" vials, in each of the four possible combinations (Table 1-A). The parents were removed after 5 days. Following emergence of the first flies, progeny were removed daily from the vials and counted for a period of 7 days. The results, which are summarized in Table 1 clearly fail to indicate that any degree of infertility exists between line I and line II flies. In a sec-

Table 1. Results of two sets (A and B) of crosses between flies of D. mauritiana line I and D. mauritiana line II.

A

| Parental Cross[a] | Number of crosses producing progeny | Mean number (± SD) of $F_1$ progeny per successful cross[b] | | |
|---|---|---|---|---|
| | | ♀ | ♂ | Total |
| $I_♀ \times I_♂$ | 11 | 16.5 ± 5.7 | 17.0 ± 6.1 | 33.5 ± 5.9 |
| $I_♀ \times II_♂$ | 13 | 16.3 ± 4.6 | 17.0 ± 4.4 | 33.3 ± 4.5 |
| $II_♀ \times II_♂$ | 12 | 17.1 ± 5.8 | 15.5 ± 6.0 | 32.6 ± 5.9 |
| $II_♀ \times I_♂$ | 11 | 18.2 ± 4.5 | 16.1 ± 3.9 | 34.3 ± 4.2 |

B

| Parental Cross[a] | Number of crosses producing progeny | Mean number (± SD) of $F_1$ progeny per successful cross[b] | | | Number of pregnant $F_1$ females[c] | Mean number (± SD) of $F_2$ progeny produced per $F_1$ female[d] | | |
|---|---|---|---|---|---|---|---|---|
| | | ♀ | ♂ | Total | | ♀ | ♂ | Total |
| $I_♀ \times II_♂$ | 18 | 20.4 ± 4.0 | 18.6 ± 4.2 | 39.0 ± 4.1 | 5 | 52.8 ± 1.8 | 52.8 ± 1.9 | 105.6 ± 1.9 |
| $II_♀ \times I_♂$ | 13 | 16.7 ± 4.5 | 16.9 ± 4.2 | 33.6 ± 4.4 | 11 | 56.7 ± 2.3 | 57.0 ± 1.8 | 113.7 ± 2.1 |

a) Twenty-five single-pair crosses of each of the combinations shown were set up in 1" x 3.75" vials.
b) Collected over a period of seven days following emergence of the first adults.
c) Each pregnant female, taken from among the $F_1$ progeny seven days after emergence of the first adults, was placed in a 6 oz. culture bottle. All of these females, representing both parental combinations, produced progeny.
d) Collected over a period of 5 days following emergence of the first adults. The greater number of $F_2$ progeny produced per $F_1$ female resulted from the use of bottles rather than vials.

ond fertility experiment, 25 single pair matings of each of the combinations, line I female X line II male, and line II female X line I male, were set up in vials (Table 1-B). The mean numbers of $F_1$ progeny were similar to those found in the first experiment. From the progeny of each type of cross, single pregnant females were placed into 6 oz. culture bottles. The number of $F_2$ progeny which emerged in each bottle over a 5 day period following the emergence of the first flies were determined. The results (Table 1-B) again failed to provide evidence for infertility between line I and line II flies.

The $F_1$ progeny of the crosses represented in Table 1-A were used to gain information on the inheritance of mtDNA in Drosophila. The $F_1$ females which were removed daily from each vial were placed into bottles containing live yeast for a period of 3 days. Ovaries were then dissected from the flies and immediately frozen. MtDNAs were isolated from collections of ovaries from females resulting from the four types of cross, and then analyzed by agarose gel electrophoresis following EcoRI digestion. The results of this experiment (Fig. 8) clearly indicate that the mtDNA of the progeny show the pattern of EcoRI fragments characteristic of that of the mtDNA of the female flies from which they were derived.

Drosophila melanogaster mtDNAs. MtDNA was isolated from flies of three different strains of D. melanogaster which originated from widely separated geographical locations: Oregon-R-Utah (Ore.), Nagasaki (Nag.), and L-M. Using agarose gel electrophoresis it was determined that each of the three mtDNAs was cleaved into four fragments by HindIII (Fig. 9). Three of the fragments (a, 8.4 kb; c, 4.8 kb; d, 0.4 kb, Fig. 9) of each mtDNA migrated to a similar position in the gel. However, the fourth (b) fragment migrated progressively further into the gel in mtDNAs of flies of the strains D. melanogaster Ore., D. melanogaster L-M, and D. melanogaster Nag. to positions indicating sizes of 5.9, 5.5 and 5.1 kb respectively. In electron microscope preparations the mean sizes of the HindIII b fragments of D. melanogaster Ore. and D. melangaster Nag. were 5.9 $\pm$ 0.2 kb (n = 30) and 5.2 $\pm$ 0.3 kb (n = 30) respectively. From analyses of data obtained using agarose gel electrophoresis and electron microscopy of EcoRI digested mtDNAs from D. melanogaster Ore. and D. melanogaster Nag. (Fig. 3 and data not shown) it was determined that, as in the case of D. mauritiana line I and line II mtDNAs, a difference in size of the A+T-rich regions of D. melanogaster Ore. and D. melanogaster Nag. mtDNAs could completely account for the noted difference in the size of HindIII restriction fragments of these mtDNAs (Fig. 4).

In experiments similar to those described for D. mauritiana mtDNAs het-

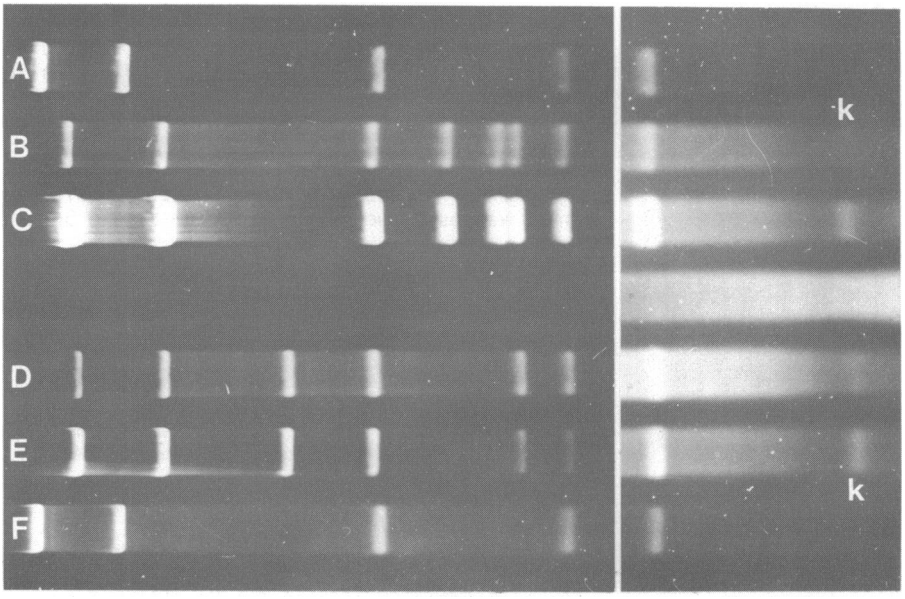



Figure 8. Fluorescence photographs showing the distribution of ethidium bromide-stained bands representing the EcoRI digestion products of Drosophila mtDNAs in a 1.0% agarose slab gel after electrophoresis. Migration was from left to right. In the left hand photograph lanes B and E contain mtDNAs from D. mauritiana line I, and D. mauritiana line II respectively. Lane C contains mtDNA from the progeny of a cross between a D. mauritiana line I female and a line II male. Lane D contains mtDNA from the progeny of a cross between a D. mauritiana line II female and a line I male. Lanes A and F contain the four EcoRI digestion products of D. melanogaster Oregon-R mtDNA as size standards (11.50, 5.37, 1.74 and 0.9 kb [22]). The photograph on the right is an underexposed print of the right end of the gel shown in the photograph on the left, to demonstrate in each D. mauritiana mtDNA digest the presence of a band (k) containing fragments of approximately 0.4 kb.

eroduplexes were constructed using 1) EcoRI fragments of D. melanogaster Ore. mtDNA and nicked circular molecules of D. melanogaster Nag. mtDNA, 2) EcoRI fragments of D. melanogaster Nag. mtDNA and nicked circular molecules of D. melanogaster Ore. mtDNA, 3) HindIII fragments of D. melanogaster Ore. mtDNA and nicked circular molecules of D. melanogaster Nag. mtDNA, and 4) HindIII fragments of D. melanogaster Nag. mtDNA and nicked circular molecules of D. melanogaster Ore. mtDNA. In electron microscope preparations of these heteroduplexes, we failed to observe single-stranded regions of any kind interspersed in double-stranded regions which represented the pairing of a strand of a restriction fragment, including the one which contains the A+T-rich re-

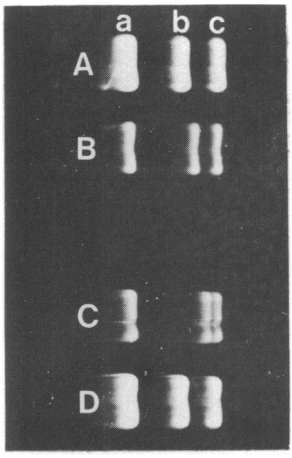



Figure 9. Fluorescence photograph showing the distribution of ethidium bromide-stained bands representing the 3 largest of the 4 fragments produced by HindIII digestion of D. melanogaster mtDNAs in a 1.0% agarose slab gel after electrophoresis. Migration was from left to right. Lanes A and D contain D. melanogaster Ore. mtDNA. Lanes B and C contain D. melanogaster L-M, and D. melanogaster Nag. mtDNAs respectively. The relative position of band b varies between strains, from 5.1 to 5.9 kb.

gion, and a strand of a circular molecule (Fig. 10).

In view of the results of these heteroduplex experiments, we further tested the possibility that the difference in size of the HindIII b fragments of D. melanogaster Ore. and D. melanogaster Nag. mtDNAs could result from the presence of one or more extra HindIII sensitive sites near the end of this fragment. The products of HindIII digestion of D. melanogaster Nag. mtDNA were examined following electrophoresis in 4% acrylamide gels. While the band expected for the HindIII d fragment (420 base pairs) was clearly discernable in these gels, no other bands representing fragments in the range 100-1000 base pairs were apparent.

D. simulans mtDNAs. MtDNA was next isolated from flies of two different strains of D. simulans: one originating from California (Cal.) and the other from Peru. Data obtained from electrophoretic analysis (Fig. 11) and electron microscopy (Fig. 3) of HindIII and EcoRI digestion products, respectively, of the two mtDNAs, again indicated a difference in size of the nucleotide sequences in the A+T-rich region of these mtDNAs. The difference in this case was, however, only 0.3 kb (Fig. 4). Heteroduplexes were constructed between nicked circular molecules of D. simulans Cal. mtDNA and 1) EcoRI fragments of D. simulans Peru mtDNA and 2) HindIII fragments of D. simulans Peru mtDNA. Examination of these heteroduplexes (Fig. 10) again failed to reveal regions of strand separation.

## DISCUSSION

The data presented establish that an overall difference in length of

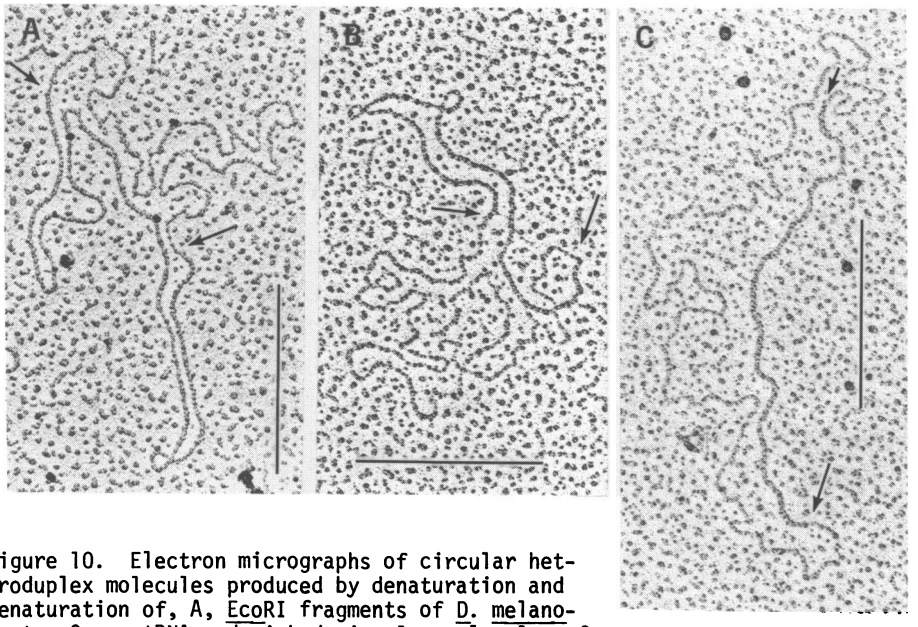



Figure 10. Electron micrographs of circular heteroduplex molecules produced by denaturation and renaturation of, A, EcoRI fragments of D. melanogaster Ore. mtDNA and nicked circular molecules of D. melanogaster Nag. mtDNA; B, HindIII fragments of D. melanogaster Ore. mtDNA and nicked circular molecules of D. melanogaster Nag. mtDNA; C, HindIII fragments of D. simulans Peru mtDNA and nicked circular molecules of D. simulans Cal. mtDNA. The arrows indicate the limits of the double-stranded regions, which in each heteroduplex correspond to the ends of the A+T-rich region-containing restriction fragment and the complementary regions of the circular molecule. The bar represents 0.5 μm.

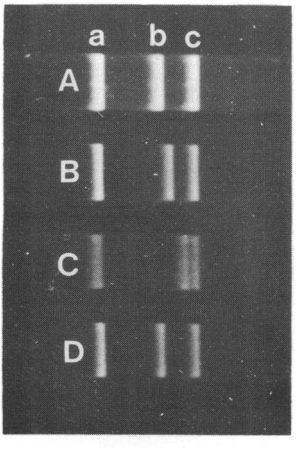



Figure 11. Fluorescence photograph showing the distribution of ethidium bromide-stained bands representing the three largest of the four fragments produced by HindIII digestion of Drosophila mtDNAs, in a 1.0% agarose slab gel after electrophoresis. Migration was from left to right. Lanes B and C contain mtDNA from D. simulans, Cal. and D. simulans, Peru respectively. Band b has migrated further into the gel in lane C than in lane B. Lanes A and D contain the EcoRI digestion products of D. melanogaster Ore. mtDNAs as size standards.

approximately 0.7 kb exists between the A+T-rich regions of mtDNA molecules of flies of the two different lines of D. mauritiana, derived from the original stock we obtained from Dr. L. Tsacas. In heteroduplexes constructed between mtDNAs of D. mauritiana line I and line II, two or three unpaired segments were seen in a centrally located portion of the A+T-rich region. The largest of these unpaired segments was common to all heteroduplexes, and its two component strands were approximately 1.1 and 0.1 kb. This suggests that one of these strands contained nucleotide sequences totalling at least 1.0 kb which were completely absent from the corresponding region of the complementary strand. Each of the other two regions of strand separation seen in D. mauritiana heteroduplexes also comprised complementary strands of different length, but one or other of these segments of strand separation were not seen in some of the heteroduplexes. A clear explanation of this observation is not possible from the data obtained. The possibility that this observation could result from the mtDNA molecules from one or both of the D. mauritiana lines being heterogeneous in regard to the nucleotide sequences within the A+T-rich region, is not supported by our observations of full pairing in renatured molecules in which both strands were derived from mtDNA of a single line.

It was noted that the sum of the differences in length of the single strands of the two or three regions of strand separation (1.2 kb to 2.0 kb, Fig. 6) found in the A+T-rich region in heteroduplexes was greater than the overall difference in length (0.7 kb) of the A+T-rich region of line I and line II mtDNA molecules. This suggests that within at least one portion of the A+T-rich region of line II mtDNA the overall length of the nucleotide sequence is greater than the corresponding nucleotide sequence of line I mtDNA.

It appears from the present data, and our previous observations (9) that the degree of base pairing which can be achieved in heteroduplexes of the A+T-rich regions of mtDNA molecules of D. mauritiana line I and D. simulans Cal. is considerably greater than in heteroduplexes of the A+T-rich regions of mtDNA molecules from the two different lines of D. mauritiana. Also, the EcoRI site which is present in D. mauritiana line I mtDNA, but not in D. mauritiana line II mtDNA, is also present in D. simulans Cal. mtDNA (Fig. 4). However, the mtDNAs of both strains of D. simulans tested contain an EcoRI site which is not found in mtDNA molecules of either of the D. mauritiana lines (Fig. 4). D. simulans and D. mauritiana are considered to be the most closely related of all the melanogaster subgroup species. This conclusion is based on taxonomic criteria, comparisons of larval salivary gland chromosome

bands, and the extent to which these species are capable of interbreeding (5, 16). In regard to the latter, crosses of D. simulans and D. mauritiana yield fertile females in each reciprocal cross. Sterile males are produced when the female parent is D. simulans but no males are produced when the female parent is D. mauritiana (16). Our data indicate that the nucleotide sequences of A+T-rich regions of D. mauritiana line I and line II mtDNA molecules have diverged to a greater degree than have the nucleotide sequences of A+T-rich regions of D. mauritiana line I and D. simulans Cal. mtDNA molecules. This suggests that among D. mauritiana flies, the line I mtDNA molecule is more primitive than the line II mtDNA molecule.

It seems clear from the data presented that the A+T-rich regions of mtDNA molecules of D. melanogaster Ore. and D. melanogaster Nag. also differ in overall length by approximately 0.7 kb (Fig. 4). In this case, however, unlike the situation for line I and line II mtDNAs of D. mauritiana, we failed to observe any regions of strand separation in heteroduplexes which included the A+T-rich regions of the two D. melanogaster mtDNAs. As it would be expected that differences in length, or of mismatch of nucleotide sequences of as little as 50 base pairs would have been detected by the technique employed (6), it seems reasonable to argue from this latter finding that the overall difference in length between the D. melanogaster Ore. and D. melanogaster Nag. mtDNAs results from many small differences scattered throughout the A+T-rich regions. As regions of strand separation were also not observed in heteroduplexes which included A+T-rich regions of mtDNA molecules of D. simulans Cal. and D. simulans Peru, a similar explanation for the 0.3 kb difference in length of the A+T-rich region of these molecules seems appropriate.

Differences in length of up to 0.75 kb, of A+T-rich region-containing restriction fragments of mtDNAs from three different strains of D. melanogaster have recently been reported by others (20). Also, differences in sensitivity to various restriction enzymes among mtDNAs from different strains of the same Drosophila species have been demonstrated. In one case, differences of two HindIII and one HaeIII cleavage sites among mtDNAs of three strains of D. melanogaster, and of one HindIII cleavage site between mtDNAs of two strains of D. virilis were found (21). In a second case a difference of one HaeIII cleavage site was found among mtDNAs of three D. melanogaster strains (20).

In the present experiments, the sensitivity to EcoRI of mtDNA molecules of the progeny of reciprocal crosses between D. mauritiana line I and line II flies indicates that these mtDNAs are mainly, if not totally, inherited from

the female parent. A similar mode of inheritance has recently been claimed for D. melanogaster mtDNA (20). Evidence for maternal inheritance of mtDNAs of vertebrate species has been obtained from the results of studies of crosses involving the closely related species Xenopus laevis and X. mulleri (7) and Equus caballus and E. asinus (15), and of crosses involving rats with mtDNAs having different sensitivities to EcoRI (4). In D. melanogaster it has been shown that the nebenkern, the sperm mid-piece component derived from mitochondria, actually enters the ovum (18). It cannot, therefore, be ruled out, as has been argued for similar reasons in rat (4) and noted by Hutchison et al. (15), that mtDNA molecules from the male parent make a minor contribution to the mtDNA complement of the organism.

We previously presented evidence from the results of heteroduplex experiments that the nucleotide sequences in the A+T-rich regions of mtDNA molecules of different Drosophila species have diverged extensively. The present data provide evidence that in different flies of the same species the A+T-rich regions of mtDNA molecules have diverged in regard to both sequence arrangement and size.

While the D. melanogaster and D. simulans flies containing mtDNA molecules with different length A+T-rich regions were from widely different geographic locations, this was not so for D. mauritiana. Further, in the case of D. mauritiana our data indicate the absence of a fertility barrier between flies containing variant A+T-rich regions. In regard to these considerations it is important to note that if, as the presently available data implies, Drosophila and other metazoan mtDNA is solely inherited from the females, and mtDNA molecules of an individual organism are homogeneous in nucleotide sequence, then mtDNA of any given two female lines will diverge simply as a function of the accumulation (fixation) of viable mutations.

We have noted previously (9) that while the A+T-rich region contains the origin of replication (11, 12) it seems unlikely from a consideration of the base composition of this region of the D. melanogaster mtDNA molecule that it contains sequences coding for proteins. Two reported failures to detect RNA transcripts which map in the A+T-rich region (1, 2) support this argument. Clearly, any function which might be proposed for the A+T-rich region must now take into account not only the extensive differences in size (8) and sequence (9) of this region among Drosophila species, but the considerable differences in size and sequence which have occurred within this region in female lines of flies which can, and presumably do in the case of D. mauritiana, interbreed.

ACKNOWLEDGEMENTS

We thank Mr. W. D. Ashworth, Jr. for technical assistance, Dr. J. M. Goddard for discussions, Drs. G. Picard and L. Tsacas for providing flies, and Dr. L. M. Okun for constructive criticism of the manuscript. This investigation was supported by National Institutes of Health Grant No. GM 18375. This work has been submitted by C. M.-R. Fauron in partial fulfillment of the requirements for the degree of Doctor of Philsophy (Biology), College of Science, University of Utah.

REFERENCES

1. Battey, J., Rubenstein, J. L. R., and Clayton, D. A. (1979) Proc. 8th Ann. ICN-UCLA Symp. Molecular and Cellular Biology: Extrachromosomal DNA, pp. 427-442, ed. D. Cummings, I. B. Dawid, P. Borst, S. Weissman, and F. Fox. Academic Press, New York.
2. Bonner, J. J., Berninger, M., and Pardue, M. L. (1978) Cold Spring Harbor Symp. Quant. Biol. 42, 803-814.
3. Bultmann, H., and Laird, C. D. (1973) Biochim. Biophys. Acta 299, 196-209.
4. Buzzo, K., Fouts, D. L. and Wolstenholme, D. R. (1978) Proc. Natl. Acad. Sci. USA 75, 909-913.
5. David, J., Lemeunier, F., Tsacas, L., and Bocquet, C. (1974) Ann. Génét. 17, 235-241.
6. Davis, R. W., Simon, M. and Davidson, N. (1971) In Methods in Enzymology, eds. Grossman, L. and Moldave, K. (Academic Press, New York), Vol. XXI, pp. 413-428.
7. Dawid, I. B., and Blacker, A. W. (1972) Developmental Biol. 29, 152-161.
8. Fauron, C. M.-R. and Wolstenholme, D. R. (1976) Proc. Natl. Acad. Sci. USA 73, 3623-3627.
9. Fauron, C. M.-R., and Wolstenholme, D. R. (1980) Nucleic Acids Res. 8, 2439-2452.
10. Fouts, D. L., Manning, J. E. and Wolstenholme, D. R. (1975) J. Cell Biol. 67, 378-399.
11. Goddard, J. M. and Wolstenholme, D. R. (1978) Proc. Natl. Acad. Sci. USA 75, 3886-3890.
12. Goddard, J. M. and Wolstenholme, D. R. (1980) Nucleic Acids Res. 8, 741-757.
13. Goldring, E. S. and Peacock, W. J. (1977) J. Cell Biol. 73, 279-286.
14. Klukas, C. K. and Dawid, I. B. (1976) Cell 9, 615-625.
15. Hutchison, C. A. III, Newbold, J. E., Potter, S. S. and Edgell, M. H. (1974) Nature 251, 536-538.
16. Lemeunier, F. and Ashburner, M. (1976) Proc. Roy. Soc. London, B193, 275-294.
17. Peacock, W. J., Brutlag, D., Goldring, E., Appels, R., Hinton, C. and Lindsley, D. C. (1974) Cold Spring Harbor Symp. Quant. Biol. 71, 434-448.
18. Perotti, M. E. (1973) J. Ultrastructure Res. 44, 181-198.
19. Polan, M. L., Freidman, S., Gall, J. G. and Gehring, W. (1973) J. Cell Biol. 56, 580-589.
20. Reilly, J. G. and C. A. Thomas Jr. (1980) Plasmid 3, 109-115.
21. Shah, D. M. and Langley, C. H. (1979) Nature Lond. 381, 696-699.
22. Wolstenholme, D. R. and Fauron, C. M.-R. (1976) J. Cell Biol. 71, 434-448.

23. Wolstenholme, D. R., Goddard, J. M. and Fauron, C. M.-R. (1979) Proc. 8th Ann. ICN-UCLA Symp. Molecular and Cellular Biology; Extrachromosomal DNA, pp. 409-425, ed. D. Cummings, I. B. Dawid, P. Borst, S. Weissman, and F. Fox. Academic Press, New York.