INTRAMOLECULAR HETEROGENEITY OF MITOCHONDRIAL DNA OF *DROSOPHILA MELANOGASTER*

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ABSTRACT

DNA from purified mitochondria of *Drosophila melanogaster* can be isolated as supercoiled molecules which when nicked have a contour length of 5.9 μm. Partial denaturation mapping shows regional heterogeneity of base composition with one early denaturing region, with a calculated GC content close to zero, extending over 20% of the genome. DNA isolated from unfertilized eggs shows nuclear and mitochondrial DNA in equal proportions; we found no evidence of other cytoplasmic species.

Confusion as to which of the satellite DNA peaks of *Drosophila* are cytoplasmic (4, 12) has been resolved in an analysis (17) which has shown that two (1.688 and 1.672 g/cm^3) of the three distinct light satellites are of nuclear origin and that the remaining one (1.680 g/cm^3) is of cytoplasmic origin. Polan et al. (18) and Bultmann and Laird (6) identified this 1.680 g/cm^3 peak as mitochondrial DNA. An additional DNA species of cytoplasmic origin (1.697 g/cm^3) has been claimed to be present in unfertilized eggs (18), and Travaglini et al. (20) report a major DNA species in unfertilized eggs to be a satellite of density 1.669 g/cm^3.

In this paper, we report additional characterization of the mitochondrial DNA molecule by denaturation mapping. We have failed to detect other cytoplasmic DNA species in unfertilized eggs (or early embryos).

MATERIALS AND METHODS

Preparation of Mitochondrial DNA

D. melanogaster (Canberra wild type) eggs or embryos were harvested in NaCl (0.7%), Triton X-100 (0.015%) and dechorionated with sodium hypochlorite (5% available chlorine) for 2 min. The eggs were homogenized (Dounce) in buffer (0.25 M sucrose, 0.03 M Tris, 1 mM EDTA, 2.5 mM $CaCl_2$, pH 7.5) and the debris was removed by filtering through nylon mesh. Nuclei were sedimented by several cycles of centrifugation at 1,000 *g* and the mitochondria were collected by centrifugation at 16,000 *g* for 20 min. After washing, mitochondria were incubated with pancreatic deoxyribonuclease (25 μg/ml) for 30 min at room temperature in 0.02 M Mg^{2+}. After incubation, EDTA (pH 8) was added to a final concentration of 0.01 M and the mitochondria were washed three times with 5 mM EDTA, 0.25 M sucrose, 0.15% bovine serum albumin (pH 8).

Mitochondria were lysed in 0.5 M NaCl, 0.01 M EDTA, pH 8, containing sarkosyl (1%). The lysate was then centrifuged to equilibrium in CsCl (ρ = 1.70 g/cm^3) at 35,000 rpm in a Spinco no. 40 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) for 60 h.

Total DNA Extraction

Total DNA was extracted as described previously (17).

Base Composition

A ^{32}P-labeled DNA copy was synthesized using *Escherichia coli* DNA polymerase I as described before (17). The DNA was hydrolyzed enzymatically and the bases were separated and counted.

Ethidium Bromide Gradients

The mitochondrial pellet was lysed into a CsCl-Ethidium bromide gradient by the method of Clark-Walker (8). Ethidium bromide was removed by extraction with CsCl-water-saturated isopropanol.

Thermal Denaturation

Thermal denaturation was carried out in teflon-stoppered cuvettes in a Gilford 2400 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio.) equipped with a Haake heating unit (Haake Inc., Saddle Brook, N. J.). The rate of temperature rise was 0.5°C/min. Absorbance (260 nm) and temperature were monitored continuously. A hyperchromicity of only 30% was routinely obtained and was accepted as the complete hyperchromic shift in the calculation of renaturation rate (3).

Renaturation Experiments

DNA samples were sheared in a VirTis homogenizer (VirTis Co., Inc., Gardiner, N. Y.) (10 min at 40,000 rpm) to a length of 0.8 μm as measured by electron microscopy. DNA in 0.1 × SSC (0.015 M NaCl, 0.0015 M Na citrate, pH 7.0) was denatured by boiling for 2 min, the sample was held at 80°C for 2 min, then 10 × SSC added to a final concentration of 1 × SSC. Renaturation was measured by decrease in optical density at 260 nm at 57°C, and analyses were carried out by the method of Britten and Kohne (5).

Analytical Ultracentrifugation

DNA samples were centrifuged to equilibrium in a Beckman Model E analytical ultracentrifuge (Beckman Instruments, Inc., Spinco Div.) at 44,770 rpm in CsCl with *Micrococcus luteus* (ρ = 1.731 g/cm^3) as marker. For alkaline centrifugation, 2 μg of *M. luteus* DNA (ρ = 1.789 g/cm^3) was added to the DNA sample which was made 0.01 M NaOH, and the initial CsCl density was adjusted to 1.745 g/cm^3.

Electron Microscopy

Mitochondrial DNA was dialysed against 0.05 M Na phosphate (pH 7.8). Denaturation buffer (0.05 M Na phosphate, 10% formaldehyde (pH 7.8) was brought to the required temperature for 5 min, and $^1/_5$ vol DNA solution was added (0.25 μg). The solution was incubated for 10 min, cooled quickly in ice, and left for 10 min. It was then spread on 0.25 M ammonium acetate, using the technique of Davis et al. (9). DNA molecules were picked up on parlodion-coated grids and rotary shadowed with platinum. Photographs were taken at a magnification of 12,800 in a Philips 200 electron microscope. Molecules at a final magnification of 76,000 were measured on a D-mac digitizing table (D-mac Ltd., Glasgow, Scotland) (0.1 mm resolution) associated with a CDC 3600 computer (Control Data Corp., Minneapolis, Minnesota).

Hybridization

Denatured mitochondrial DNA which was in the open circular or linear form was fixed to 47-mm nitrocellulose filters ([Sartorius Filters, Inc., San Francisco, Calif.], pore size 0.45 μm) in 2 × SSC (10), and small filters cut with a cork borer (7.7 mm diameter) contained 0.5 μg of mitochondrial DNA. Filters were then incubated for 1$^1/_2$ h at 37° in 0.02% Ficoll (Pharmacia Fine Chemicals, Piscataway, N. J.) and 0.02% polyvinyl pyrrolidone. 100 μl (1.8 μg) of ^{32}P-mitochondrial DNA (5 × 10^4 cpm/μg in 6 × SSC) and appropriate amounts of heterologous DNA were boiled for 2 min to ensure denaturation; the filters were added and incubated for 20 h at T_m − 25°C. Filters were bulk-washed five times in 2 × SSC for 30 min, dipped in water, dried, and counted.

DNA Estimation

Trays were placed in population cages for 1 h to induce flies to lay any previously fertilized eggs. Fresh trays were put in for a further hour, removed, and the eggs were allowed to develop for the appropriate length of time. The eggs were collected, dechorionated, and washed several times in 16% sucrose. They were homogenized in cold 0.25 M perchloric acid, and left for 30 min at 4°C. The homogenate was centrifuged for 10 min at 10,000 rpm and the supernate discarded. The pellet was suspended in 2 ml of 0.5 M perchloric acid and extracted three times at 70° C for 20 min. Between extractions, the supernate was removed and pooled. The DNA in the combined supernate was estimated by the method of Burton (7).

Unfertilized Eggs

Unfertilized eggs were collected from the progeny of a cross between *y/y* virgins and attached-XY males (*$Y^SX \cdot Y^L$, In (1) EN, $KSy \cdot KLy^+$*). The F_1 flies were examined before being placed in population cages, and any vials showing evidence of nonvirginity or nondisjunction were discarded. Samples of the unfertilized eggs, checked over a 48-h incubation period, showed no evidence of embryo development.

RESULTS AND DISCUSSION

We found in contrast to Travaglini and Schultz (20) or to Polan et al. (18) that it was possible to isolate supercoiled mitochondrial DNA molecules in either ethidium bromide-CsCl or CsCl gradients. The mitochondrial DNA shows a single homogeneous peak in CsCl at a buoyant density of 1.680 g/cm^3, and in alkaline CsCl there are two peaks of approximately equal amounts at densities of 1.740 and 1.735 g/cm^3 in agreement with results of Polan et al. (18). In the electron microscope, CsCl-isolated DNA showed supercoiled molecules, open circle and linear molecules. The open circular molecules were measured (Fig. 2*a*) using ϕX174 replicative form (1.68 μm) (1) included in the same grid as a length standard, and were found to be 5.99 ± 0.04 μm. This is consid-

erably longer than other estimates of 5.3 μm (18) or 5.5 μm (6), but in neither of these determinations was an internal length standard used. Wolstenholme (22) obtained a length of 6.1 μm for circular molecules in lysates of *D. melanogaster* eggs.

Analysis of a [^{32}P]DNA copy of mitochondrial DNA gives a base composition of A:39.6, G:11.0, T:38.6, C:10.6. This GC content (21.7%) agrees with the composition predicted from density (20.5%) (15).

We have confirmed the complex melting profiles reported (18, 6); about 15% of the DNA melts at 65°C, 15% at 71°C, and the remainder at 78°C in 1 × SSC. A similar profile at lower temperatures is obtained in 0.1 × SSC or 4.7 M sodium perchlorate. Since this DNA sample consisted of a mixture of only linear and open circular molecules, the melting transitions are not explicable in terms of different T_m's of closed and open circular molecules (23).

Renaturation rates showed no evidence of a highly repeated DNA component but conformed closely to second order kinetics. A $C_0t^1/_2$ of 3 × 10^{-2} mol s per liter was obtained in 1 × SSC (equivalent to 1.7 × 10^{-2} mol s per liter in 0.12 M PO_4) in agreement with the data of Bultmann and Laird (6).

Partial Denaturation

The three discrete transitions in the thermal denaturation profile indicate that there are regional variations in base composition. If the mitochondrial DNA is sheared to 0.8 μm, buoyant density analysis resolves two peaks (1.679 and 1.685 g/cm^3), the less dense peak presumably containing AT-rich early melting DNA (Fig. 1). We have further confirmed that regional heterogeneity exists in the molecule by partial denaturation mapping.

Electron microscope examination showed some denaturation at 40°C, and the amount and number of denatured sites increased with increasing temperature (Table I). At 52°C (Fig. 2*d*), molecules were completely denatured. At 40°C, most molecules showed a single denaturation site, involving about 8% of the genome. At 42°C (Fig. 2*b*), this region extended to some 20% of the molecule, and a number of other small regions melted. At higher temperatures (Fig. 2*c*), there was an increasing number of melted regions (Table I) but the large denaturation site was still obvious and accounted for 25% of the mitochondrial genome. At 52°C (Fig. 2*d*), when most molecules were completely melted, single-stranded circular molecules were observed.

Denaturation maps were constructed for the different temperatures (Fig. 3). As the temperature at which molecules were incubated was increased, the total length of the molecules decreased. The fully melted, single-stranded circles were only 0.52 of the length of the double-stranded molecules. This differential results from the collapse of the single-stranded regions in the formaldehyde buffer, and the denaturation map was corrected by representing the length of single-

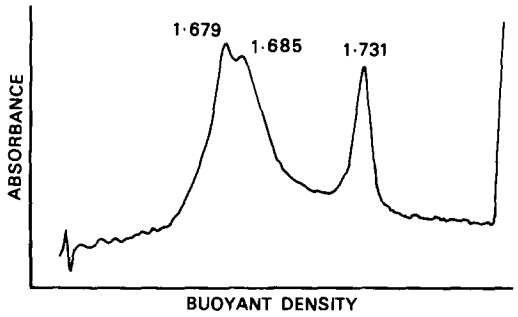

FIGURE 1 Analytical CsCl gradient of sheared mitochondrial DNA.

TABLE I

The Relationship between the Number of Melted Regions in D. melanogaster Mitochondrial DNA and Temperature

		Melted regions per molecule						
Temperature	Molecules scored	0	1	2	3	4	5	6
°C		*% of molecules*						
40	75	57	25	15	3			
42	16	3	41	38	16	2		
44	64		2	44	42	12		
46	104			4	23	43	24	6

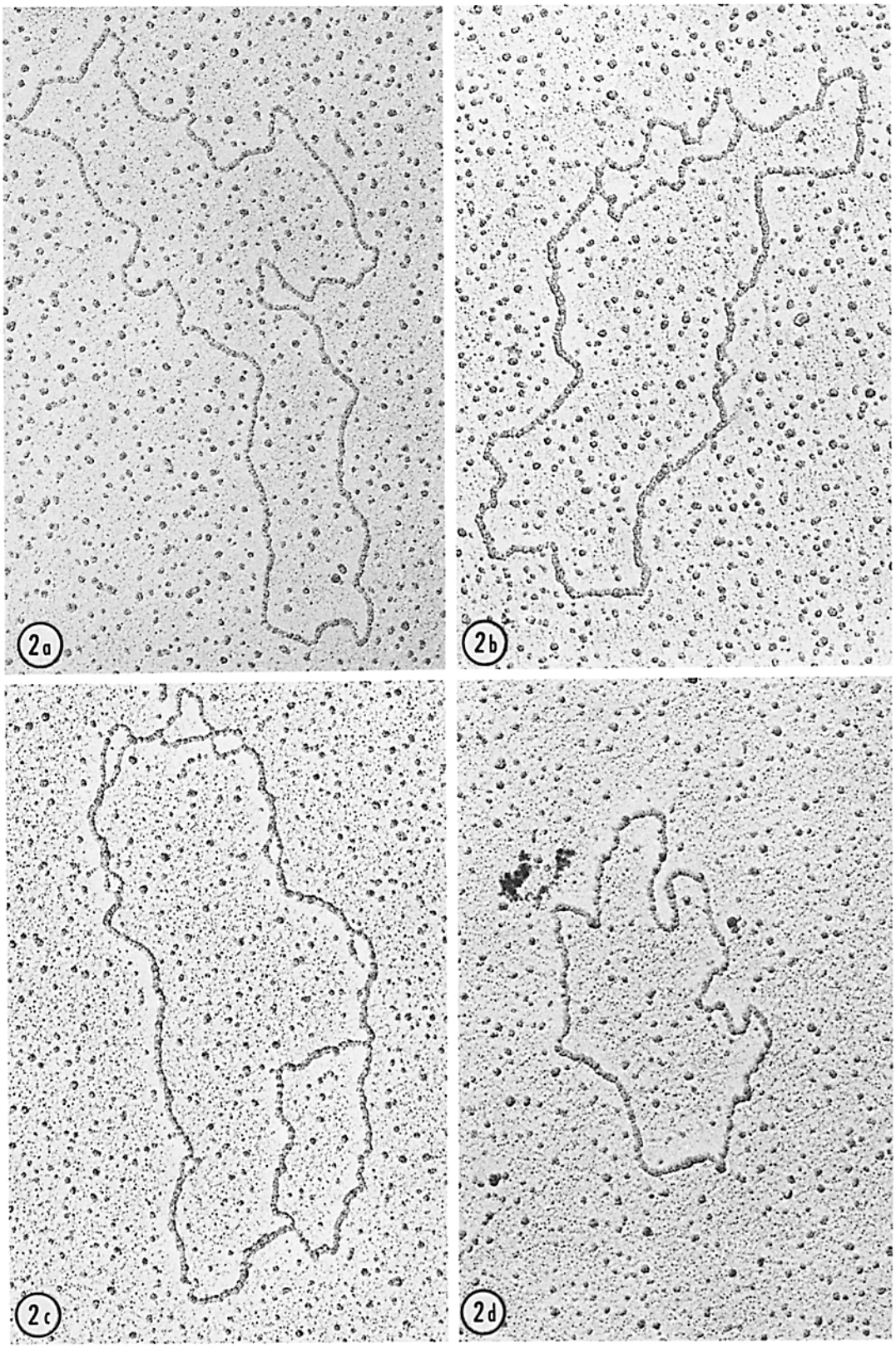

FIGURE 2 Mitochondrial DNA molecules from partial denaturation experiment. (*a*) 25°C, open circular molecule; (*b*) 42°C, showing a single denatured region; (*c*) 46°C, showing several denaturation sites; (*d*) 52°C, single-stranded molecule. (*a*), (*c*), and (*d*), × 56,000; (*b*) × 52,000.

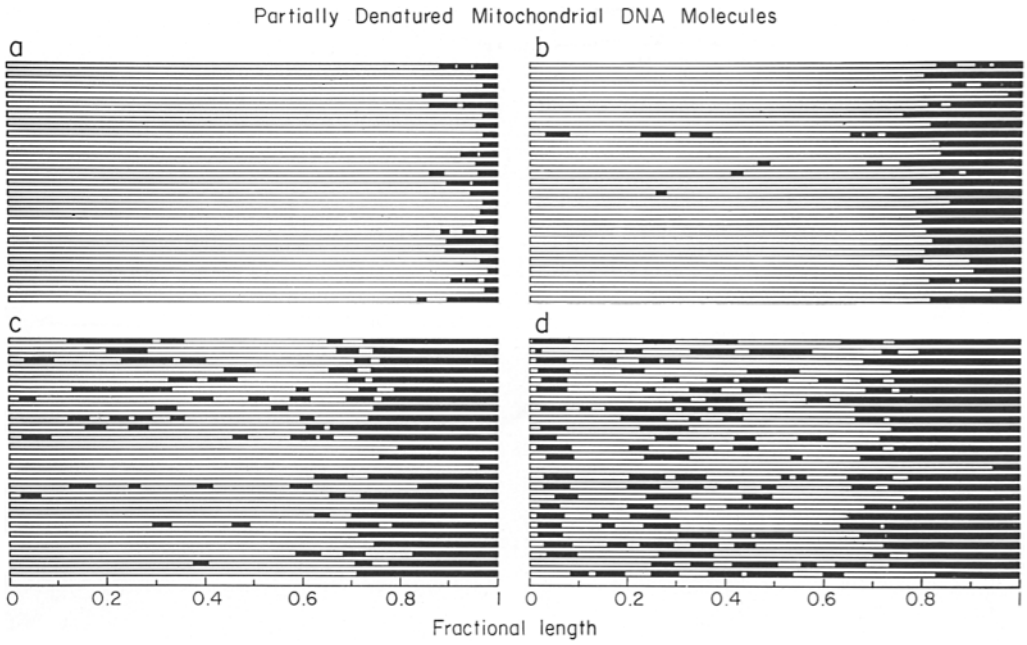

FIGURE 3 Denaturation maps of *D. melanogaster* mitochondrial DNA. The lengths of the denatured regions were corrected for single-stranded collapse, the molecule lengths normalized, and each circular molecule was represented as a linear rod, the right terminus being one end of the large denatured region, (*a*) 40°C; (*b*) 42°C; (*c*) 44°C; (*d*) 46°C.

stranded regions in double-strand length equivalents. Wolstenholme et al. (22) found that the ratio of the length of single-stranded to the length of double-stranded molecules in similar experiments with rat liver mitochondrial DNA was 0.55.

The partial denaturation pattern that we have observed differs markedly from that found for rat liver mitochondrial DNA (22) where only a few small regions were differentiated from the rest of the genome. *D. melanogaster* mitochondrial DNA is unusual in its base composition (21.7% GC) and is one of the most AT-rich mitochondrial DNAs known. The early melting regions could, in large part, account for the low buoyant density. The melts at 65° and 71°C in SSC correspond to GC contents of zero and 5%, respectively (14). These regions, accounting for approximately 30% of the genome, would have such a limited coding capacity that it is probable that they do not code for proteins and may not even be transcribed. Laird et al. (13) have claimed, from electron microscopy, that 60–80% of the molecule participates in coupled transcription-translation. AT-rich regions have been found in yeast mitochondrial DNA (2), and it is likely that they do not have a protein-coding function.

Although our renaturation results did not show any highly repeated DNA in the mitochondrial genome, we have investigated the relationship of the molecule to the highly repeated AT-rich nuclear satellites of *D. melanogaster* (17). None of the satellites competed with the hybridization of ^{32}P mitochondrial DNA to filter bound mitochondrial DNA (Table II), although we have demonstrated the satellite DNAs to be effective competitors to homologous labeled complementary RNA.

DNA in Unfertilized Eggs

Mitochondrial DNA is a major component (50%) of DNA extracted from unfertilized eggs (Fig. 4). This is also the case in very young embryos (<1 h old), and, as embryos develop, the proportion of mitochondrial DNA decreases until in 12-h embryos it comprises only 4% of the total DNA (Fig. 5).

We have determined the DNA content of unfertilized eggs to be 0.5×10^{-9} g/egg. These eggs were free from contaminating fertilized eggs which might contain a large amount of DNA (see Materials and Methods). As the mitochondrial DNA is half the total DNA, there must be ~10,000 copies of the mitochondrial genome in each egg. Light

TABLE II

Competition Hybridization of Mitochondrial DNA and Nuclear Satellite DNAs

	DNA in solution	Competing DNA	DNA filter	Blank filter
			cpm bound	
(*a*)				
	1.8 μg ^{32}P mit-DNA	–	17,037	187
	"	1 μg cold mit-DNA	11,375	695
	"	10 μg 1.686 satellite DNA	16,260	382
	"	10 μg 1.672 satellite DNA	15,301	307
	0.9 μg ^{32}P mit-DNA	–	8,711	147
	"	1 μg cold mit-DNA	4,427	132
	"	6 μg cold mit-DNA	964	72
	"	10 μg 1.672 satellite DNA	9,242	164
	"	10 μg 1.686 satellite DNA	9,447	189
(*b*)				
	1.8 μg ^{32}P mit-DNA	–	13,172	819
	"	10 μg 1.705 satellite DNA	11,528	522
	0.9 μg ^{32}P mit-DNA	–	5,663	172
	"	11 μg 1.705 satellite DNA	6,336	114

0.5 μg of mitochondrial DNA (mit-DNA) was loaded onto each filter, with either 0.9 or 1.8 μg of ^{32}P mitochondrial DNA in solution (150 μl) and appropriate amounts of cold DNA added as competitor. Hybridization was carried out at either 56°C (*a*) or 72°C (*b*) as described in Materials and Methods.

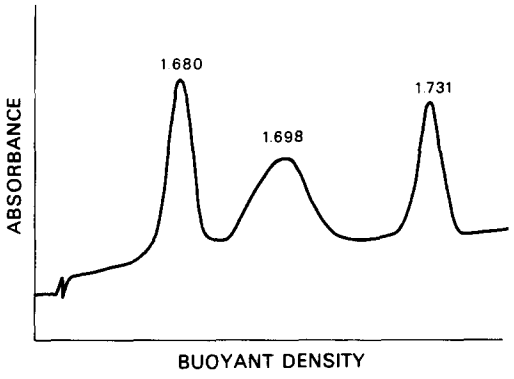

FIGURE 4 Analytical CsCl gradient of total DNA extracted from a lysate of unfertilized eggs. Figures refer to densities in g/cm^3; 1.731 is *M. luteus* DNA.

and electron microscope observations have shown the ooplasm to be rich in mitochondria (11).

The nonmitochondrial DNA banded as a single broad peak (Fig. 4) in CsCl gradients at slightly higher than nuclear main band density, but was far in excess of the amount expected. The broadness of the band can be attributed to low molecular weight of the DNA, as can the slight shift in density. The unreplicated haploid DNA value for *D. melanogaster* is 0.18×10^{-12} g (19), and we would expect only four times this amount in the unfertilized egg instead of the observed 0.25×10^{-9} g, i.e., 50% of 0.5×10^{-9} g/egg. The 300-fold excess of nuclear DNA over the expected value of a single replicated diploid genome may originate from the surrounding nurse cells which contain up to 512 times the haploid amount of DNA. Painter (16) showed that, in the growth of ova before fertilization, the cytoplasm of the egg is the recipient of large amounts of chromatin or its derivatives. The chromatin is introduced by engulfment or indirect absorption of nurse cells and their nuclei, or from the breakdown of the germinal vesicle. Partitioning of the cell contents into nuclear and cytoplasmic fractions revealed only DNA species present in developing embryos and no new species. We found no evidence of a sharp 1.697 g/cm^3 cytoplasmic species (18), nor did we find excessive amounts of the major nuclear satellites (21) of density 1.672, 1.686, and 1.705 g/cm^3.

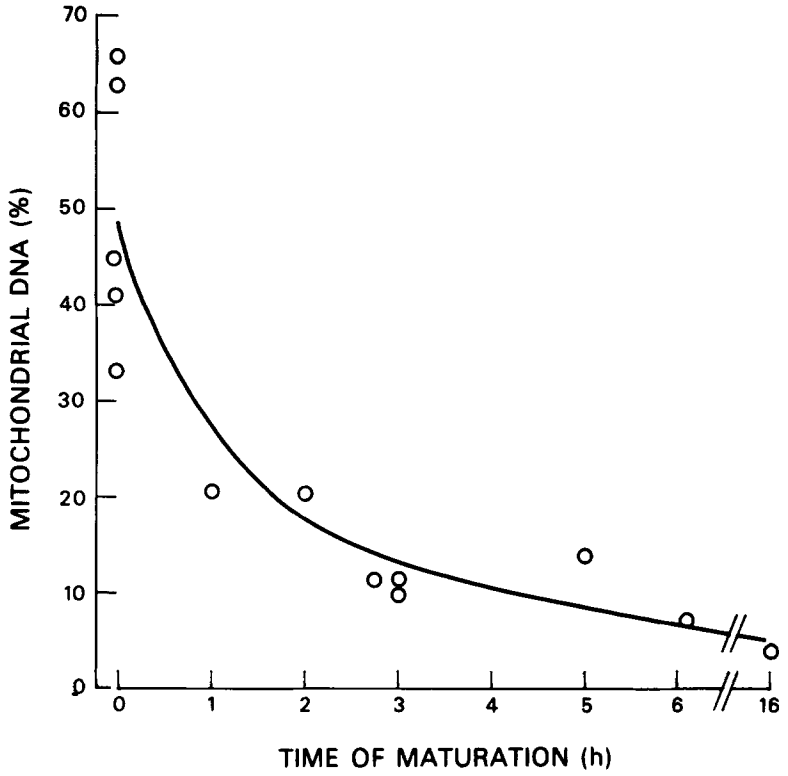

FIGURE 5 Dependence of proportion of mitochondrial DNA on age of embryos. Total DNA was extracted from embryos after various times of development and analysed by analytical centrifugation in CsCl gradients.

CONCLUSION

The mitochondrial genome in *D. melanogaster* differs from other animal mitochondrial genomes in having an extensive region which appears to be devoid of GC base pairs and which presumably has no coding function. We find no support for earlier reports of cytoplasmic DNA species, in either unfertilized eggs or early embryos, other than the 1.680 g/cm^3 mitochondrial DNA.

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