STUDIES ON MITOCHONDRIA

II. Mitochondrial DNA of Thoracic Muscles of *Schistocerca gregaria*

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ABSTRACT

The buoyant densities of the nuclear and mitochondrial DNA from the thoracic muscles of *Schistocerca gregaria* were found to be 1 702 and 1.689 g/cm^3, respectively, corresponding to guanine plus cytosine (G + C) content of 42.2 and 30%. A preliminary treatment of the mitochondrial pellet with DNase (25°C, 20 min) is necessary to eliminate the contaminating nuclear DNA. The mitochondrial DNA renatures readily after heat denaturation and incubation at 65°C. The DNA released from the mitochondrial pellet by osmotic shock consists of circular open and closed molecules with a contour length around 5 μ. The instability of insect mitochondria in in vitro preparations is discussed.

INTRODUCTION

The mitochondrial genetic system has been extensively studied in several animal tissues (1–6). However, very few studies have been undertaken on insect tissues. Insect thoracic muscles, which include flight muscles, are rich in mitochondria and, therefore, constitute an interesting system for the study of mitochondrial growth. These mitochondria undergo a very rapid growth which in certain cases is under hormonal control (7–9). Mitochondrial protein synthesis has been studied in thoracic muscles from *Locusta migratoria* (10, 11) and from *Manduca sexta* (12). Kleinow and his collaborators (13–15) have characterized the mitochondrial ribosomes and RNA from *Locusta* thoracic muscles. *N*-formyl-methionine peptides have also been isolated from bee thoracic muscle mitochondria (16).

Insect mitochondrial DNA has been detected by autoradiography in the ovaries of *Drosophila* (17). Van Bruggen et al. (18) have tried to isolate the mitochondrial DNA from the thoracic muscles of *Musca domestica* but without much success. Nevertheless, they could show that the DNA released from mitochondria by osmotic shock consisted of circular molecules with a mean contour length of 5.2 μ. Fansler et al. (19), working on the poly(deoxyadenylate-deoxythymidylate) (dAT) of *Drosophila*, have recently reported the presence of a DNA band at 1.685 g/cm^3 which, they think, is of mitochondrial origin.

As a continuation of our previous work on the variations of mitochondrial DNA during the development of thoracic muscles in *Schistocerca gregaria* (20), we now report the isolation and partial characterization of this mitochondrial DNA.

MATERIALS AND METHODS

The rearing of *S. gregaria* and the methods for the isolation of mitochondria have been previously described (20, 21). The mitochondrial pellet was gently resuspended in 0.25 M sucrose, 0.002 M Tris-HCl,

0.005 M $MgCl_2$, and 100 μg/ml of DNase I (Worthington Biochemical Corp., Freehold, N. J.) at a final pH of 7.4 so as to obtain a protein concentration of 4–6 mg/ml. This suspension was incubated either at 2°C for 45 min or at 25°C for 20 min. The incubation was stopped by the addition of cold 0.25 M sucrose, 0.002 M Tris-HCl, and EDTA 0.01 M, pH 7.4. The mitochondria were pelleted and the pellet was washed twice with the same medium. Total thoracic muscle or the nuclear pellets (500 *g* × 10 min) were used for the isolation of total DNA.

The DNA was isolated by the method of Marmur (22) except that the steps requiring the ethanol precipitation were omitted. In the case of the mitochondrial DNA, the sodium lauryl sulfate treatment of the mitochondrial pellet was complemented with a pronase treatment (100 μg/ml, 1 hr, 25°C). The total DNA was further purified on a methylated albumin kieselguhr (MAK) column (23).

The Tm values were measured in standard saline citrate (SSC), ph 7.0 in a Gilford 2000 spectrophotometer (Gilford Instrument Laboratories, Oberlin, Ohio) and the temperatures were monitored with a YSI thermistor probe (Yellow Springs Instrument, Yellow Springs, Ohio) inserted in one of the cells. The absorption data were plotted on probability paper as described by Knittel et al. (24) or treated with a computer.[1] The buoyant density in CsCl was determined according to Mandel et al. (25) with a Beckman E ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at 44,770 rpm at 25°C for 20–24 hr. *Rhodospirillum rubrum* DNA, whose density in relation to *Escherichia coli* DNA was earlier reported by us (26), was used as reference. The UV spectra of denatured DNA was analyzed by the method of Hirschman and Felsenfeld (27). For denaturation studies, the DNA in SSC was heated for 10 min at 100°C and quickly immersed in an ice bath. Renaturation was then carried out in 2X SSC at 15°C.

For liberating the mitochondrial DNA molecules, the mitochondrial pellet without DNase treatment was lysed in 8 M or 4 M ammonium acetate by osmotic shock as described by Van Bruggen et al. (18). The hypophase consisted of deionized water. The fragments were picked up on carbon-covered grids and shadowed with platinum at an angle of 8° with a Balzer rotary evaporator (Balzers High Vacuum Corp., Santa Ana, Calif). The grids were examined with a Philips 300 or an Hitachi electron microscope.

RESULTS

Nuclear DNA

The purification of the nuclear DNA on a MAK column was necessary before the Tm determination since much of UV-interfering material remained in the DNA extract even after extensive dialysis Fig. 1 presents a Tm curve plotted on probability paper (24). It has a Tm of 86 8°C corresponding to a G + C content of 43%. This Tm varied from 86.1° to 87.0°C with a mean of 86.6°C in at least five different extracts of nuclear DNA. This corresponds to a G + C content of 41–43% with a mean of 42.2%, assuming that no unusual bases are present. The 260/280/232 ratio of these preparations varied between 1.9–2.2 (260/280) and 1.85–2.0 (260/232). The absorption hyperchromicity shift was from 36 to 41%.

In CsCl, this DNA bands at a density of 1.7027 g/cm³ corresponding to a G + C content of 43.5% (Fig. 2 *a*). After heat denaturation, the band shifts to a density of 1.718 g/cm³ (Fig. 2 *b*). After a 1½ hr renaturation in 2X SSC at 65°C, the buoyant density at 1.716 is still 0.014 g/cm³ higher than that of native DNA (Fig. 2 *c*). A 6 hr renaturation time, does not change the buoyant density which stays at 1.716 g/cm³. This band is resistant to ribonuclease, pronase, or amylase treatments but disappears with DNase treatment, indicating thereby that it is indeed DNA (Fig. 2 *d*). The UV spectra of the denatured DNA analyzed by the method of Hirschman and Felsenfeld gave

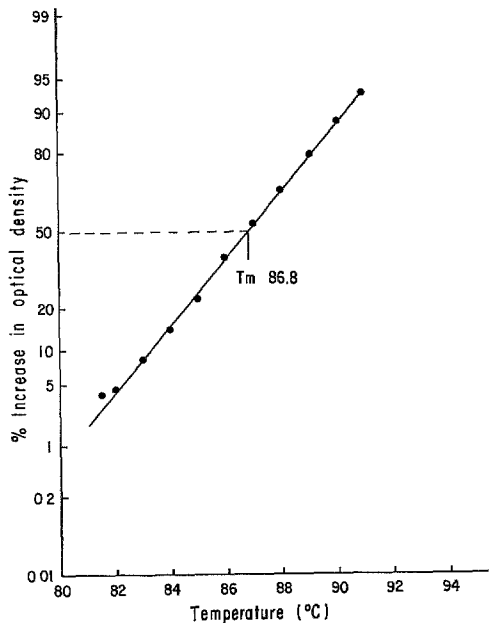


FIGURE 1 Tm curve of the locust thoracic muscle DNA plotted as described by Knittel et al. (24).

[1] Tanguay, R., and P. Morissette. Manuscript in preparation.

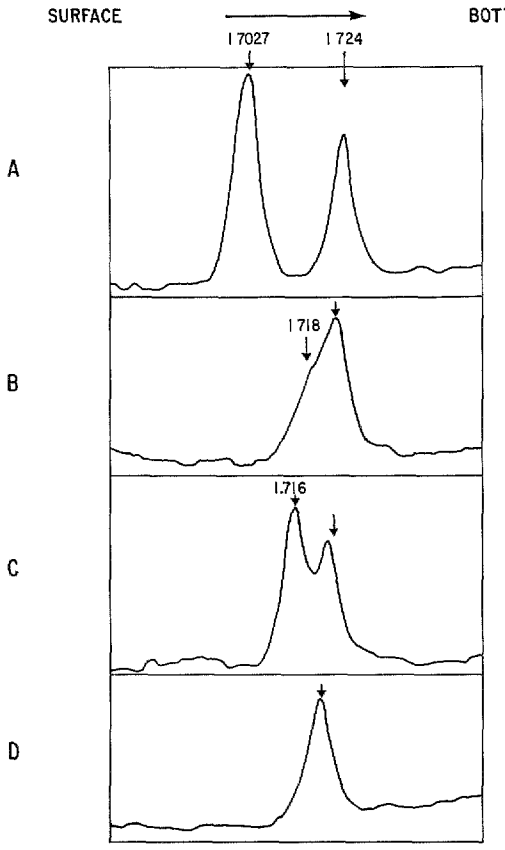


FIGURE 2 Densitometer tracings of UV absorption photographs of the locust thoracic muscle nuclear DNA in the analytical ultracentrifuge. (*a*) native DNA; (*b*) DNA denatured for 10 min at 100°C DNA; (*c*) DNA denatured for 10 min at 100°C and then renatured for 1½ hr in 2× SSC solution at 65°C; (*d*) native DNA incubated with DNase.

an AT value of 56%, i.e., a G + C content of 44%.

Mitochondrial DNA

Unfortunately, due to limited availability of experimental material, we could not isolate enough DNA from mitochondria to carry out Tm and UV analysis The passage of this DNA extract through a MAK column gave a distinct but very small fraction eluting at a molarity of 0.8 M NaCl. The base composition of this DNA was, therefore, determined only in CsCl gradients. The recovery of mitochondrial DNA was, however, very low after DNase treatment of the mitochondria. Since the results obtained with this DNA were reproducible, further purification through a MAK column was not carried out.

Fig. 3 *a* presents the density profile of the DNA extracted from a mitochondrial pellet not treated with DNase. One large peak at 1.702 g/cm³ and a small but consistent peak at 1.690 g/cm³ can be seen. The proportion of this peak varied from preparation to preparation When the mitochondrial pellet is treated for 45 min at 2°C with DNase, the DNA extract still shows the presence of two bands, one at 1.702 and the other at 1.689 g/cm³ (Fig. 3 *b*). There is, however, an enrichment

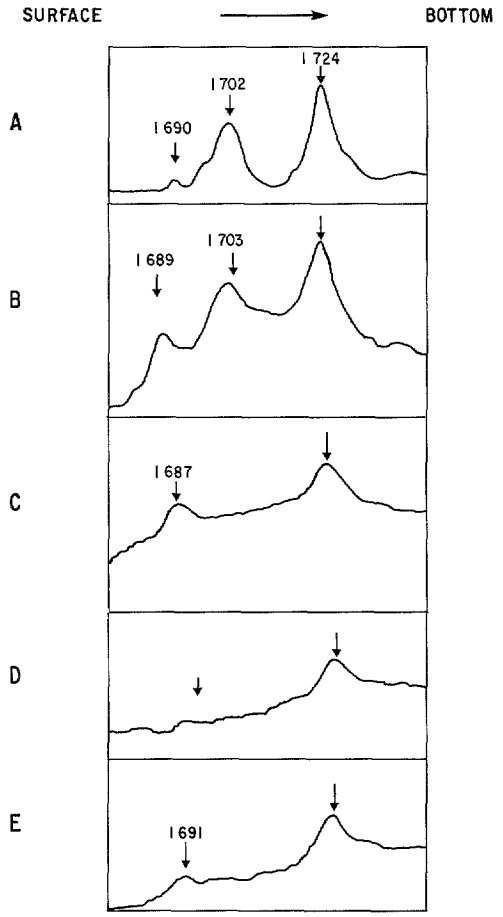


FIGURE 3 Densitometer tracings of UV absorption photographs in the analytical ultracentrifuge of the locust thoracic muscle mitochondrial DNA obtained from the mitochondria. (*a*) not treated with DNase; (*b*) treated with DNase at 2°C for 45 min; (*c*) treated with DNase at 25°C for 20 min; (*d*) the mitochondrial DNA extract was incubated with DNase; (*e*) mitochondrial DNA was denatured and then renatured in 2× SSC solution at 65°C for 4 hr.

TABLE I

Buoyant Densities and Base Composition of Nuclear and Mitochondrial DNA from S. gregaria Thoracic Muscles

	Tm →	G + C	Density →		G + C	G + C*
	°C	%	g/cm³		%	%
Nuclear DNA	86.6	42.2	1.7027	(Native)	43.5	44.0
			1.718	(Denatured)		
			1.716	(Renatured)		
Mitochondrial DNA	—	—	1.689	(Native)	30.0	—
			—	(Denatured)		
			1.691	(Renatured)		

* As determined by the method of Hirschman and Felsenfeld (27).

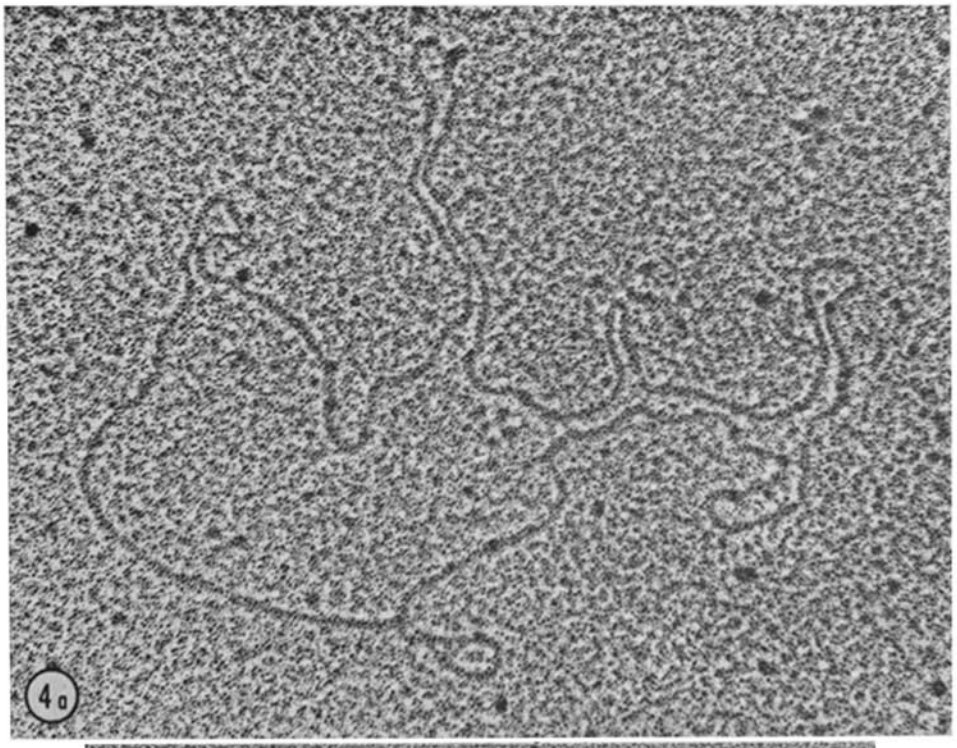
of the 1.689 g/cm³ band compared to the corresponding DNA band of the preparation obtained from mitochondrial pellet not treated with DNase. After a 20 min DNase treatment of the mitochondrial pellet at 25°C, there is only one band left at a density of 1.689 g/cm³ (Fig. 3 *c*). The yield of DNA extracted from this pellet is, however, much lower than that from a nontreated pellet. All the experiments in which we tried to isolate mitochondrial DNA after an incubation of the mitochondrial pellet at 37°C failed, since we could not detect a band in CsCl gradient. In order to make sure that this band was DNA, the extract was incubated with DNase. In this case, the 1.689 g/cm³ band disappears, indicating its DNA nature (Fig. 3 *d*). If this last extract is denatured and renatured for 4 hr in 2X SSC at 65°C, a small band reappears consistently at a density of 1.690–1.691 g/cm³ (Fig. 3 *e*). In spite of high background on the tracings resulting from technical difficulties, this small band was reproducible in at least three different preparations. Table I shows the buoyant densities and the G + C content of mitochondrial DNA and nuclear DNA from the thoracic muscles of *S. gregaria*

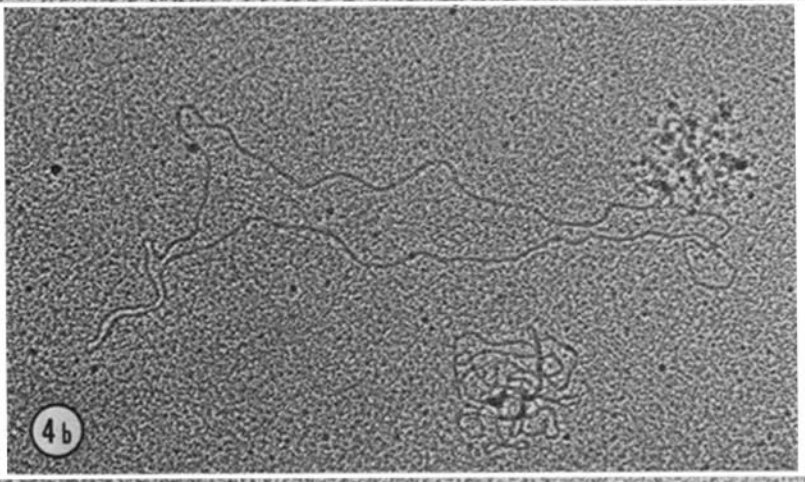
Figs 4 *a–c* show three typical circular DNA molecules. Open circles were mostly found, but occasionally closed circles as indicated by the presence of supercoiled molecules were also seen. The molecule in Fig. 4 *a* has a contour length of 4 74 μ. Much longer linear DNA molecules, presumably of nuclear origin, were also seen in this lysed extract.

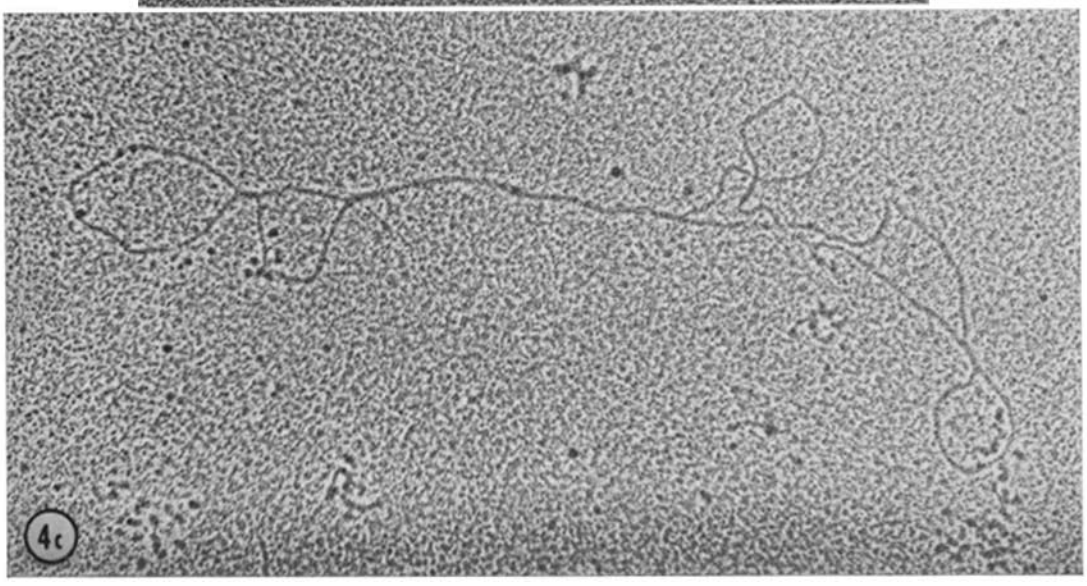
DISCUSSION

In a previous paper (20) we reported the kinetics of hydrolysis into acid-soluble fragments of exogenous DNA added to a mitochondrial preparation, and we concluded that a treatment of the mitochondria with DNase at 25°C was necessary to degrade the contaminating nuclear DNA. We further demonstrated that a low temperature treatment was not sufficient to completely degrade the contaminating DNA, whereas at high temperature (37°C) the kinetic data suggested that the mitochondria were lysed or became permeable to the added DNase. The present report fully confirms these findings. With DNase treatment at 2°C, there is a slight decrease in the amount of contaminating nuclear DNA (1.702 g/cm³) (Fig. 3 *b*) but a lot of it is still left. On the other hand, after a 20 min DNase treatment at 25°C, the nuclear DNA band disappears. The lower yield of DNA after this treatment is probably due to the instability of these mitochondria in vitro. Van den Bergh (28, 29) had previously stressed the high instability of insect mitochondria in his studies on the phosphorylation and respiratory control properties of these mitochondria. The fact that we could not get any band in CsCl after a treatment at 37°C also provides a further argument in favor of mitochondrial instability Van Bruggen et al. (18) were also unable to isolate mitochondrial DNA from the thoraces of *Musca domestica*. The instability of certain types of mitochondria during the in vitro incubation has also been shown in other organisms (30–33).

FIGURE 4 DNA released from the mitochondria of *S. gregaria* thoracic muscles by osmotic shock *a*, × 100,700, *b*, × 55,350; *c*, × 68,880.







The fact that there is an enrichment in the 1.689 g/cm³ band after a DNase treatment argues against the possibility that this DNA could be of nuclear origin (satellite). The G + C content of this DNA varies between 30 and 31% compared with 42.2% for the nuclear DNA. After heat denaturation and renaturation, the density of the mitochondrial DNA returns to the original native density (1.690 g/cm³), indicating that it is renaturable like most mitochondrial DNA from multicellular organisms (34, 35). The G + C content of the nuclear DNA found by the Tm (42.4%) and the buoyant density (43.5%) determinations are in good general agreement with one another, and also fall within the limits given by Mandel and Marmur (36). The small difference between two values could, however, be due to the presence of a small amount of methylated bases, since Wyatt has reported, in a very closely related species, *Locusta migratoria*, the presence of 0.2% methylcytosine (37).

The DNA liberated from thoracic mitochondria lysed by osmotic shock contains circular molecules of around 5 μ. Van Bruggen et al., using the same technique, found circular molecules of 4.6 μ–5.6 μ in *Musca* mitochondria. Finally, the buoyant density value for *S. gregaria* mitochondrial DNA (1.689 g/cm³) is close to that found by Fansler et al. (19) in *Drosophila*. In both cases, the mitochondrial DNA has a lower density than the corresponding nuclear DNA. More results, however, are necessary before it is possible to make any generalization concerning insect mitochondrial DNA which might be useful in evolutionary relationships as discussed by Rabinowitz and Swift (6). The low stability of the insect mitochondria, on the other hand, imposes some limitations for future studies.

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