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Electron microscopic observation of mitochondrial DNA isolated from a human kidney: circular dimers in histologically normal organ mitochondria*

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Abstract

Circular DNA isolated from human kidney mitochondria was studied by electron microscopy. I. Mean contour length of monomers of the mitochondrial DNA was 4.96 $\pm$ SE 0.28 /μ 2. The complex molecules (oligomers) of mitochondrial DNA were observed in frequency of 6.2 per cent. Among them circular dimers accounted for two per cent of all circular DNA molecules. 3. Circular DNA fibers with an intermediate perimeter between the monomer and dimer, and with a contour length shorter than 3 μ were occasionally observed. 4. Some discuions were made on the emergence of the circular dimer.

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ELECTRON MICROSCOPIC OBSERVATION OF MITOCHONDRIAL DNA ISOLATED FROM A HUMAN KIDNEY: CIRCULAR DIMERS IN HISTOLOGICALLY NORMAL ORGAN MITOCHONDRIA

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The DNA in mitochondria of many higher organisms is in the form of double-stranded circular molecules with a contour length of 4.5 to 5.5 μ corresponding to a molecular weight of 9 to 10 million daltons (1-5). Besides the simple circular molecules, there were observed complex circular molecules of twice or several times the size of 5 μ (dimers, trimers, etc.) (6-19). The naturally occurring dimers are tentatively classified in two forms, unicircular double length molecules (circular dimers) and catenanes (catenated dimers) made up of interlocked 5-μ monomers (6-8). Among them, the circular dimers have not been observed in normal tissues (9, 10, 20). In human, especially, the circular dimers have been observed only in leukemic leukocytes (7, 20, 21) and solid tumors (21).

In this report, electron microscopic data on the mitochondrial DNA isolated from a histologically normal human kidney are presented. The circular dimers, which have been reported only in leukemic leukocytes (7, 20, 21) and solid tumors (21), are observed in about 2 per cent of the non-tumorous kidney mitochondrial DNA.

MATERIALS AND METHODS

Preparation of mitochondria: Mitochondria were isolated 2 hr post-mortem from the kidney of a 54-year old patient, died of hepatoma associated with liver cirrhosis, which was provided by a pathologist of our school. Mitochondria were prepared essentially according to the method of HOGEBOOM (22) as reported in a previous paper (23). Namely, the renal cortex, obtained after removing the capsule, pelvis, calices and medulla from the kidney, was minced and washed with 0.25M sucrose solution (containing 10 mM Tris-HCl and 0.1 mM EDTA, pH 7.6). The minced cortex was homogenized in the 0.25M sucrose solution with a teflon homogenizer, and the pH of the homogenate was adjusted to 7.4 by adding 5 N KOH. The homogenate was centrifuged at 60×g for 10 min, and the supernatant was superimposed on a equal volume of 0.34M sucrose solution

and centrifuged at 700×g for 10 min. Upper, three fifths of the supernatant was collected and centrifuged at 7,000×g for 10 min. The supernatant was decanted. The residue consisted of two layers, greenish-brown top layer and light brown, trace bottom layer. The greenish-brown top layer, constituting bulk of the pellet, was carefully separated from the bottom layer and suspended in a 0.25M sucrose solution (free of Tris-HCl and EDTA). The suspension was centrifuged at 7,000×g for 10 min, and the greenish-brown layer was carefully separated. The above process of suspension and centrifugation was repeated a few times until the residue became a homogeneous greenish-brown pellet.

The final preparation was suspended in saline-EDTA (0.15M NaCl, 0.1M EDTA, pH 8.0).

Isolation of mitochondrial DNA: Mitochondrial DNA was isolated essentially according to Marmur's method (24). Outline of the procedures is presented as follows. Mitochondria suspended in saline-EDTA was centrifuged at 10,000×g for 10 min. The residue was suspended in saline-citrate (0.15M NaCl, 0.015M sodium citrate). Twenty-five per cent sodium lauryl sulfate was added to make a final concentration of 2%. The mixture was incubated at 60° for 10 min and then cooled to room temperature. Five-M perchlorate (neutralized) was added to a final concentration of 1 M, and the whole mixture was shaken with an equal volume chloroform-isoamyl alcohol (24 : 1) for 30 min and centrifuged at 3,000 rev./min for 15 min. Upper aqueous phase containing nucleic acids, was carefully pipetted off into a tube. Two volumes of ethanol were layered on the aqueous phase, gently mixed, and left standing overnight at 0~4°. Nucleic acids collected by centrifugation at 3,000 rev./min for 20 min was dissolved in saline-citrate. The solution was shaken again with an equal volume of chloroform-isoamyl alcohol for 30 min, centrifuged, and the aqueous phase was collected. The above deproteinization process was repeated a few times. The nucleic acids precipitated with ethanol after deprotinization were dissolved in saline-citrate and treated with heat-treated RNase 1 A (50 μg/ml) at 37° for 30 min. After cooling to 0°, the mixture was deproteinized. The DNA so purified was precipitated from the deproteinized upper aqueous phase with two volumes of ethanol, and solubilized in a small volume of saline-citrate.

Electron microscopic observation: Protein monolayer technique of KLEINSCHMIDT (25) modified by FREIFELDER and KLEINSCHMIDT (26) was mostly used for spreading mitochondrial DNA. Namely, DNA dissolved at a concentration of about 2 μg per ml in a solution of 1 M ammonium acetate and 0.01% cytochrome c and added with formalin in final concentration of 0.5% ran down an inclined clean glass slide onto a hypophase of 0.3M ammonium acetate containing 0.5% formalin. The protein-DNA film was picked up on carbon-coated collodion films mounted on copper grids. Rotary shadow-casting was conducted on the grids with platinum-palladium (80 : 20, about 20 mg in weight) at a shadow angle of 6~10° at a mean distance of about 7 cm in a Hitachi evaporating unit (11, 25). The grids were then examined in HU-11 C electron microscope (Hitachi Ltd.). Micrographs were taken at 75 KV on Fuji film (FG), at an optical magnification of 10,000. Electron optical magnification was checked by simultaneous observa-

tion of standard carbon replicas of diffraction grating with 1.05 μ spacing. Contour length of DNA molecules was measured on the four- or five-time enlarged prints with a map-measuring device (24).

RESULTS

Pathological findings: The kidney used for the preparation of mitochondrial DNA did not show any noteworthy pathological change. No metastasis of liver carcinoma was detected.

Electron microscopic observation: Electron micrographs of the mitochondrial DNA-preparation showed circular DNA fibers in about 84 per cent of the sum-total length of the entire DNA fibers and linear fibers in about 16 per cent. The length of each linear fiber was mostly shorter than 5 μ. Even considering involuntary selection of circular DNA on photography, the above numerical values mean that contamination of nuclear DNA and splitted mitochondrial DNA are of little significance. Complete open circular DNA molecules were less than 22 per cent of the total circular DNA molecules. The histogram of the distribution of lengths of the circular DNA molecules are illustrated in Fig. 1. Circular dimers, trimers, and circular DNA fibers with an intermediate perimeter and with a contour

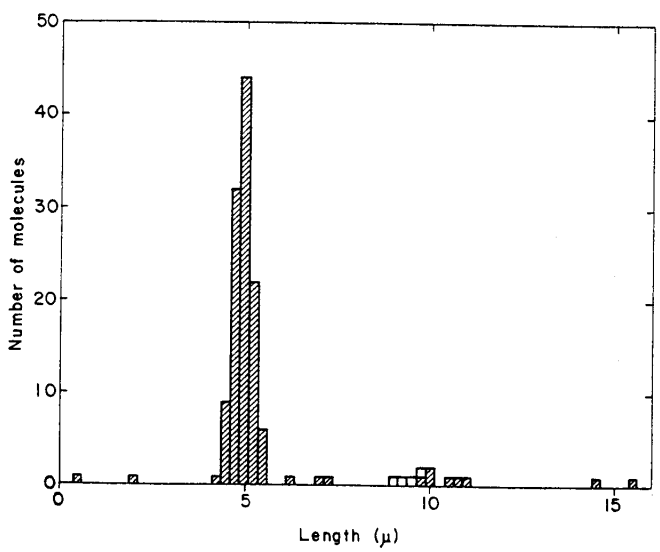

Fig. 1 Distribution of lengths of circular DNA molecules isolated from human kidney mitochondria. White columns indicate circular dimers. Hatched columns include monomers, catenated dimers, trimers and intermediate length circular DNA molecules.

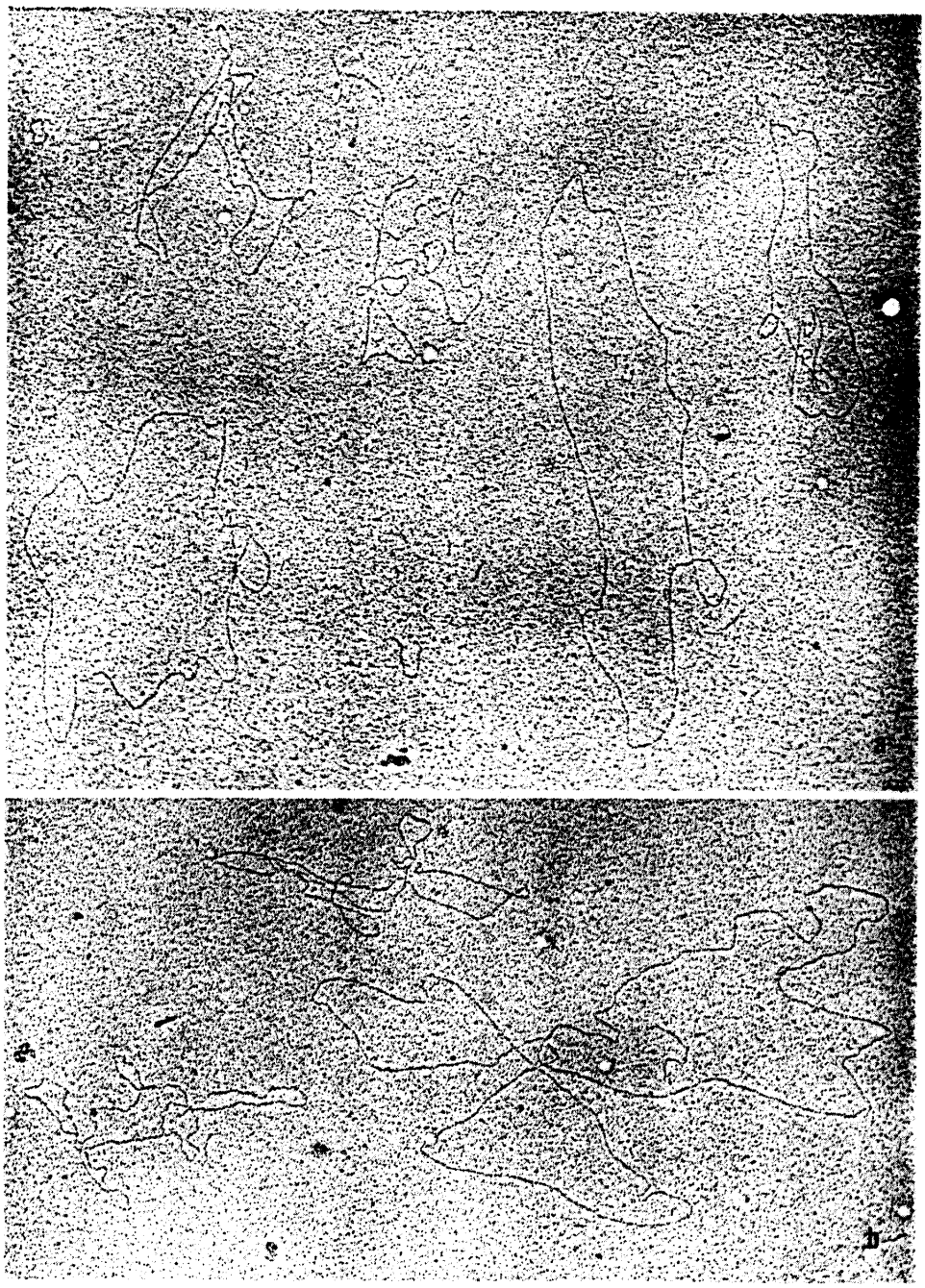

Fig. 2 Electron micrographs of circular DNA molecules isolated from human kidney mitochondria. Rotary shadowing with platinum-palladium (80 : 20). a. Monomers. b. A catenated dimer and monomers. (× 40, 000.)

length shorter than 3 μ were occasionally observed. Mean contour lengths of the monomers, dimers and trimers were 4.96 ± SE 0.28 μ, 9.85 ± SE 0.62 μ and 15.0 μ, respectively. Scoring the frequency of complex mito-

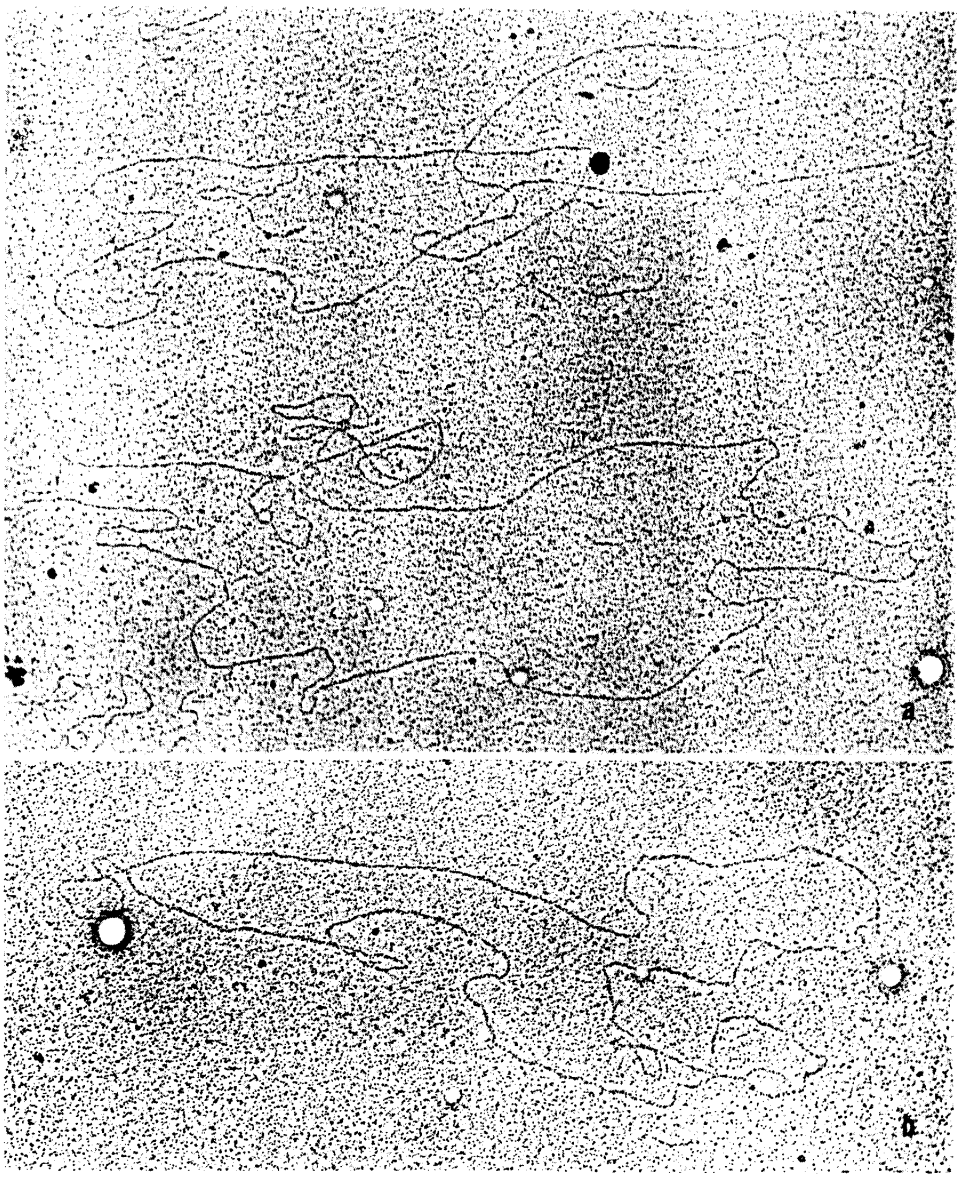

Fig. 3 Electron micrographs of circular DNA molecules isolated from human kidney mitochondria. Rotary shadowing with platinum-palladium. a. A catenated dimer and trimer, consisting of an open circular dimer interlocked with a monomer. b. A circular dimer. (× 40, 000.)

chondrial DNA was systematically performed at the microscope by direct observation on the fluorescent screen, according to the method of CLAYTON *et al.* (9). The result is illustrated in Table I. Circular dimers accounted for 2 per cent of circular DNA molecules isolated from human kidney

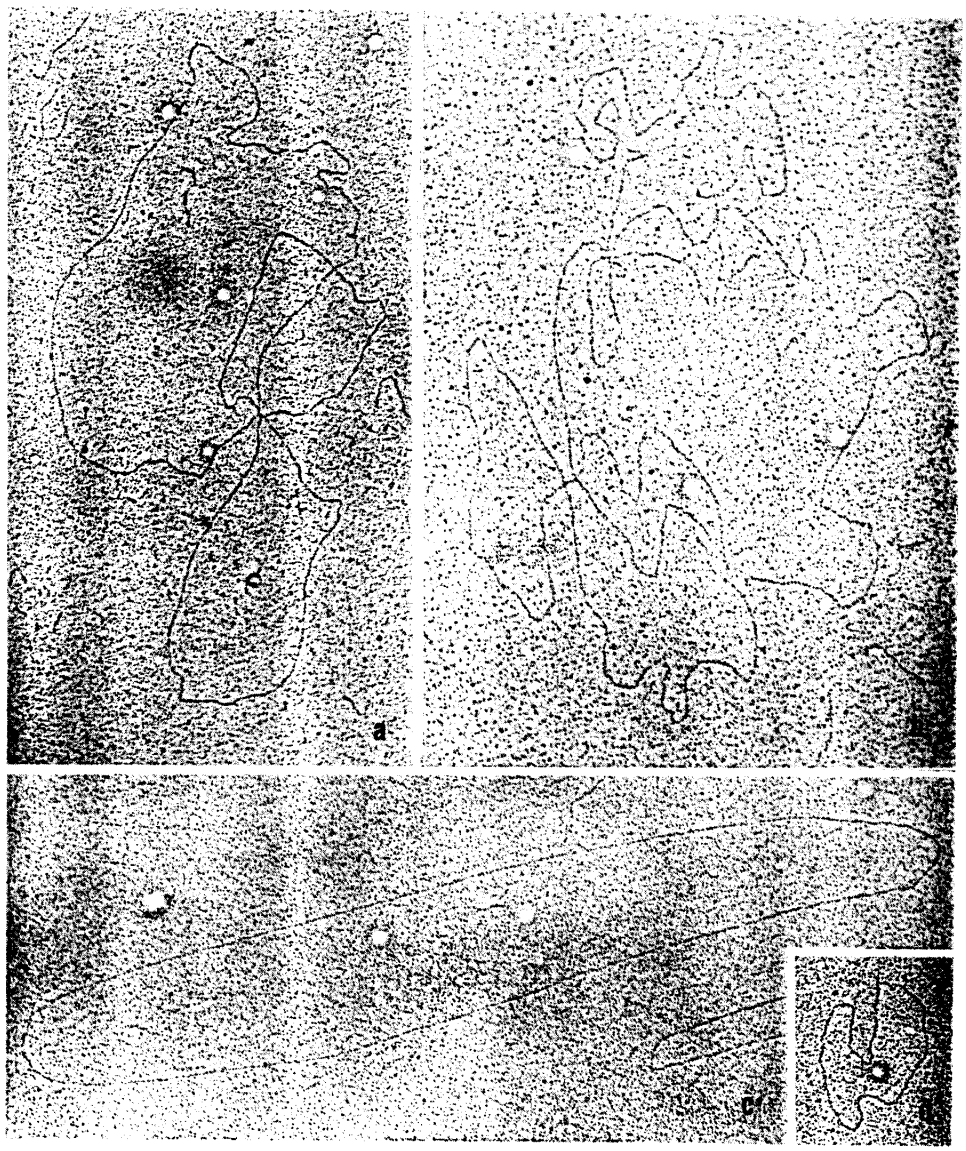

Fig. 4 Electron micrographs of circular DNA molecules isolated from human kidney mitochondria. a. A catenated dimer. b. A trimer, consisting of three interlocked monomers. c. A DNA circle (contour length, 7.2μ) having an intermediate length between monomer and dimer. d. A small size DNA circle (contour length, 2.1μ). ($\times$ 40,000.)

mitochondria. Electron micrographs of monomers, oligomers, and circular DNA fibers with an intermediate perimeter and with a contour length shorter than 3 μ were illustrated in Fig. 2~4.

TABLE 1 DISTRIBUTION OF CIRCULAR DNA FORMS OF MITOCHONDRIA ISOLATED FROM THE HUMAN KIDNEY

DNA forms	Number	Frequency (%)
Monomers	585	83.0
Oligomers	44	6.2
Catenated dimers	28	4.0
Circular dimers	14	2.0
Trimers	2	0.3
Hypertwisted, ambiguous forms	77	10.9

DISCUSSIONS

Electron microscopic data on mitochondrial DNA isolated from human kidney were presented in this paper. Mean contour length of the mitochondrial DNA was 4.96 μ in the monomer form, which is about the same as that of mitochondrial DNA previously observed in the same organ of the other mammals (4, 9) or in the other organs, tissues or cells (1-19). Complex forms of the mitochondrial DNA (6-10) were also observed in the preparation.

As reported in preceding papers, catenanes are most commonly observed in the complex forms and occur at a frequency of 2 to 9 per cent of mitochondrial DNA molecules (9, 10). The circular dimers, on the other hand, have been observed only in the mitochondrial DNA isolated from cultured mouse fibroblasts (L cells) (10, 28, 29) and neoplastic cells such as human leukemic leukocytes (7, 21, 27), rat ascites hepatoma (AH 130 strain) (14), C3H mouse ascites tumor induced by Schmidt-Ruppin strain of Rous sarcoma virus (15, 18), and human solid tumors (21), but not from normal tissues (9). CLAYTON *et al.* (20) referred the circular dimer to an "abnormal" form, because it occurred in leukemic leukocytes and did not occur in the appropriate controls. Recently, they suggested a significant relationship between the presence of the abnormal mitochondrial DNA form and neoplasia in man (21).

We revealed in the present study that the circular dimer occurred in about 2 per cent of the total number of circular DNA molecules in the mitochondria isolated from the histologically normal kidney of a patient, who died of hepatoma associated with cirrhosis, but extrahepatic metastasis

was not detected anywhere. From the data presented in this paper, abnormality of the emergence of the circular dimer in human mitochondria and relatively high emergence of the dimer in neoplasia cannot be denied, but the possibility of a direct correlation of the emergence of it with neoplasia in man may be ruled out. This supposition can also be supported by occasional emergence of the circular dimer in the mitochondria isolated from adult rat liver (14, 15) and from beef liver (30). NASS (10) reported that the circular dimer and higher oligomers were reversibly generated in mouse L cell mitochondria by keeping cells in stationary phase of growth, by treatment of cells with cycloheximide, and by amino acid starvation. In the present case, as the liver functions were markedly disturbed a few month before death, metabolic disorder of the patient whose kidney was used in this experiment was thought to be remarkable. The patient was given 2.5g rolitetracycline nitrate (pyrrocycline N) for 5 days in total before death. No other antibiotics and antitumor drugs were administered. Referring to the above Nass' data, it might be thought that the metabolic disorder of the patient or administered rolitetracycline nitrate had a bearing on the emergence of the circular dimer, though it should be backed by direct experimental data.

It has been reported that small circular DNA's are present in neoplastic cells (HeLa cells (6, 31)) and neoplastic cell mitochondria (rat ascites hepatoma (11), C3H mouse ascites tumor (14, 18) and human hepatoma (17)). In our experiment, too, there were occasionally observed unusual circular DNA such as DNA with intermediate perimeter between the monomer and dimer, and with a contour length shorter than 3 μ, though the significance of their presence is not clear.

SUMMARY

Circular DNA isolated from human kidney mitochondria was studied by electron microscopy.

1. Mean contour length of monomers of the mitochondrial DNA was $4.96 \pm$ SE $0.28\ \mu$.

2. The complex molecules (oligomers) of mitochondrial DNA were observed in frequency of 6.2 per cent. Among them circular dimers accounted for two per cent of all circular DNA molecules.

3. Circular DNA fibers with an intermediate perimeter between the monomer and dimer, and with a contour length shorter than 3 μ were occasionally observed.

4. Some discussions were made on the emergence of the circular dimer.

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