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Abstract

An electron microscopic observation was made on the DNA's extracted from purified HeLa cell nuclei, mitochondria, and the whole cell, and fractionated by ethidium bromide-cesium chloride density gradient method or sucrose density gradient method. Nuclear DNA presents mainly long linear DNA derived from fragmented chromosomal DNA. In addition to this, the existence of small circular DNA molecules measuring 0.32 -1.78 μ, was confirmed. Mitochondrial DNA was mainly circular DNA, which measured 4.87 μ in the mean value of the contour lengths in the highest frequency group, and small circular DNA molecules, measuring 0.3-1.01 μ in contour length, were also found in an extremely low frequency.

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CIRCULAR DNA'S FROM HELA CELL NUCLEI AND MITOCHONDRIA

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Circular DNA's are known to be widespread among living organisms, such as in the mitochondria (1—18), chloroplasts (19), some of the oncogenic viruses (20—23), bacteriophages (24—26), and bacterial plasmids (27, 28). Mitochondrial DNA's from a variety of organisms with a few exceptions (29—32) have been shown to be closed circular duplex DNA molecules measuring about 5μ in contour length. RADLOFF *et al.* (4) have developed a dye-buoyant-density method with ethidium bromide for the isolation of closed circular duplex DNA, and applying this method they found that HeLa cells contain, in addition to the closed circular mitochondrial DNA of mean length of 4.81 μ, a heterogenous group of smaller DNA molecules and a paucidisperse group of multiples of the mitochondrial length. However, as they prepared DNA mainly from whole HeLa cell extracts and partly from the mitochondria, the origin of the small circular DNA molecules is not clear. In the present investigation we describe circular DNA's isolated from purified nuclei, mitochondria, and the whole of HeLa cells, and demonstrate the existence of small circular DNA molecules in the HeLa cell nuclei.

MATERIALS AND METHODS

HeLa cells: HeLa cells were grown in dilution bottles (5.5×12 cm) in YLE medium containing 20 % bovine serum for 1 week before the cells were harvested.

Isolation of HeLa cell nuclei and mitochondria: After replacing the culture medium with 0.25 M sucrose solution containing 10 mM Tris-HCl buffer, pH 7.6, and 0.1 mM EDTA, the cultured cells were gently scraped from the flasks with a rubber policeman and transferred with a wide mouth pipet to a centrifuge tube, washed twice with the sucrose solution, homogenized with teflon homogenizer, immediately added with 0.1M $MgCl_2$ to a final concentration of 2 mM and fractionated by HOGEBOOM's method (33) to isolated crude nuclear fraction

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and mitochondrial fraction.

The nuclei were purified by the modification of CHAUVEAU's method (34, 35) as follows. The crude nuclear fraction obtained by HOGEBOOM's method was suspended and homogenized in 20 vol. of 2.2M sucrose solution containing 1.8 mM $CaCl_2$ and 5 mM Tris-HCl, pH 7.4, centrifuged at 40,000 x g for 60 min. The residue was suspended in 0.25 M sucrose solution containing 1.8 mM $CaCl_2$, layered over 0.3 M sucrose solution containing 1.8 mM $CaCl_2$, centrifuged at 800 x g for 10 min. The residue was washed again with 0.25 M sucrose containing 1.8 mM $CaCl_2$.

The mitochondria were further purified from the mitochondrial fraction by sucrose density gradient fractionation.

Extraction of DNA: DNA was isolated by a modification of MARMUR's method (36), as briefly as follows. The nuclei or mitochondria were washed once with 10 volumes of saline-EDTA (0.15 M NaCl plus 0.1 M ethylenediamine tetraacetate, pH 8.0). The nuclei were suspended in 5 volumes of saline-EDTA and the mitochondria in 2 volumes of saline-EDTA. The suspensions were lysed by addition of an equal volume of 2 % SDS (sodium dodecyl sulfate) and by placing the mixture in 60°C water bath for 10 min, and then cooled to room temperature. Five M perchloric acid was added to a final concentration of 1 M to the viscous, lysed suspension and the whole mixture was shaken with an equal volume of chloroform-isoamyl alcohol (24 : 1) in a flask for 30 min at 4°C. The resulting emulsion was separated into 3 layers by centrifugation at 5,000 x g for 10 min. The upper aqueous phase was carefully pipetted off into a flask, added with an equal volume of isoamyl alcohol (24 : 1), and the deproteinization procedure was repeated again. The upper aqueous phase was pipetted off into a test tube, on which 2 volumes of ethyl alcohol was gently layered. By gently mixing with a glass rod, long fibrous DNA educed in alcohol was spooled on the glass rod, which was tentatively called nuclear fibrous DNA. After removing the fibrous DNA, the rest was kept overnight at −10°C and precipitated by centrifugation. The pellet thus obtained was called nuclear pellet DNA. The both DNA's were resolved in dilute saline-citrate (0.015 M NaCl plus 0.0015 M trisodium citrate), and added with one tenth volume of 10-fold concentrated saline-citrate (1.5 M NaCl plus 0.15 M trisodium citrate).

Ethidium bromide-CsCl density gradient method: RADLOFF *et al*'.s method (4) was employed for the isolation of closed circular DNA. A mixture of purified nuclear or mitochondrial DNA's 50—80 μg/ml in buoyant CsCl, 3.00 ml of 10 mM Tris-HCl, pH 7.6, 1.566 gm/ml CsCl, 10 μg/ml ethidium bromide, was centrifuged for 48 hours at 37,000 rpm, 20°C. Prior to drop collection, the centrifuge tubes were examined in a darkened room with 365 mμ light, and photographed. Then 6 drops were collected in each test tube, added with 0.5 ml of standard saline citrate (0.15 M NaCl plus 0.015 M trisodium citrate, pH 7.6) and the optical density of the collected samples was measured at the wave length of 260 mμ for DNA and 285 mμ for ethidium bromide.

Sucrose density gradient centrifugation: Linear gradients of 5—20 % sucrose solution in standard saline citrate were prepared in 5 ml tubes for the SW 39

swinging rotor. About 0.2 ml of the DNA solution (176 μg/ml) was carefully delivered onto the gradient surface, and centrifuged at 24,000 rpm for 20 hours at 25°C. Six drops were collected in each test tube, added with 0.5 ml of standard saline citrate, and the optical density of the samples was measured at 260 mμ.

Electron microscopy: Specimens for electron microscopy were prepared by the method of FREIFELDER and KLEINSCHMIDT (37), rotary shadowed with platinum paladium, and were examined in a Hitachi HU 11D electron microscope. Electron micrographs were made at a magnification of × 10,000. The magnified factor was checked with a grating replica.

Chemicals: The ethidium bromide was obtained by the courtesy of Dr. D. E. GRIFFITHS from Boots Pure Drug Co., Ltd, Nottingham, England. Cesium chloride was of optical grade obtained from Nakarai Chemical Co., Ltd. The sodium dodecyl sulfate (SDS) was obtained from Katayama Chemical Co., Ltd. All other chemicals were of reagent grade.

RESULTS

Fractionation of DNA by ethidium bromide cesium chloride density gradient method and electron microscopy

a. *Nuclear DNA*

In the fractionation of the nuclear fibrous DNA fraction by ethidium bromide-cesium chloride density gradient method, fibrous DNA bound with larger amount of ethidium bromide was fractionated in the upper band (Fig. 1). Electron microscope observation of peak fraction No. 39 revealed long linear DNA as shown in Photo 1. On the other hand, in fraction No. 36 small circular DNA was observed at low frequency intermingling with long linear DNA. Fractionation pattern of the nuclear

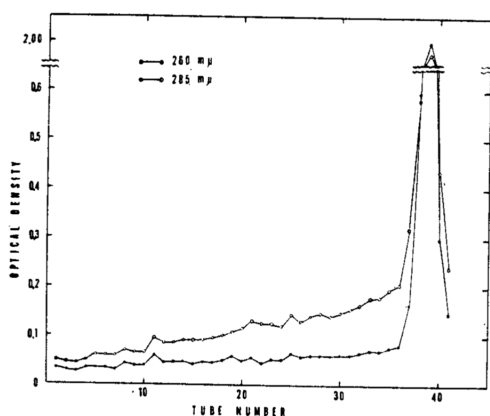


Fig. 1 Ethidium bromide-cesium chloride density gradient fractionation of HeLa cell nuclear DNA which spooled on the glass rod.

pellet DNA fraction is shown in Fig. 2. The peak fraction No. 46 contained mainly short linear DNA derived from fragmented chromosomal DNA. Fraction No. 43, which corresponds to a little upper layer than mitochondrial circular DNA layer, contained small circular DNA and linear DNA fiber (Photo 2). Photo 3 presents electron micrographs of selected molecules of small circular DNA's, measuring 0.32—1.78 μ in contour length, which were found in DNA isolated from nuclear fraction.

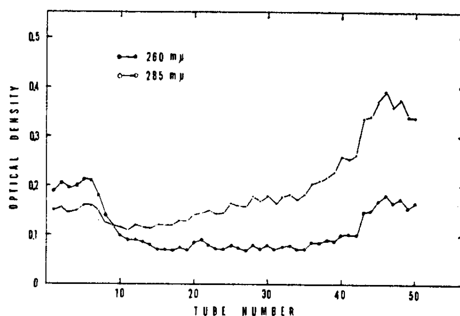


Fig. 2 Ethidium bromide-cesium chloride density gradient fractionation of HeLa cell nuclear DNA obtained in the pellet by centrifugation after removing fibrous DNA that spooled on the glass rod.

b. *Mitochondrial DNA*

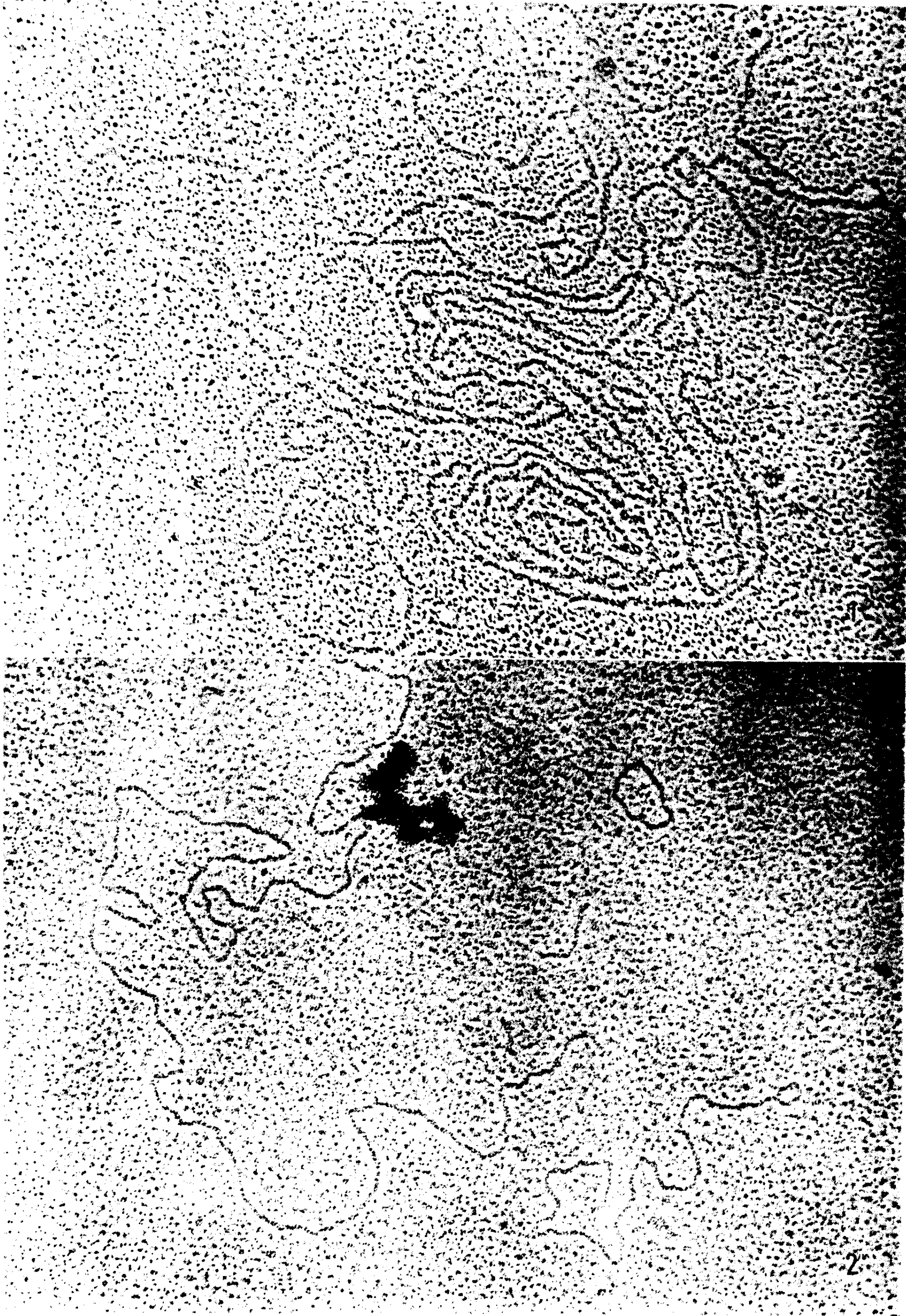
Circular DNA's, measuring 4.87 $\mu \pm 0.47$ μ in the mean value of the contour lengths in the highest frequency group, were observed as presented in Photo 4. Dimer molecules were also detected. In addition to these, small circular DNA molecules, measuring 0.3—1.01 μ in contour length, were also found in extremely low frequency. These are considered to be derived from the mitochondria, though the contamination from the nuclei cannot completely be overruled.

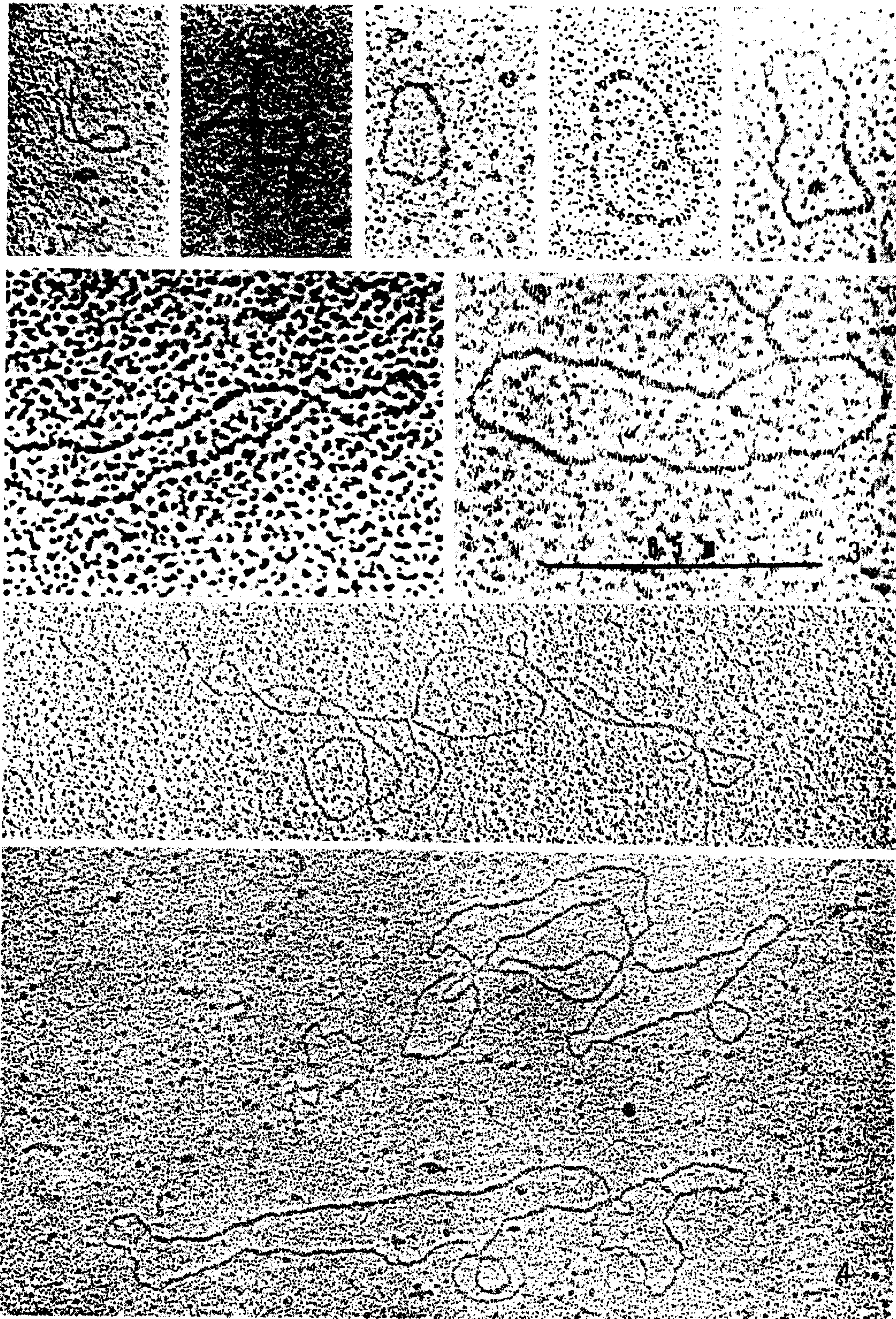
Fractionation of DNA by sucrose density gradient method and electron microscopy

DNA extracted from the whole HaLa cells was divided into two fractions by the method described previously: the one was the fibrous DNA fraction which spooled on the glass rod, and the other was the pellet DNA fraction precipitated by centrifugation after removing the fibrous DNA fraction. The pellet DNA fraction was further fractionated by sucrose density gradient method. The frationation pattern is presented in Fig. 3.

Photo 1. HeLa cell nuclear DNA obtained by ethidium bromide-cesium chloride density gradient fractionation of nuclear fibrous DNA fraction (Fraction No. 39 in Fig. 2) (×55,000).

Photo 2. HeLa cell nuclear DNA obtained by ethidium bromide-cesium chloride density gradient fractionation of nuclear pellet DNA fraction (Fraction No. 43 in Fig. 2). A small oircular DNA and fragmented linear DNA are observed (× 55,000).


1
2


0.5 μ
3
4

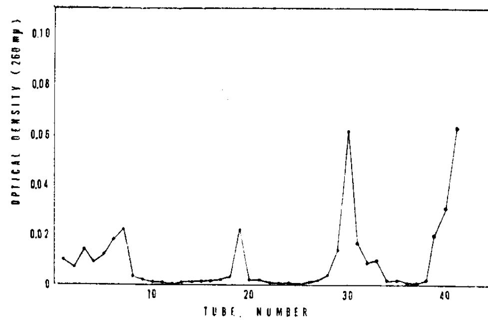


Fig. 3 Sucrose density gradient fractionation of HeLa cell DNA obtained in the pellet by centrifugation after removing fibrous DNA which spooled on the glass rod.

In the peak fraction No. 7, small circular DNA molecules were concentrated as shown in Photo 5.

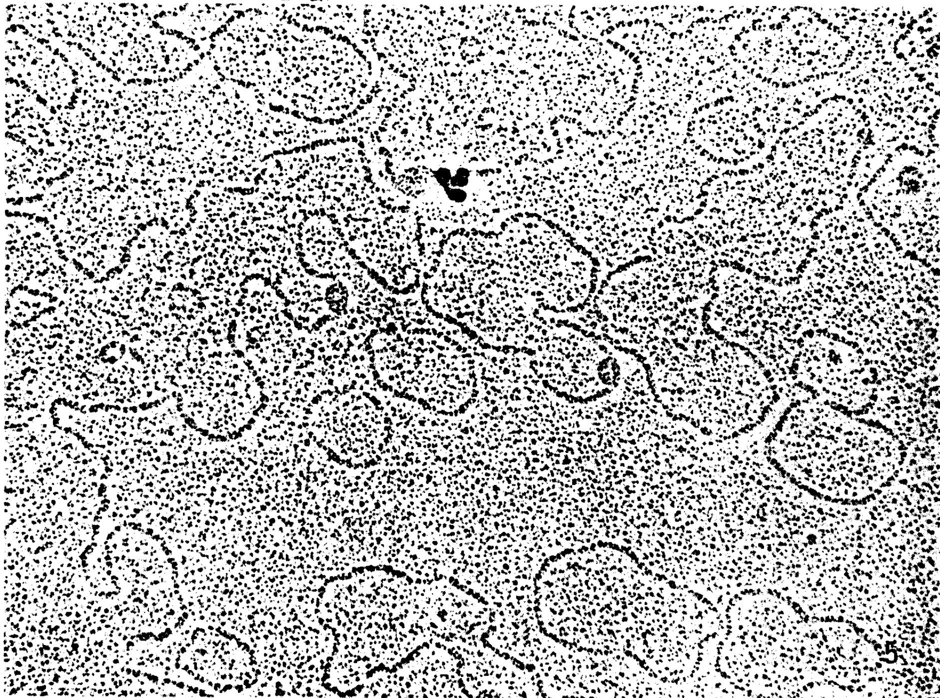


Photo 5. Small circular DNA's and linear DNA's obtained by sucrose density gradient fractionation of pellet DNA fraction from HeLa cells (Fraction No. 7 in Fig. 3) (× 48, 700).

Photo 3. Selected molecules of small circular DNA, measuring 0.32—1.78 μ in contour length, which were found in DNA isolated from nuclear fraction (× 77, 600).

Photo 4. Circular DNA molecules isolated from HeLa cell mitochondria (× 50,000).

Frequency distribution of the contour lengths of small circular DNA molecules

Cumulative frequency distributions of the contour lengths of small circular DNA molecules of submitochondrial size isolated from HeLa cell nuclei, mitochondria, and the whole cell are presented in Fig. 4. The frequency distribution of the small circular DNA molecules is only cumulative and does not necessarily represent the frequency occurrence in the HeLa cell.

Fig. 4 Cumulative frequency distributions of the contour lengths of small circular DNA molecules of submitochondiral size isolated from HeLa cell nuclei, mitochondria, and the whole cell.

DISCUSSION

The dye-buoyant method with ethidium bromide segregates only closed circular duplex DNA molecules, and the molecules that contain even one single-strand scission intermingle with a large excess of linear DNA (4). In our results of the fractionation of circular DNA by the dye-buoyant method, small circular DNA molecules were found with some contamination of linear DNA. However, they were considerably concentrated in a peak fraction by sucrose density gradient method. These small circular DNA molecules are considered to be really derived from the nuclei, because the contamination of mitochondrial circular DNA, measuring about 5 μ, was not found in the DNA isolated from the nuclear fraction.

In our previous studies on the properties of mitochondrial DNA from a variety of normal and cancer cells of human and animal origin, we have reported that cancer cell mitochondria contain circular DNA molecules similar or a little shorter in the mean value of the contour length of the highest frequency group than that of normal cells, and have a wider and heterogenous distribution in the contour length of DNA molecules with higher frequency of dimer and oligomer molecules. (8, 9, 14—18). In addition to this, small circular DNA molecules, measuring about 1—4 μ or

even smaller, were found in every tumor cell mitochondria examined (8, 9, 14—18). Small circular DNA molecules were also found in HeLa cell mitochondrial DNA in low frequency.

Recently, the presence of a satellite DNA has been demonstrated in the nuclear DNA (38, 39), which has been shown to be distinct from mitochondrial DNA (38) and hybridize with ribosomal DNA (39). However, GIACOMONI and CORNEO (40) reported that HeLa cell satellite DNA does not act as a template for the synthesis of ribosomal DNA. The small circular DNA described in the present paper seems to be different from the satellite DNA, but a comparative study on both DNA's will be necessary to clarify their properties. The biological significance of the small circular DNA molecules and their correlation with malignant changes have to await further investigation.

SUMMARY

An electron microscopic observation was made on the DNA's extracted from purified HeLa cell nuclei, mitochondria, and the whole cell, and fractionated by ethidium bromide-cesium chloride density gradient method or sucrose density gradient method. Nuclear DNA presents mainly long linear DNA derived from fragmented chromosomal DNA. In addition to this, the existence of small circular DNA molecules measuring 0.32—1.78 μ, was confirmed. Mitochondrial DNA was mainly circular DNA, which measured 4.87 μ in the mean value of the contour lengths in the highest frequency group, and small circular DNA molecules, measuring 0.3—1.01 μ in contour length, were also found in an extremely low frequency.

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