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Electron microscopic observation of polyoma DNA component I isolated by CsCl-ethidium bromide density gradient centrifugation

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Summary

The DNA extracted from a purified preparation of polyoma virus was separated into two fractions situated in either light or dense bands by CsCl-ethidium bromide density gradient centrifugation. The electron microscopic examination of the DNA in each band showed the molecular configuration of linear and circular DNA, respectively. It was previously considered that the DNA in the dense band consisted component I (closed supercoiled circular DNA) of polyoma virus, however this band contained a number of open circular DNA molecules which increased the length of circumference. The influence of dye binding on the molecular configuration of closed supescoiled circular DNA is discussed.

Introduction

Artificial changes in the biologic activity or morphologic appearence of nucleic acids in the course of extraction should be avoided for accurate investigation of their nature.

This study was attempted to observe ultrastructural morphologic changes of polyoma DNA molecules isolated by CsCl-ethidium bromide density gradient centrifugation.

Ethidium bromide is capable of intercalative binding between DNA base paisr (1). The amount of dye that can be bound with the closed circular DNA is smaller than the amount of dye binding with the linear or nicked circular DNA. The buoyant density of DNA-dye complex is inversely related to the amount of dye bound (2, 3). On the basis of these findings, Radloff *et al.* (4) effectively isolated closed circular DNA from nicked circular DNA of polyoma virus by CsCl-ethidium bromide density gradient centrifugation. This method has been widely used for the study of covalently closed circular DNA and may be applied for the purification of supercoiled circular DNA. However, the binding of the dye to DNA has been shown to influence its physical state in such ways as reducing the number of superhelical turns (2, 5), nicking the DNA strand (6), or increasing the length of the DNA molecules (7, 8). These effects of dye binding are determined as morphologic changes in the molecular configuration of DNA by electron microscopic observation. Freifelder (8) observed by electron microscopy a lengthening of linear λ b 2 and nicked SV 40 DNA by treatment with ethidium bromide.

In this study we found that the polyoma DNA molecules in the dense band, component I, separated by CsCl-ethidium bromide density gradient centrifugation were mostly open circular, and their lentgh was increased.

Materials and Methods

1) *Virus and cell cultures*

Large plaque variants (Lp) which were cloned by plaque purification from 4 B 5–6 strain of SE-polyoma virus (9) maintained in our laboratory were used. Kidney cells from 12- to 14-day-old Swiss mice were cultured in Roux bottles containing 50 mℓ of Eagle's MEM supplemented with 10% Tryptose phosphate broth and 10% calf serum.

2) *Growth of the virus*

Primary confluent cultures of the mouse kidney cells were infected with 0.5 mℓ of virus suspension at a multiplicity of 0.1 plaque forming unit/cell. After 1 hr at 37°C, the infected cultures were incubated with Eagle's MEM without serum for 7 days at 37°C.

3) *Concentration and purification of the virus*

On the 7th day after infection, dead cells and debris were removed by low speed centrifugation and suspended in 0.1 M Tris-HCI buffer pH 8.5 or Dulbecco's PBS containing receptor destroying enzyme (RDE) (100 μg/mℓ) in one-fiftieth of the original medium. This suspension was incubated for 30 min. at 37°C to release the virus from cell debris. After incubation, the suspension was centrifuged for 15 min. at 8,000×g. Virus particles in the supernatant fluid were collected onto the KBr cushion and purified further by CsCl density gradient centrifugation as described by Roblin *et al.* (10). The virus material thus obtained was dialysed against a large volume of Tris-HCl buffered saline (0.01 M Tris-HCl, 0.15 M NaCl and 0.01 M EDTA, pH 7.5). An electron micrograph of the virus particles banded at a density of 1.32in CsCl is shown in Fig. 1.

4) *Extraction of viral DNA*

Sodium dodecylsulphate (SDS) was added to the dialysed virus suspension at a concentration of 0.5%. This suspension was heated at 50°C for 30 min. to break down

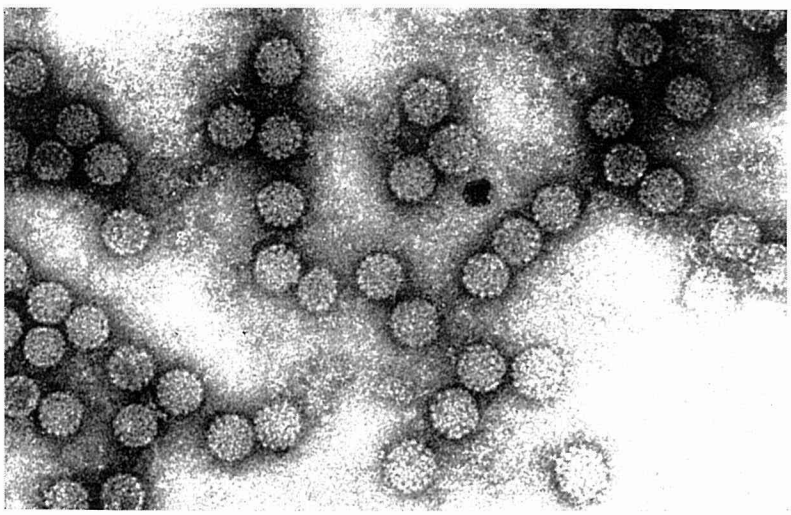

Fig. 1 Electron micrograph of polyoma virus particles banded at a density of 1.32 in CsCl density gradient centrifugation.

the virus (11). After incubation, an equal volume of water-saturated phenol was added and the mixture was shaken by hand at room temperature for 5 min. The phenol and the aqueous phases were separated by centrifugation at 3,000 rpm for 5 min. The phenol extraction was repeated 3 times. The phenol dissolved in the aqueous phase was removed by shaking with ether and the aqueous phase was dialysed against a large volume of Tris-HCl buffered saline.

5) *CsCl-ethidium bromide density gradient centrifugation*

CsCl-ethidium bromide density gradient centrifugation was carried out by the method of Radloff (4). Three mℓ of DNA solution containing 1.566 g/mℓ of CsCl and 100 μg/mℓ of ethidium bromide were centrifuged in a RPS 65 TA rotor in a Hitachi 65 P preparative ultracenrifuge at 43,000 rpm at 20°C for 48 hr.

6) *Electron microscopy*

Specimens were prepared by a modification of the formamide methd of Davis (12). The spreading solutions consisted of 0.2 mℓ DNA solution (5 μg/mℓ), 0.05 mℓ cytochrome C (1 mg/mℓ), 0.05 mℓ 1.0 M Tris-HCl buffer pH 8, 6 containing 0.1 M EDTA and 0.2 mℓ formamide. A quantity of 250 μℓ of the spreading solution was spread onto distilled water. The films were picked up on collodion-coated specimen grid and shadowed with platium-palladium. The uranyl staining was omitted. The electron micrographs were made at a magnification of ×6,000 wih a Hitachi HS-7 D electron microscope. The negative films of the electron micrograph were enlarged ×10 on a Nikon profile projector and were traced on tracing paper. Lengths were then measured with a curvemeter.

Results

The phenol-extracted polyoma DNA was centrifuged in CsCl-ethidium bromide density gradient. After centrifugation, the centrifuge tubes were examined in a darkened room for the DNA bands using ultraviolet rays (wave length 360 mμ). As shown in Fig. 2, two distinct bands were observed. The tube was punctured at the bottom and band was collected on small vials. The each fraction was extensively dialysed against a large volume of Tris-HCl buffered saline containing 0.01 M EDTA for 48 hrs. Immediately after dialysis, specimens for electron micrography were prepared from each of the dialysed light and dense bands. Observations with an electron microscope showed that the light band contained linear DNA molecules of various lengths and a few circular DNA molecules (Fig. 3 a), and the dense

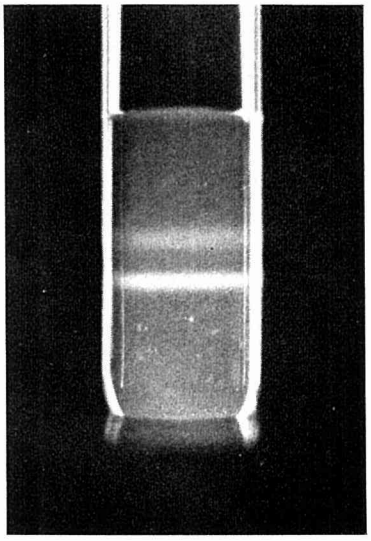

Fig. 2 A photograph of light and dense bands in CsCl-ethidium bromide density gradient of phenol extracted polyoma DNA. Three mℓ of DNA solution containing 1.566 g/mℓ of CsCl and 100 μg/mℓ of ethidium bromide were centrifuged in a RPS 65 TA rotor in a Hitachi 65 P preparative ultracentrifuge at 43,000 rpm at 20°C for 48 hr.

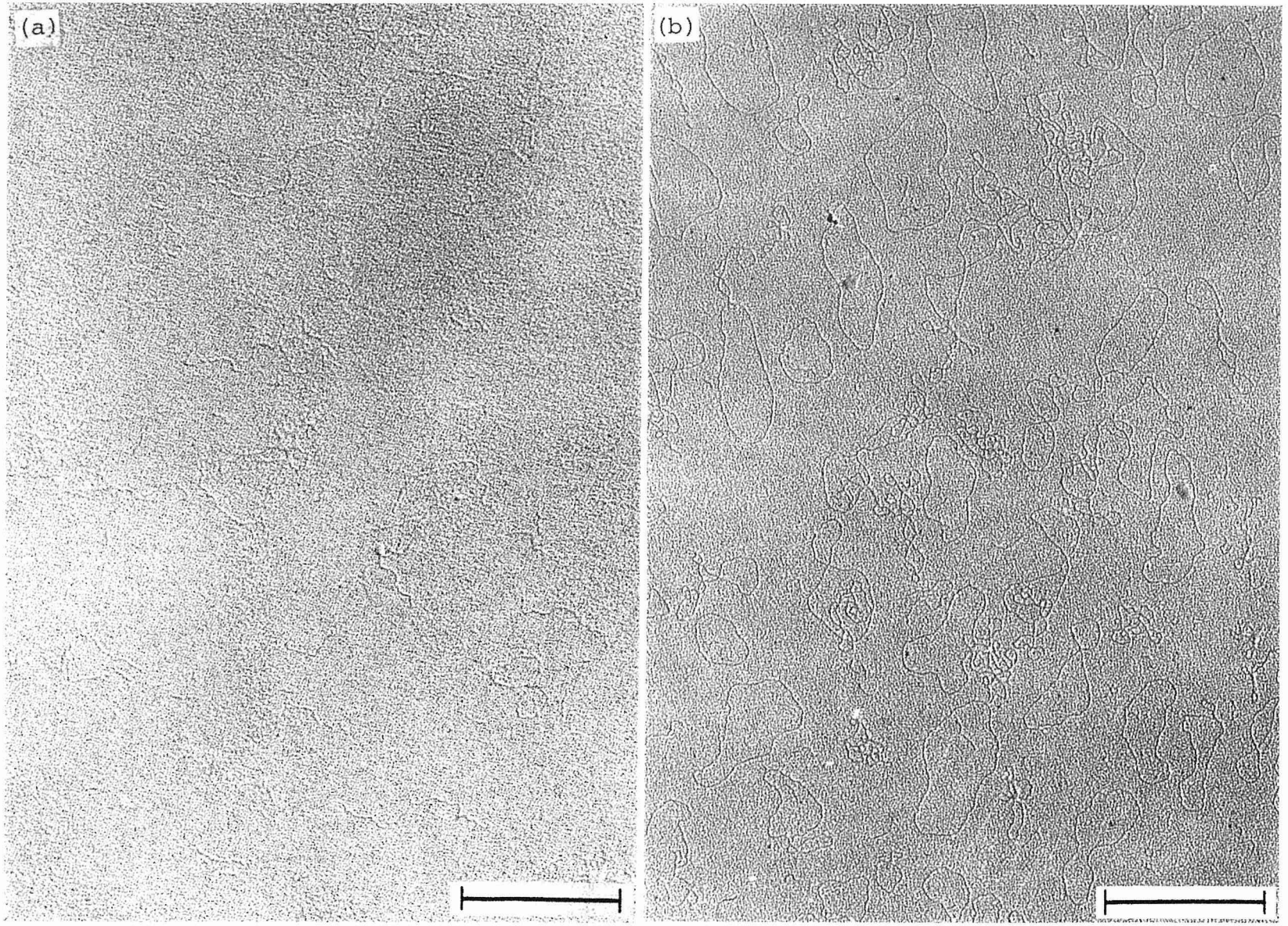

Fig. 3 Electron micrograph of polyoma DNA isolated by CsCl-ethidium bromide density gradient centrifugion, × 26,000.
(a) DNA from the light band, (b) DNA from the dense band of Fig. 2. The scale represents 1 μ.

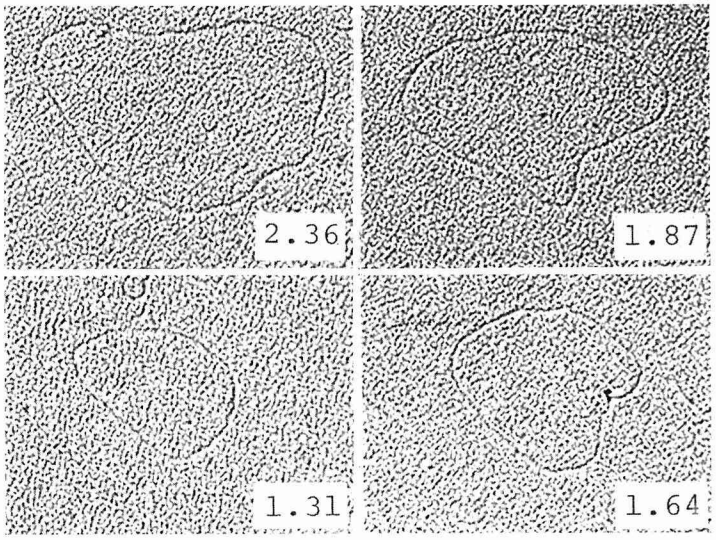

Fig. 4 Electron micrograph of open circular DNA of polyoma virus obtained from the dense band in CsCl-ethidium bromide density gradient, ×42,800. Each open circular molecule was selected from the same negative film of electron micrograph. The number gives the length in microns of the molecule.

band almost entirely consisted of circular DNA molecules (Fig. 3 b). These indicated that CsCl-ethidium bromide density gradient centrifugation is useful for the isolation of closed circular DNA.

The DNA molecules in the dense band were a mixture of supercoiled and open circular DNA. The supercoiled circular DNA had a reduced number of superhelical turns to various extents and the molecular size of the open circular DNA varied. Fig. 4 presents micrograph illustations of the differences of molecular size among the open circular DNAs. Fig. 5 shows the frequency distribution of the circumferences obtained from 441 molecules of open circular DNA. The circumferences of the molecules were distributed from 1 to 2.5 μ. Molecules with a circumferenc of between 2.0 and 2.17 μ were most frequent. The open circular DNA derived from closed supercoiled circular DNA of polyoma virus has been shown to have the length of 1.35 μ by several investigators (13, 14, 15). Fig. 5 indicates that about 90 percent of the 441 open circular DNA, which were obtained from the fraction of dense band, were obviously increased in length comparing with previous reports (13, 14, 15).

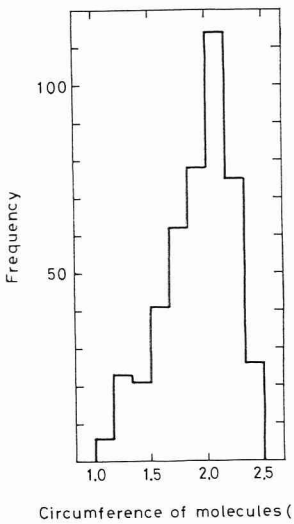

Fig. 5 Frequency distribution of the circumferences of open circular polyoma DNA molecules observed in the dense band in CsCl-ethidium bromide density gradient.

Discussion

Polyoma virus DNA which is a closed supercoiled circular duplex having 12–15 helical turns (5, 16), is designated as component I. Physico-chemical studies have shown that component I is converted to open circular DNA by binding with a small amount of dye, but that the number of the helical turns increases with a higher concentration of dye (2). Radoloff *et al.* (4) clearly separated componet I from nicked circular DNA of polyoma virus by density gradient centrifugation with CsCl and ethidium bromide of a high concentration. The former was situated in the dense band, and the latter in the light band. Centrifugation with CsCl-ethidium bromide density gradient of DNA which was extracted from a purified preparation of polyoma virus gave clearly two bands of DNA (Fig. 2) as described by Radoloff (4). However, the molecular configuration of DNA in the dense band differed from the supercoiled circular form of component I. The majority of DNA contained in the dense band converted to open circular DNA and showed a reduced number of superhelical turns (Fig. 3 b), and the circumference of the open circle increased (Fig. 5). Ethidium bromide is a candidate that might be responsible for the molecular configuration of supercoiled DNA. Dye-bound-DNA is easily nicked by illumination (6). Freifelder (8) observed by electron microscopy that the circumferences of nicked SV 40 DNA increased by treatment with this drug. It was probable that the appearance of the open circular DNA with an increased circumference in the dense band might be an artifact resuling from the binding of ethidium bromide with supercoiled circular DNA.

Although CsCl-ethidium bromide density gradient centrifugation is useful for the purification of component I, artifacts due to dye-binding should be considered when the nature of extracted DNA is investigated.

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