INTERFERENCE OF ETHIDIUM BROMIDE WITH THE FORMATION OF SUPERCOILED MITOCHONDRIAL DNA

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1. Introduction

Ethidium bromide induces in *Saccharomyces cerevisiae* a rapid change to a respiratory deficient phenotype [1] and the synthesis of an abnormal mitochondrial DNA [2]. Inhibition of the synthesis of the mitochondrial DNA by ethidium bromide has been reported for *Physarum polycephalum* [3] and for mouse L-cells [4]. In this report we describe that (1) in cultured human liver cells (Chang) ethidium bromide prevents the *de novo* formation of supercoiled mitochondrial DNA, (2) existing supercoiled mitochondrial DNA is degraded, and (3) normal synthesis of supercoiled mitochondrial DNA is restored after removal of the drug.

2. Materials and methods

The growth conditions for the serially propagated cell line derived from human liver (Chang) were described previously [5, 6]. The cell line was found to be free from mycoplasma contamination [5]. Thymidine-methyl-^{3}H (26 Ci/mmole) and thymidine-2-^{14}C (60 mCi/mmole) were obtained from The Radiochemical Centre Amersham, England. Cytoplasmic extracts were prepared by lysing the cells ($\sim 10^7$) in 0.9 ml of 0.001 M EDTA, 0.001 M spermidine, 0.01 M Tris-HCl pH 7.6, 0.5% Triton X-100. After standing for 30 sec (0°) the suspension was briefly agitated with a Vortex mixer and the nuclei were removed by centrifugation at 2000 *g* for 10 min. The lysates were made 1% SDS (0.1 M NaCl, 0.001 M EDTA, 0.01 M Tris-HCl pH 7.6) and fractionated by means of sedimentation through linear sucrose gradients (15–30% sucrose in 0.1 M NaCl, 0.001 M EDTA, 0.01 M Tris-HCl pH 7.6, 0.5% SDS, Spinco SW 27, 25,000 rpm, 25°, 12 hr). All further analytical methods have been described in detail [5].

3. Results and discussion

No supercoiled mitochondrial DNA (39 S) [7] was detectable by means of sedimentation through neutral sucrose gradients in a cytoplasmic extract of cultured liver cells labeled with thymidine-methyl-^{3}H in presence of 1 μg/ml ethidium bromide (fig. 1). Density equilibrium centrifugation in ethidium bromide-CsCl-gradients [8] also revealed no incorporation of the radioisotope into closed circular DNA. Ethidium bromide seems to completely inhibit the *de novo* synthesis of this DNA species. However, the incorporation of thymidine-methyl-^{3}H into nuclear DNA proceeded at a rate of about 70% of that of the control cultures for at least 24 hr at this ethidium bromide concentration.

Cells in logarithmic growth phase were prelabeled with thymidine-methyl-^{3}H and then exposed to ethidium bromide (1 μg/ml) for 26 hr. The cytoplasm of these cells and a cytoplasm of control cells were fractionated by sedimentation through sucrose gradients. The fractions of the gradients corresponding to sedimentation values between 43 S and 35 S were pooled and further analyzed by density equilibrium centrifugation in ethidium bromide-CsCl-gradients. The 39 S DNA isolated from control cultures separated in these gradients into a heavy band (HB) containing

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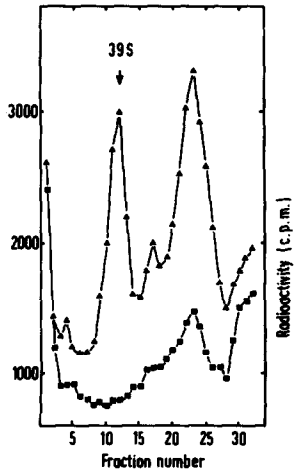
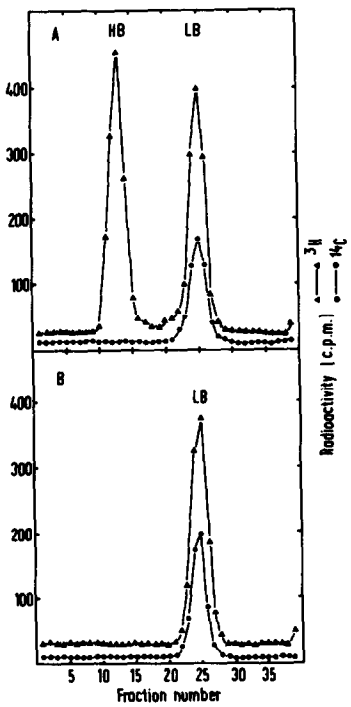


Fig. 1. Sedimentation of labeled cytoplasmic DNAs through sucrose gradients. Cells were labeled with thymidine-methyl-^{3}H (5 μCi/ml) for 12 hr in presence of 1 μg/ml ethidium bromide (■——■). A sister culture labeled in the absence of ethidium bromide was used as a control (▲——▲). Cytoplasmic extracts were prepared, layered on top of a sucrose gradient and centrifuged as described in sect. 2. The gradients were fractionated into 1 ml fractions. Aliquots were used to determine the acid precipitable radioactivity. The direction of sedimentation is from right to left.



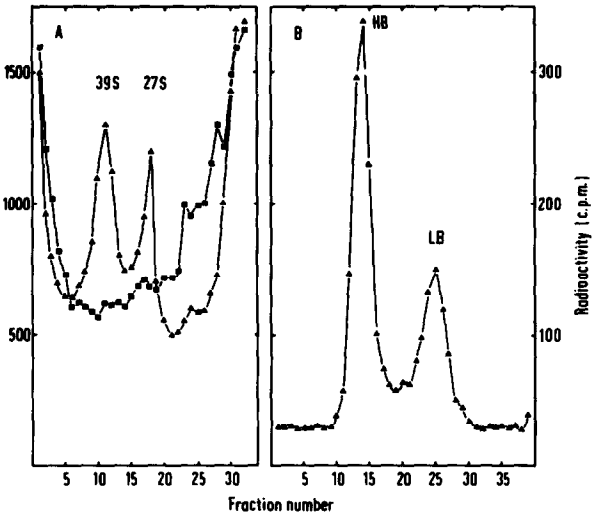


Fig. 3. Restoration of the synthesis of supercoiled (39 S) mitochondrial DNA after exposure to ethidium bromide. Cells were grown in presence of 1 μg/ml ethidium bromide for 24 hr. The medium was then replaced by a medium which did not contain ethidium bromide. This medium was again replaced twice with fresh medium at 1 hr intervals. Cells were labeled with thymidine-methyl-^{3}H (5 μCi/ml) for 6 hr. Cytoplasmic extracts were prepared and analyzed as described in fig. 1. Frame A: Sedimentation of cytoplasmic labeled 4–10 hr (■——■) and 24–30 hr (▲——▲) after removal of ethidium bromide. Frame B: Buoyant density centrifugation (ethidium bromide-CsCl-gradient) of the 39 S DNA labeled 24–30 hr after removal of the drug. Centrifugation conditions as described in fig. 2.

Fig. 2. Buoyant density centrifugtaion of 39 S DNA in ethidium bromide-CsCl-gradients. Cells (two sister cultures) were labeled with thymidine-methyl-^{3}H (5 μCi/ml) for 24 hr. Subsequently one culture was grown for 26 hr in presence of 1 μg/ml ethidium bromide, the other was grown in a normal medium. The cytoplasms were fractionated as described in fig. 1. "39 S" DNA (35 S–43 S) was precipitated with 2 vol. of ethanol and analyzed in ethidium bromide-CsCl-gradients (starting density 1.560 g/ml, 200 μg/ml ethidium bromide, Spinco SW 50-L, 30,000 rpm, 20°, 60 hr. Frame A: ^{3}H-DNA from the control culture (▲——▲). Frame B: ^{3}H-DNA from the culture grown in presence of ethidium bromide (▲——▲). ^{14}C-labeled nuclear DNA was added as a density marker for the light band (●——●). The field is directed to the left.

closed circular DNA, and into a light band (LB) which consisted of nicked and linear DNA (fig. 2A). The "39 S" DNA obtained from the cytoplasm of cells exposed to ethidium bromide did not yield a heavy band. All of the radioactivity banded at the position of nicked or linear DNA (fig. 2B). Therefore ethidium bromide does not only inhibit the *de novo* synthesis of supercoiled mitochondrial DNA but also causes a degradation of existing supercoiled mitochondrial DNA.

Cells once exposed to ethidium bromide (1 μg/ml) resume the synthesis of supercoiled mitochondrial DNA with a lag period during subsequent growth in the absence of the inhibitor. No incorporation of thymidine-methyl- ^{3}H into 39 S DNA occurred during the time interval 4–10 hr after removal of ethidium bromide (fig. 3A). When the cells were cultured for a prolonged time in the absence of ethidium bromide (24 hr, labeling 24–30 hr), labeled 39 S DNA was found in the cytoplasm (fig. 3A). Furthermore this 39 S DNA yielded in ethidium bromide-CsCl-gradients a heavy band characteristic of closed circular duplex DNA (fig. 3B).

These results suggest that the degradation of supercoiled mitochondrial DNA by ethidium bromide is a reversible process in mammalian cells.

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