CLOSED CIRCULAR DNA ASSOCIATED WITH YEAST MITOCHONDRIA

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1. Introduction

DNA with a contour length of 2 μ (mol. wt. 4 X 10^6 dalton) is the most abundant circular class detected in yeast (1–5% of the total DNA) [1,2]. It has the buoyant density of nuclear DNA (1.698–1.701 in CsCl), it is not γ-DNA [3]. It has been often found in mitochondrial fractions [4–6], and many identical 2 μ circles may be present in a single cell [6]. The cellular localization and biological function of this DNA are not known. It was reported [5] that these molecules were associated with a particulate band (possibly peroxisomes) when a curde mitochondrial preparation was fractionated on a sucrose gradient. The buoyant density of the 2 μ circles and their presence in a cytoplasmic respiratory deficient (ρ^-) mutant lacking mitochondrial DNA [7] suggest nuclear origin. However, the possibility still remains that the 2μ DNA represents a part of the mitochondrial genome not affected by the conversion to ρ^- state and with a C+G content higher than the mean value (17%) [8]. No other class of DNA has been identified in yeast, even though cytoplasmically inherited genes not associated with the ρ factor are known [9,10]. The results reported here show that the in vivo labelling of the DNA circles of nuclear density is inhibited by cycloheximide in analogy with the behavior of the nuclear [11–13] and not the mitochondrial DNA. Contrary to some reports [14], however, our circular DNA fraction also contains DNA of mitochondrial density.

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** *Abbreviations*: CH, cycloheximide; EBr, ethidium bromide.

2. Materials and methods

[^{3}H]Adenine (11.2 Ci/mmole) and Aquasol were obtained from New England Nuclear, EBr** from Calbiochem, CsCl from The Harshaw Chemical Co., CH (Acti-dione) was The Upjohn Co. product.

Cultures of the haploid strain of *Saccharomyces cerevisiae* (275-10b) were grown in 100 ml of 3.5% potassium lactate, 1% yeast extract, 2% Bactopeptone, in shaken conical flasks at 30°C. Cells were either labelled for many generations with 300 μCi of [^{3}H]adenine or alternatively pretreated for 15 min with 20 mg of cycloheximide and then labelled with 300 μCi of [^{3}H]adenine for 5 hr in the presence of antibiotic.

About 2 g of cells (wet weight) were harvested by centrifugation at 4°C and washed twice with distilled water and once in the homogenization medium (20 mM Tris–Cl, 1 mM EDTA, 0.5 M sorbitol, 1.27 mM EBr, pH 7.5).The cells were then suspended in 1.5 ml of cold homogenization medium, mixed with 7 g chilled 0.5 mm glass beads and shaken 15 sec in a special small flask on a Braun homogenizer (B. Braun, Melsungen, West Germany) with CO_2 cooling. Subsequent operations were carried out at 4°C and under red light [5]. Unbroken cells, nuclei and debris were removed by centrifugation at 2000 *g* for 20 min and the crude mitochondrial fraction pelletted at 17 500 rev/min in a Spinco no. 30 rotor with one subsequent wash in the homogenization medium. The pellet was resuspended into 5 ml of 0.1 M EDTA, 0.15 M NaCl, 2.5% SDS, 1.27 mM EBr, pH 8.0, transferred to a dark bottle and lysis carried out at 60°C for 10 min. The solution was made 1 N with respect to NaCl and kept on ice for 1 hr. The heavy precipitate was spun down and the supernatant dialyzed in the dark against

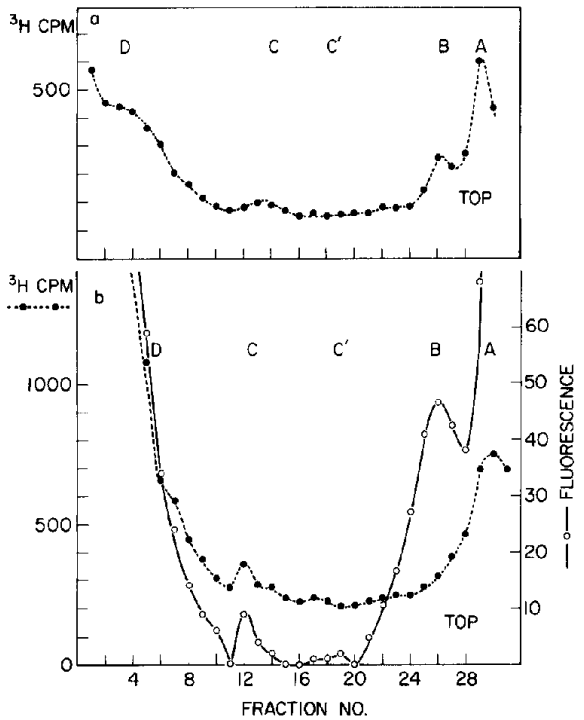

Fig. 1. EBr-CsCl equilibrium density gradient of nucleic acids extracted from a crude mitochondrial fraction; cells labelled with [^{3}H] adenine (details in sect. 2). Labelling: a) in the absence of CH; b) in the presence of 200 μg/ml of CH. (•...•...•) ^{3}H cpm, (o-o-o) fluorescence, arbitrary units.

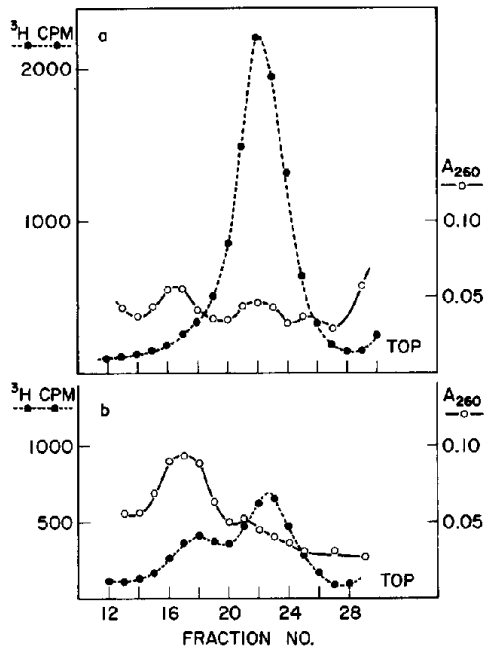

Fig. 2. CsCl equilibrium density gradient of pooled fractions from gradient in fig. 1b (labelling in the presence of CH) after extraction of EBr with isoamyl alcohol. (•--•--•) ^{3}H cpm, (o—o—o) absorbance at 260 nm: a) fractions 28–31 (band A, fig. 1b); b) fractions 24–27 (band B. Fig. 1b).

SSC (0.15 M NaCl, 0.015 M Na citrate, pH 7.0) for several hours. The gradient was prepared by mixing 1.8 mg EBr, 5 ml of the dialyzed solution and 4.7 g of CsCl. The mixture was poured into 10 ml centrifuge tubes, overlayed with paraffin oil and centrifuged in a Spinco no. 40 rotor at 33 000 rev/min for 60 hr at 18°C. Gradients were manually fractionated into 30 fractions of 20 μl. Aliquots of 20 μl from each fraction were mixed with 0.8 ml H_2O and 10 ml Aquasol, and the ^{3}H radioactivity was measured on a Beckman LS-250 scintillation counter. For the fluorescence measurement (fig. 1b), the 200 μl fractions from the EBr-CsCl gradient were diluted to 2 ml with distilled water and fluorescence read on a Turner no. 110 fluorometer with excitation wavelength between 500–550 nm and the secondary filter absorbing below 575 nm (zero was arbitrarily set on the fraction with minimal reading, i.e. fraction 20 in fig. 1b). For ^{3}H counting, 200 μl aliquots of dilute fractions were mixed with 10 ml Aquasol and radioactivity measured. The peak fractions from the EBr-CsCl gradients were pooled, concentrated if needed, extracted three times with isoamyl alcohol to remove ethidium bromide and dialyzed against SSC. The DNA contained in these samples was analyzed on CsCl equilibrium density gradients which were prepared by mixing 6 g of solid CsCl with 5 ml of the respective fraction. Centrifugation and fraction collection was as described above, 200 μl fraction were mixed with 0.8 ml of water, absorbance at 260 nm determined on a Gilford 240 spectrophotometer and the whole fraction was then used for ^{3}H counting.

3. Results

Separation of DNA molecules on an EBr-CsCl gradient (fig. 1a) is partly due to differences in innate buoyant density but mainly due to the different extent of EBr-DNA binding. The closed circular DNA

(band C) binds less EBr and appears therefore denser on the gradient. The upper bands contain linear and open circular molecules (bands A and B). The heavy band D at the bottom of the gradient is RNA not removed by our procedure. With the exception of band D, our results are very similar to those reported by Clark-Walker [5] and by Hollenberg et al. [14]. If labelling is done in the presence of cycloheximide, incorporation into band B is blocked (fig. 1b). However, the unlabelled nuclear DNA is still banding at the position expected, fraction 26, as shown by the fluorescence curve. The fluorescence, measured and plotted in arbitrary units, is proportional to the concentration of nucleic acids.

To verify that our peaks (A, B of fig. 1b) correspond to the identically indexed peaks of Hollenberg et al. (fig. 1b of ref. [14] and to two bands A of Clark-Walker (fig. 2 of ref. [5], we extracted the EBr from the respective fractions with isoamyl alcohol and recentrifuged the DNA on CsCl gradient (fig. 2). The A_{260} profile of fig. 2a shows that peak A contains the two types of DNA (nuclear density 1.699 and mitochondrial density 1.683) in approximately a 1:1 ratio. Only the mitochondrial peak is visible on the radioactivity curve, however, as expected from labelling in the presence of cycloheximide. Since peaks A and B (fig. 1b) are overlapping, peak A, if clearly separated, would contain linear and open circular forms of mitochondrial DNA. Peak B (see fig. 2b) consists mainly of nuclear DNA, again with cross contamination from peak A. These patterns are in complete agreement with results of Hollenberg et al. and of Clark-Walker. Slight incorporation into nuclear DNA in the presence of cycloheximide is expected due to incomplete inhibition of protein synthesis by cycloheximide and completion of rounds of DNA replication as discussed in [12,13].

We analyzed in a similar fashion peaks C from gradients 1a and 1b. As expected, the major component in the peak of closed circular DNA is of nuclear density (fig. 3a). A smaller peak of mitochondrial DNA is also seen. Fig. 3b shows the components of band C when labelling is done in the presence of cycloheximide. Under these conditions, incorporation was preferentially into the circular mitochondrial DNA and the labelling of the heavier DNA was blocked by cycloheximide. We have further analyzed the intermediate region C′ (fig. 1a). The major component is still nuclear DNA but the ratio between the two DNAs has changed in favor of the mitochondrial component (compare fig. 3c with fig. 3a). This region may contain the replicative intermediates of mitochondrial DNA as found with rat liver preparations [15].

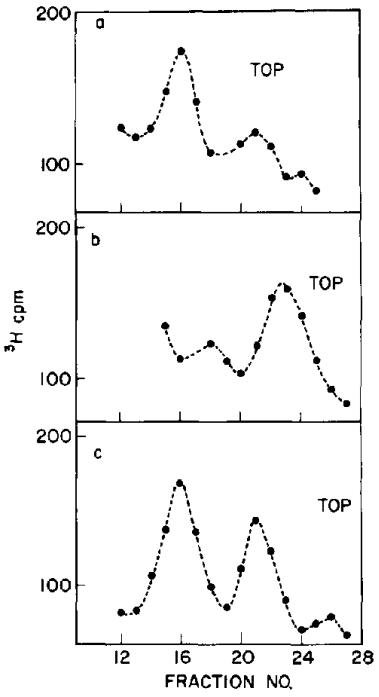

Fig. 3. CsCl equilibrium density gradient of pooled fractions from gradients in fig. 1 after extraction of EBr with isoamyl alcohol: a) fractions 11–15 (band C, fig. 1a), labelling in the absence of CH; b) fractions 11–15 (band C, fig. 1b), labelling in the presence of CH; c) fractions 16–21 (band C′, fig. 1a), labelling in the absence of CH.

We have tried without success to separate the crude mitochondrial fraction into two particulate fractions (fig. 5.2 of ref. [5]). Such a purification step would be very useful due to the claim [5] that particles containing only mitochondrial DNA (ρ = 1.673) can thus be obtained. The failure to remove the nuclear DNA contamination from the mitochondrial fraction by sucrose gradient centrifugation has been reported by others [16] and confirmed in this laboratory.

4. Discussion

Our observation that the in vivo labelling of small DNA circles prepared from yeast mitochondria can be blocked by CH does not obviously answer unequivocally the question whether this DNA is nuclear or cytoplasmic. It represents, however, a third property (together with its buoyant density and its conservation in the ρ^- mutants) which this DNA shares with the nuclear DNA. We consider it therefore likely that this DNA represents either a nuclear contaminant (in contrast to the exclusion of this possibility by Clark-Walker [5]) or a DNA molecule exported from the nucleus into cytoplasm with some unknown biological function. Since this DNA differs in the three above mentioned attributes from the mitochondrial DNA (ρ = 1.683), we do not think it is a part of the organelle's genome. Chloroplast DNA synthesis of *Chlamydomonas* is also insensitive to cycloheximide in comparison to its nuclear counterpart [17]. To our knowledge, no organelle DNA has been found with replication tightly coupled to protein synthesis on cytoplasmic ribosomes (postulated site of the CH action). This renders rather unlikely the possibility that the 2 μ DNA is an unidentified genome of some cytoplasmic organelle [5] but the possibility that it is an episome which becomes integrated into nuclear DNA before replication is harder to rule out.

In contrast to others [14], we have succeeded in preparing small amounts of circular DNA of mitochondrial density which seems to be enriched in the imtermediate band of the EBr-CsCl gradient. We are investigating whether this species represents the elusive 25–26 μ circles [14] postulated for the intact mitochondrial genome.

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