MICROBODY-ASSOCIATED DNA IN *CANDIDA TROPICALIS* pK 233 CELLS

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1. Introduction

Microbodies synonymous with peroxisomes or glyoxysomes are cytoplasmic organelles distinct from mitochondria which have their own DNA and are regarded as genetically independent semi-autonomous organelles [1]. Microbodies usually multiply by budding outgrowth of the endoplasmic reticulum [2]. However, in normal alkane-grown *Candida* yeasts, they multiply by division together with a remarkable increase in catalase activity [3,4]. In our previous studies by electron microscopy DNA molecules of about 15 μm in contour length were found to be released from microbodies by osmotic shock [5], and DNA fibrils were detected in matrix of microbodies [6]. Several other investigators have also suggested the existence of DNA in microbodies of yeast and various other organisms, although the possibility of contamination of their preparations with nuclear DNA was not completely ruled out.

This paper provides physicochemical evidence that the microbody fraction from normal alkane-grown *Candida tropicalis* cells contains a DNA that is different from nuclear and mitochondrial DNAs.

2. Materials and methods

2.1. *Cultivation of yeast*

The culture conditions of the hydrocarbon-utilizing yeast, *Candida tropicalis* (Castellani) Berkhout pK 233 [7] were essentially the same as the those described previously [4] except for addition of 0.5 μCi/ml [8-^{3}H]adenine (25 Ci/mmol, The Radiochemical Centre, Amersham) to the medium. Cells were harvested after 13 h incubation, which was critical for preparing a microbody fraction with as little contamination of mitochondria as possible [8].

2.2. *Preparation of microbodies*

The cells (wet wt 10 g) were digested with 'zymolyase' [9] to obtain protoplasts [4]. Microbodies were prepared from the protoplasts using discontinuous sucrose density gradient centrifugation as in [8]. Aliquots of each fraction of the gradient were taken for measuring the activities of catalase, D-amino acid oxidase and cytochrome *c* oxidase, and protein, and for radioactivity assay. Enzyme activities were assayed by the methods in [8] and protein was determined by the method in [10] using bovine serum albumin as standard. For radioactivity assay, aliquots were incubated in 0.5 N KOH overnight at 37°C, collected on nitrocellulose membranes, washed 3 times with 5% trichloroacetic acid, and then with ethanol and ethyl ether, and dried. Radioactivity was counted in toluene-based scintillator fluid.

2.3. *Extraction and purification of microbody DNA*

Microbody-rich fractions from a discontinuous sucrose density gradient, which were identified by their high activities of catalase and D-amino acid oxidase, were combined, adjusted to 14% sucrose

with 50 mM potassium buffer (pH 7.5), and centrifuged at 20 000 × *g* for 15 min. The precipitate consisting mostly of microbodies was resuspended in 0.85 ml TES (50 mM Tris–HCl, pH 7.4, 5 mM EDTA, 50 mM NaCl). This microbody suspension was treated with 0.5% SDS and 1 mg/ml pronase at room temperature overnight, and extracted with phenol. The resulting aqueous phase was dialyzed against TES at 4°C for 24 h.

2.4. *Extraction and purification of nuclear and mitochondrial DNAs*

When *C. tropicalis* pK 233 cells were grown on glucose, only a few microbodies appeared in the cells [3]. Therefore, to reduce contamination with microbody DNA, nuclear and mitochondrial DNAs were prepared from these cells. Nuclear DNA was prepared from a protoplast lysate, and mitochondrial DNA from the crude mitochondrial fraction (10 000 × *g* pellet) [8] isolated from the same organism. The DNA was extracted with SDS–phenol and then with chloroform–isoamyl alcohol [11]. DNAs extracted were incubated for 1 h with RNase A (0.1 mg/ml) and RNase T_1 (30 μg/ml), deproteinized, dialyzed against TES for 24 h, and precipitated with ethanol. The preparation of nuclear or mitochondrial DNA thus obtained was further purified by cesium chloride (CsCl) isopycnic centrifugation in a Hitachi RP 65T rotor at 40 000 rev./min for 44 h at 20°C and DNA was collected and dialyzed against TES.

2.5. *Separation of microbody-associated, nuclear and mitochondrial DNAs by CsCl isopycnic centrifugation*

^{3}H-Labelled microbody-associated DNA, and unlabelled nuclear and mitochondrial DNAs were mixed in 4 ml TES, and brought to a density of 1.700 g/ml with CsCl. The solution was centrifuged under the conditions above. Fractions (120 μl) were collected, and used to measure absorbance, refractory index and radioactivity, as described above but with no alkaline treatment.

2.6. *Determinations of the buoyant density and GC content of nuclear and mitochondrial DNAs*

Nuclear and mitochondrial DNAs extracted from a lysate of *C. tropicalis* pK 233 cells were subjected to analytical density gradient centrifugation in a Hitachi, model 282, ultracentrifuge at 45 000 rev./min for 20 h at 25°C as in [12]. The ultraviolet absorption was scanned with a Hitachi absorption scanner. DNA of *Micrococcus luteus* (ρ = 1.731 g/ml) was used as a density marker. The GC content was determined according to the procedure in [13].

3. Results and discussion

Figure 1 shows distribution in the discontinuous sucrose density gradient of activity of each of 3 marker enzymes, protein and alkali-resistant, acid-insoluble radioactivity of the 20 000 × *g* precipitate obtained

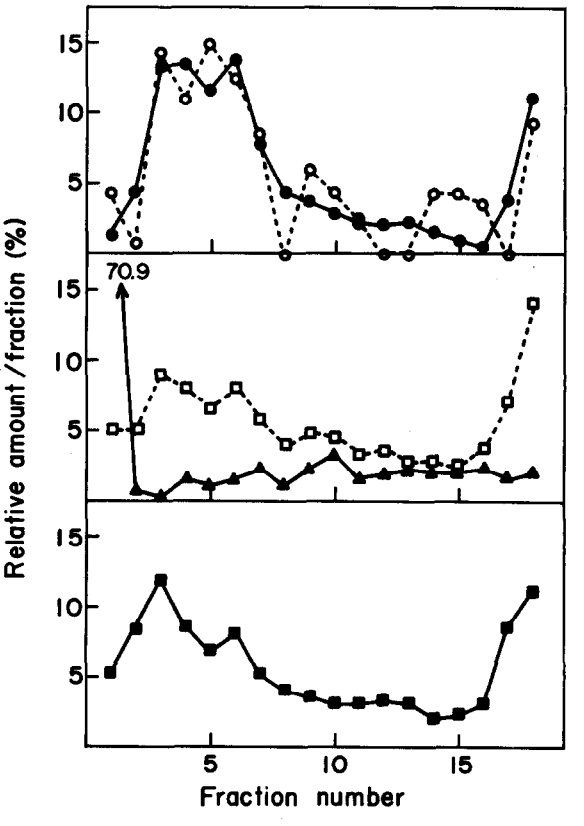

Fig.1. Sucrose density gradient centrifugation profiles of enzyme activities, protein and radioactivity from the 20 000 × *g* precipitate of lysate from *C. tropicalis* pK 233 cells. The ordinate shows the relative enzyme activities, protein and radioactivity recovered from the gradient. (●—●) Catalase; (○- - -○) D-amino acid oxidase; (▲—▲) cytochrome *c* oxidase; (□- - -□) protein; (■—■) radioactivity.

from a lysate of *C. tropicalis* cells grown on normal alkanes medium. Main peaks of the activity of both catalase and D-amino acid oxidase, as marker enzymes of microbodies, were found in fractions 3–6 with most of the protein. Since there was little activity of cytochrome *c* oxidase, a marker of mitochondria, in these fractions, they were concluded to consist mostly of microbodies, only slightly contaminated with mitochondria (less than 1% of the amount applied on the basis of cytochrome *c* oxidase activity). The peak of radioactivity was also in fractions 2–6, suggesting the presence of DNA in the microbody fractions.

The CsCl centrifugation profiles of labelled DNA extracted from microbodies (fractions 3 and 4, fig.1) and unlabelled nuclear and mitochondrial DNAs are shown in fig.2. Two peaks of A_{260} appeared in fractions 23 and 29, representing nuclear and mitochondrial DNAs, respectively. The buoyant densities of these two types of DNAs were determined to be 1.704 and 1.691 g/ml, respectively, by analytical centrifugation, and their GC contents were 44% and 31%, respectively, coinciding well with their buoyant densities.

The bulk of the radioactivity of RNA sedimented to the bottom of the gradient, but a distinct peak of

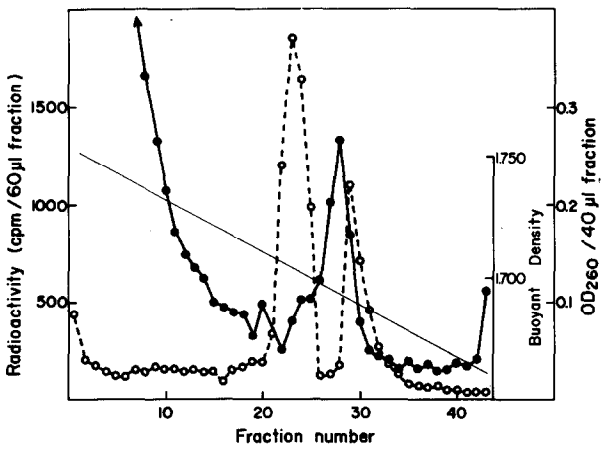

Fig.2. CsCl isopycnic centrifugation of ^{3}H-labelled microbody-associated DNA and unlabelled nuclear and mitochondrial DNAs extracted from *C. tropicalis* pK 233 cells. The labelled DNA was prepared from fractions 3 and 4 of sucrose density gradient shown in fig.1. (●—●) radioactivity; (o- - -o) A_{260}; (——) buoyant density.

labelled DNA appeared between the peaks of nuclear and mitochondrial DNAs. The buoyant density of this labelled DNA was calculated to be 1.693 g/ml by reference to those of the other 2 unlabelled DNAs. A shoulder of radioactivity was detected in the position of nuclear DNA, possibly due to contamination of the microbody fraction with nuclear DNA. About 98% of the total alkali-resistant, acid-insoluble radioactivity applied to the gradient was recovered in fractions 22–31. A closely similar CsCl profile was obtained when labelled DNA from fractions 5 and 6 in fig.1 was analyzed (data not shown).

Thus, 3 classes of DNAs were identified in *C. tropicalis*, one of which appeared to be a new class of DNA associated with microbodies.

Microbody-associated DNA has been found in cellular fractions from *Euglena gracilis* [14] and in a glyoxysome fraction from germinating *Pinus ponderosa* seeds [15]. More intensive studies of this type of DNA were made by Clark-Walker on *Saccharomyces* yeast [16]. He isolated a circular DNA with the same buoyant density as that of nuclear DNA from mitochondrial preparations and postulated that this DNA might be associated with peroxisomes rather than mitochondria of yeast cells. Recently, he has again isolated such a type of DNA, named *o* (omicron) DNA, from cytoplasmic particles in lysates of a petite mutant *Saccharomyces* yeast lacking mitochondrial DNA [17]. This DNA was shown to be closed circular in form and heterodispersed with a length ranging from 1.9–5.8 µm. On the basis of its location in the sucrose gradient, he suggested that it would originate from peroxisomes. A similar type of DNA has been isolated from *Saccharomyces* yeast and, physicochemically and morphologically characterized under the name of 2 µm DNA [18]. However, a 2 µm circular species of DNA is concluded to be located in the nucleus [19].

A novel type of DNA detected in our present study appears to be different from *o*DNA or 2 µm DNA in physicochemical and morphological properties [5], on the one hand, and from nuclear DNA in buoyant density, on the other. It is possible, therefore, that in addition to nuclear and mitochondrial DNAs, *C. tropicalis* cells contain a third type of DNA that is considered to be derived from microbodies. This assumption is supported by autoradiographic

and cytochemical studies for electron microscopy [20].

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