CHARACTERIZATION OF A NEW CLASS OF CIRCULAR DNA MOLECULES IN YEAST.

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This paper describes the isolation of a pure population of covalently closed circular twisted DNA molecules from yeast. These molecules are homogeneous in size, that is consist of monomers of 2.2μ and of multiple length oligomers of n x 2.2μ . While no data rule out the mitochondrial origin of this DNA, its actual intracellular localization remains unknown ; it displays the same buoyant density as the main nuclear DNA and therefore is not the heavy nuclear satellite DNA (δ - DNA described by Moustacchi and Williamson (1966)) ; although circular molecules represent only 1 to 5 % of the total DNA, they can be prepared in sizable and reproducible amounts by a method based on the use of mechanical disruption of yeast cells rather than lysis by snail gut juice.

MATERIAL AND METHODS

The haploid wild type yeast D 243 - 2B - R_1 ad_1 (φ^+ respiratory sufficient) was used. Cultures were grown as previously described (Guerineau et al., 1968a) and harvested during the transition phase. The cells were washed with buffer (0.6 M sorbitol, 0.001 M EDTA, 0.15 % serum albumin, 0.05 M Tris, pH 7.2).

All operations were performed at 0° to 4°C. To each gram (wet weight) of cells, 3 grams of glass beads (400μ diameter, previously washed with the same buffer) were added. The cells were disrupted in a refrigerated vibrogen (Bühler) at maximum speed for 7 minutes. The whole homogenate was separated from the glass beads by washing them with 4 ml of buffer. The pH of the homogenate was adjusted to 7.2 with 0.1 N NaOH. The cell walls and the remaining glass beads were sedimented at 1.5 x Kg for 10 minutes. The supernatant fraction was then centrifuged at 16 x Kg for 10 minutes. The pellet was resuspended in 2 ml of buffer, treated in a Potter homogenizer and centrifuged for 10 minutes at 1.5 x Kg. The pellet was discarded, and the supernatant fraction was again centrifuged for 15 minutes at 16 x Kg. This last pellet is called the " 16 x Kg fraction".

A "16 x Kg fraction", corresponding to 5 g (wet weight) of cells, was resuspended in 1 ml of SSC (0.15 M NaCl, 0.015 M sodium citrate) and lysed with Brij (final concentration 0.5 %). CsCl (1.2 g per ml) was added to the lysate. The mixture was kept in ice for 2 hours, then centrifuged at 20 x Kg for 20 minutes in order to clarify the solution. The DNA was fractionated by equilibrium centrifugation in a CsCl-ethidium bromide (CsCl-EB) gradient. The initial buoyant

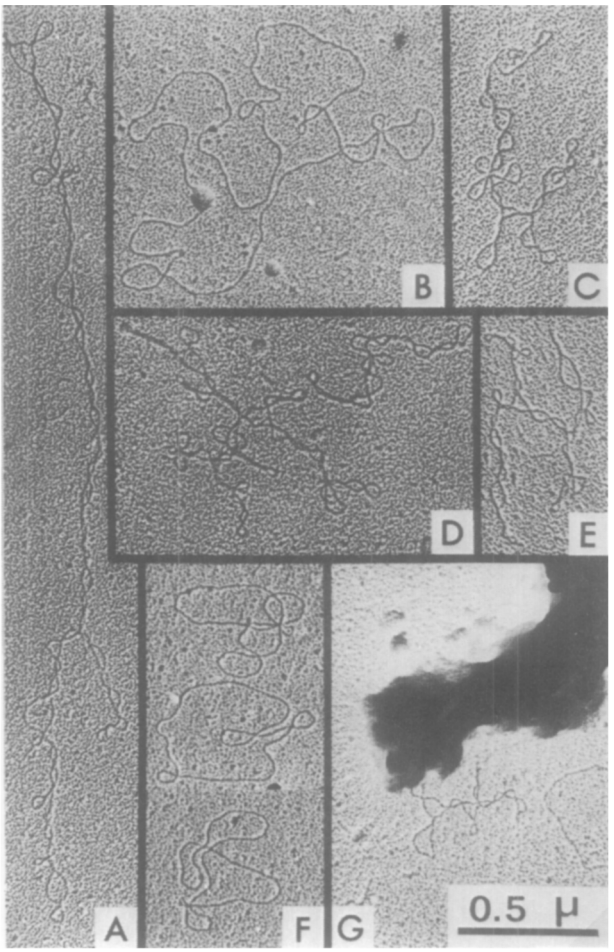

PLATE I

ELECTRON MICROGRAPHS OF YEAST CIRCULAR DNA MOLECULES OF BUOYANT DENSITY f = 1,698 g/ml.
The molecules were recovered from the heavier peak of the CsCl-EB gradient (Fig. 1).
Circles spread without ethidium bromide : A, pentamere of 11 μ ; C, circle of 3 μ ; D, monomere and trimere ; E, dimere.
Circles spread with ethidium bromide : B, trimere ; F, 3 monomeres.
Osmotic shock : G, twisted circle of 2 μ associated with a membraneous fragment.

density was adjusted to 1.580 g cm^{-3}, with a dye concentration of 300µg/ml. Centrifugation was performed in a Spinco rotor SW 50 , or a fixed angle rotor 40, at 35,000 rpm, 15°C, for 60 hours.

After dilution in 1 ml of 0.05 M sodium citrate, the fluorescence of each fraction (0.1 ml) was measured as previously described (Le Pecq and Paoletti, 1966). The fractions containing the circular DNA were pooled and the dye was removed by dialyzing against SSC for 2 days at 4°C. A dialysis bag containing 5 to 10 mg of salmon sperm DNA was then added to the dialysis bath to remove the dye completely. Electron microscopy was done according to the spreading technique of Kleinschmidt and Zahn (1959). Measurements of the contour length of circles were done in presence of a proper concentration of EB. This releases the twists and make determinations more accurate (Plate I).

RESULTS

When the DNA, extracted from a "16 x Kg fraction", was centrifuged to equilibrium in a CsCl-EB gradient, the fractionation pattern shown on Fig. 1 was obtained. Two bands were present : the lighter one consisted of linear and nicked circular DNA (Guerineau et al., 1968b) while the heavier band consisted of closed circular DNA as demonstrated below (plate I). The latter band represents

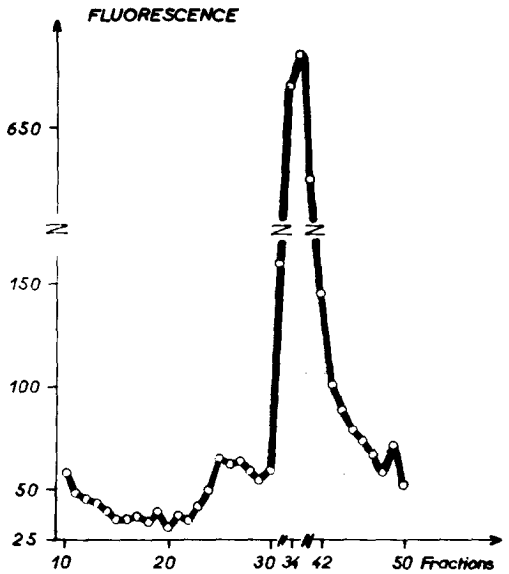

FIG. 1

ETHIDIUM BROMIDE CsCl GRADIENT CENTRIFUGATION OF DNA EXTRACTED FROM THE 16 Kg FRACTION IN ROTOR SW 40.

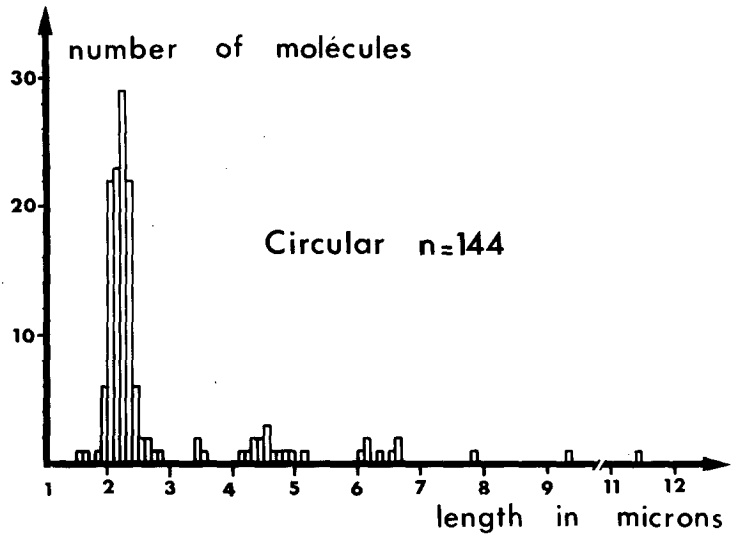

FIG. 2
LENGTH DISTRIBUTION HISTOGRAM SHOWING FREQUENCIES OF THE CIRCLES.

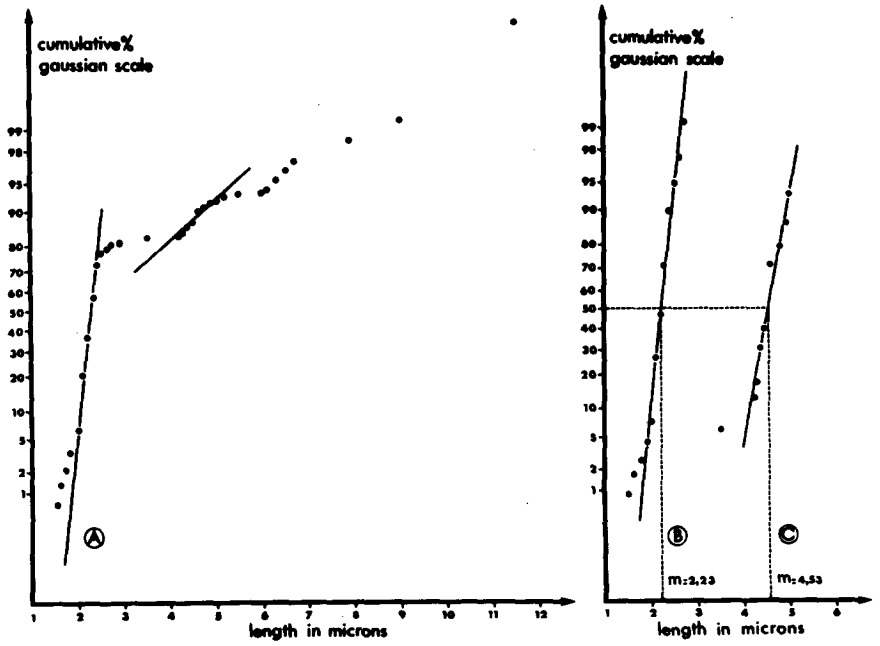

FIG. 3
CUMULATIVE DISTRIBUTION FUNCTION ANALYSIS OF THE LENGTH OF THE CIRCLES. A- of all the circles, B- of the circles from 1.5 to 2.87 μ C- of the circles from 3.52 to 5.13 μ

about 4 % of the total "16 x Kg fraction" DNA. Better than 98 % of the molecules in the heavy band are circles which do not display a catenated structure. The histogram of the length distribution of these DNA molecules is shown on Fig.2, and the analysis of the cumulative distribution is shown on Fig.3. The molecules are distributed in three classes of length : 2.2μ, 4.5μ, and 6.3μ ; that is, consist of monomers, dimers and trimers. Most of the circles display a supercoiled structure. The number of cross-overs for the highly twisted circles is of about 5.5 per μ for monomers, dimers as well as trimers.

In a different preparation, the circular molecules released from a "16 x Kg fraction" by osmotic shock (plate I) were found to be mostly free, although some remained attached to membrane structures. These molecules showed 5 to 6 cross-overs per μ and were thus indistinguishable from the covalently closed circular molecules obtained from the CsCl-EB gradient.

The buoyant density of each of the two bands obtained in the CsCl-EB centrifugation was identical and equal to 1.698 g/ml (Fig.4). Thus the circular DNA presently described has the same buoyant density as nuclear DNA.

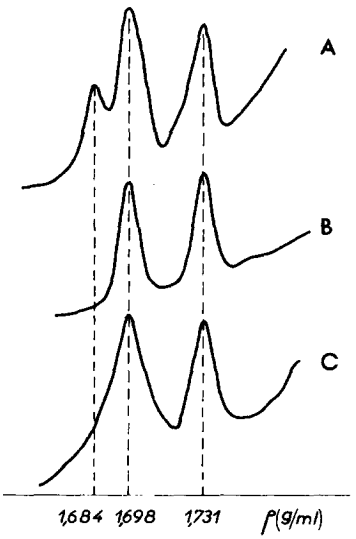

FIG.4

CsCl DENSITY GRADIENT ANALYTICAL ULTRACENTRIFUGATION OF DNA BEFORE AND AFTER FRACTIONATION IN AN ETHIDIUM BROMIDE CsCl GRADIENT

A, Unfractionated DNA from 16 Kg fraction. B, Fractions 23 to 28 (heavier peak) of EB-CsCl gradient (see Fig.1) after removal of the dye. C, Fraction 31 (lighter peak) of EB-CsCl gradient (see Fig.1) after removal of the dye. Marker DNA (ρ = 1.731) from Micrococcus.

The patterns obtained after CsCl-EB centrifugation were essentially identical whether the DNA was extracted from cultures in logarithmic, transition or stationary growth phases. Furthermore, identical results were obtained with a diploid strain.

DISCUSSION

This work reports the isolation from yeast of highly twisted closed circular DNA molecules displaying the same buoyant density as the main nuclear DNA

These molecules have been partially described, through the use of electron microscopy by Sinclair et al. (1967) (modes around 1.95μ), by Avers et al. (1968), Billheimer and Avers (1969) (modes around 2.0 to 2.5 and 5.6 to 6.0μ) and by Hollenberg et al. (1970) (modes of 2μ). The work by Shapiro et al. (1968) also suggested the existence of this type of DNA in yeast (see Fig. 11 in their paper) although no circles with a length of 2μ were shown in their histogram.

None of these authors isolated this DNA in sizable amount ; the isolation has presently been achieved through the use of a technique involving a crucial intermediary step , the preparation of a fraction enriched in membrane and mitochondrial fragments, followed by lysis directly in the mixture used for the CsCl-EB centrifugation (SSC-Brij-CsCl-EB).

The buoyant density of the circles was determined in the analytical ultracentrifuge and compared with the density of bulk nuclear DNA which had been submitted to a similar treatment (Fig. 3) : no difference was found between the DNAs ; this result rules out the possibility that these circles consist of a nuclear satellite DNA (ρ = 1,705 mg/ml) as might have been thought according to the report that circles could be heavier (1 to 2 mg/ml) than bulk nuclear DNA (Hollenberg et al., 1970).

The histogram (Fig. 2) shows that the population of circles consists of monomers units (2.2μ) and of several classes of oligomers with a length which is a multiple (up to 3) of the monomer length. Similar oligomeric patterns have been previously observed in the mitochondrial DNA of eukaryotic cells, human leukemic cells (Clayton and Vinograd, 1969), other malignant cells (Riou et al. , personal communication), mouse fibroblasts (L cells) (Nass, 1969), and in kinetoplastic DNA from ethidium bromide treated Trypanosoma (Riou and Delain, 1969).

Our data do not provide any evidence as to the actual intracellular localization of the circles. In experiments not reported here, we observed that, when our "16 x Kg fraction" was centrifuged in a sucrose density gradient, circles

were distributed in several fractions, some of which did not contain mitochondrial DNA.

Sinclair et al. (1967) observed the presence of circles of about 1.95μ average length (unreported buoyant density) in preparations of total yeast DNA and of partially purified mitochondrial DNA. These authors reported that the circles were lost after further purification of the mitochondrial DNA in CsCl. This result argues in favor of the nuclear origin of circles with a buoyant density equal to that of nuclear DNA. Billheimer and Avers (1969) came to the opposite conclusion : they were able to enrich from 5.3 % to 30 % the proportion of circles of nuclear DNA buoyant density (1.5-2.5μ), by eliminating through DNAse action on mitochondria any contaminating DNA of nuclear origin.

Whatever their localization and origin, the circles of density ρ=1,698 g/ml seem to be closed in vivo and tightly associated with membrane structures (plate I). It is not yet certain, however, whether this association reflects a physiological situation or an extraction artefact.

So far, two other populations of circles have been described in yeast : one consists of 25μ twisted circles which could not be extracted and therefore their buoyant density could not have been determined (Hollenberg et al, 1970); the other population consists mainly of open circles very heterogenous in size (up to 8μ) with the buoyant density of mitochondrial DNA (Sinclair et al., 1967 ; Avers et al.,1968 ; Shapiro et al., 1968 ; Guerineau et al., 1968b ; Billheimer and Avers, 1969). The latter population cannot be purified from the bulk mitochondrial DNA. The circles described in this paper differ from the two previous populations in that they are homogeneous in size, and have the same buoyant density as nuclear DNA. Moreover, the small heterogenous circles of mitochondrial origin are absent during the stationary phase, while the circles described here can be extracted with the usual yield from stationary phase cultures.

The physiological role of these new circles is unknown. Although a viral origin cannot be ruled out, it seems unlikely that similar viral DNA could be found in different strains, especially since no yeast-specific virus has, as yet, been described.

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