LARGE SCALE ISOLATION OF RIBOSOMAL DNA FROM GIANT SURFACE CULTURES OF *PHYSARUM POLYCEPHALUM*

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Received 10 September 1979

1. Introduction

In recent years, the ribosomal DNA (rDNA) of *Physarum polycephalum* has become an attractive system to study genome organization [1–4]. The linear, extrachromosomal rDNA can be isolated from nucleoli either as deproteinized DNA or as a transcriptionally active nucleoprotein complex [5]. Recently, a highly phosphorylated non-histone protein has been characterized which binds to specific sites on the rDNA and stimulates transcription of rRNA [6]. These observations illustrate the attractiveness of this system, which stems from the fact that a defined set of genes can be isolated in its native form, i.e., with structural and regulatory proteins still present. Such a relatively simple system will hopefully facilitate the biochemical investigations of genetic regulation in eucaryotes.

However, studies of this system have been hampered by the fact that large scale isolation of nucleoli and rDNA is tedious and results in low recoveries of rDNA or in impure preparations of nucleoli. Here we report a new method for growing *Physarum polycephalum* in very large surface cultures and an improved procedure for rapidly isolating nucleoli as well as rDNA.

2. Methods and results

Large surface cultures were prepared in the following way: plastic Bio-assay dishes (235 X 235 X 8 mm; Nunc Products, 8 Algade, DK-4000 Roskilde) were fitted with square polystyrene rods (5 X 5 mm, varying length) to form a supporting grid for the filter papers (fig.1). Soaking the polystyrene rods in acetone makes them stick well to the plastic of the dish. The closed dishes were sterilized with a germicidal ultraviolet lamp at 0.6 W/cm^2 from the top and the bottom, each time for 2 h. Filter paper (Schleicher and Schuell, CH-8714 Feldbach, type 576, 24 cm diam.) was cut to fit in the dishes and sterilized at 200°C for 15 min. *Physarum polycephalum* (strain M_3c VIII) was grown in shake flask cultures in N+C medium [7].

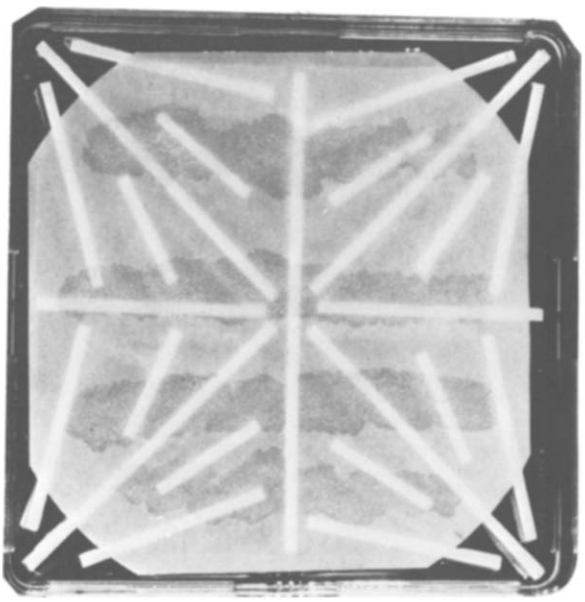

Fig.1. Arrangement of the polystyrene rods and the stripes of the plasmodia. Details are explained in the text.

For the preparation of surface cultures, microplasmodia were harvested in mid-exponential growth by centrifugation at 50 X *g* for 2 min in a MSE Mistral 4L centrifuge. The pelleted microplasmodia were gently suspended with 2 vol. of sterile water, and 5 ml aliquots of the suspension were spread in 4 stripes on the filter papers as shown in fig.1, using a wide mouth 5 ml pipette. Cultures were then starved for 90 min in the dark [8]. After starvation they were fed with ~200 ml N + C medium containing 50 μg/ml streptomycin, 100 unit penicillin/ml and 1 μCi [^{3}H]thymidine/ml. The plates were then incubated at 27°C in the dark.

To isolate nucleoli macroplasmodia were harvested 18–20 h after feeding. The cultures were briefly washed by dipping the filter papers into 50 mM Tris–base. Two stripes (½ culture) were scraped into 200 ml ice-cold solution N (250 mM sucrose, 1 mM $CaCl_2$, 0.5% Triton X-100, 10 mM Tris–HCl (pH 7.5)) [9] and homogenized for 1 min at 120 V in a Waring Blendor using the 1 l cup. At 45 s 2 ml 1 M $CaCl_2$ solution was added. Homogenates of 12 large surface cultures were combined, filtered through 2 layers of milk filters (FLAWA A.G., CH-9230 Flawil SG) and centrifuged in 4 1.25 l cups at 1875 X *g* for 20 min in an MSE Mistral 4L. The 4 pellets were suspended to 200 ml total vol. with solution P (250 mM sucrose, 10 mM $CaCl_2$, 10 mM Tris–HCl (pH 7.5)) and 60 ml Percoll (Pharmacia Fine Chemicals, Uppsala) were added. The suspension was carefully mixed, distributed into two 250 ml plastic Sorvall cups and centrifuged for 15 min at 2350 X *g* in a Sorvall HS-4 rotor. During centrifugation the nucleoli are pelleted, while the slime forms a layer on the surface. The nucleolar pellets were carefully suspended in 45 ml solution P and the nucleoli were repelleted by centrifugation at 1500 X *g* in an MSE Mistral 4L. All the above manipulations were at 4°C.

To isolate rDNA, nucleoli from 24 large surface cultures were suspended by homogenization in 18 ml total vol. solution P in a Potter-Elvehjem homogenizer. The resulting suspension was equally distributed into 6 cellulose nitrate tubes (Beckman no. 305050) and incubated at 65°C for 10 min [10]. After lowering to 45°C, 300 μl proteinase K (Boehringer, Mannheim; 10 mg/ml) and 300 μl sodium dodecyl sulfate (20%, w/v) were added. After 2 h incubation, 4.5 g CsCl were added to each tube and carefully dissolved. The solution was then spun at 40 000 rev./min 20°C, in a Beckman SW 50.1 rotor for 30 min. The resulting scum, containing most of the proteins, was removed and the refractive index of the solution was adjusted to 1.450.

The tubes were then centrifuged at 36 000 rev./min for 17 h in a Sorvall TV 865 rotor at 20°C in a Sorvall OTD-50 ultracentrifuge. Fractions (0.2 ml) were collected through the bottom of the tubes and 15 μl aliquots were processed for scintillation counting. Corresponding fractions from all gradients were pooled as indicated in fig.2, dialyzed for 3 h at room temperature against 5 l 10 mM Tris–HCl (pH 8.0), 1 mM EDTA. To check the purity of the

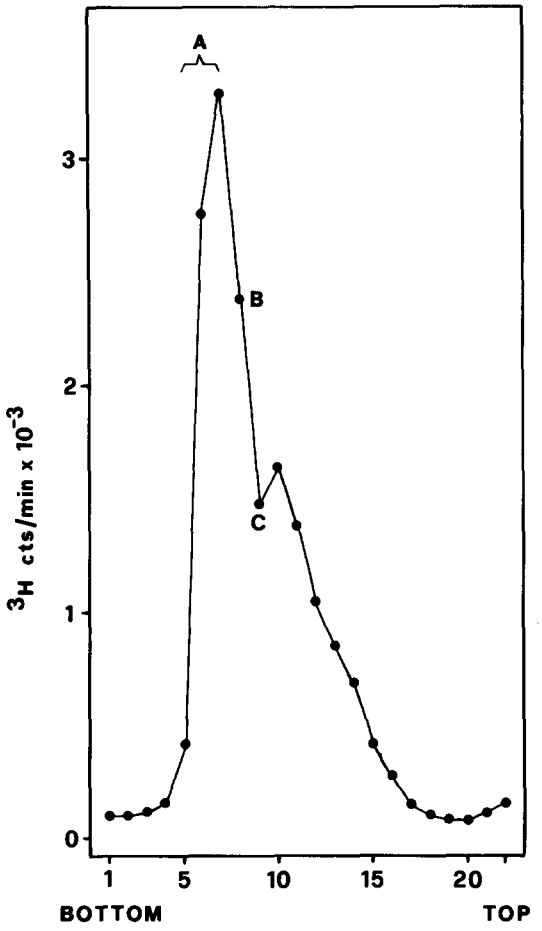

Fig.2. CsCl density gradient of isolated DNA from nucleoli. The fractions designated (A,B,C) were processed individually as in the text.

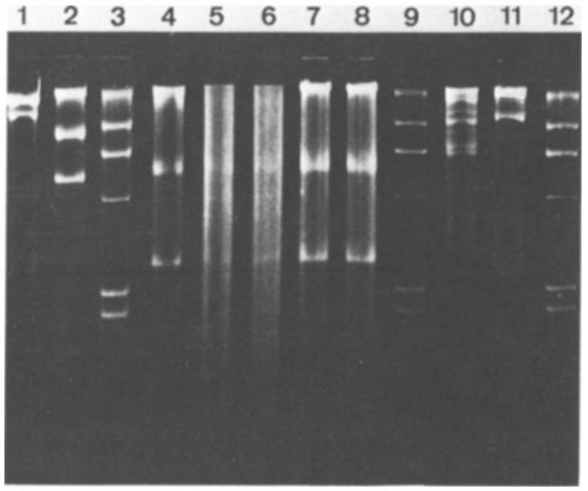

Fig.3. Demonstration of purity of the isolated rDNA. Restriction enzyme analysis of fractions A, B, C (fig.2) on a 1% agarose slab gel. Aliquots of pool A (pure rDNA) were digested with *Bam* HI (slot 1), *Hin*dIII (slot 2), *Eco* RI (slot 4), *Hpa* I (slot 10) and *Pst* I (slot 11); *Hpa* I and *Pst* 1 digests are partial. Slots 7,8: *Eco* RI restricted DNA from pool B; the fraction is slightly contaminated with chromosomal DNA. Slots 5,6: *Eco* RI restricted DNA from pool C: this fraction contains mainly chromosomal DNA. Slots 3, 9,12: *Hin*dIII digested λ DNA as marker.

isolated rDNA, aliquots of the pools were digested with restriction endonucleases and analyzed on agarose slab gels (fig.3).

3. Discussion

The above methodology allows the efficient purification of considerable amounts of rDNA within a reasonable amount of time. The rDNA obtained with this procedure can be used for restriction endonuclease analysis without further purification (fig.3). In our hands, 100–150 μg rDNA can readily be prepared in 1 batch from 24 surface cultures (= 12–15 mg total DNA). Since rDNA comprises only ~2% of the total nuclear DNA its purity is crucially dependent on the purity of the nucleolar preparation. This, in turn, is critically determined by the Ca^{2+} concentration in the homogenization buffer. Too low concentrations of Ca^{2+} lead to low recovery, whereas slightly too high concentrations greatly increase the proportion of contaminating nuclei. However, contaminating chromosomal DNA (ρ = 1.702 g/cm^2) can be efficiently separated from rDNA (ρ = 1.714 g/cm^2) in a CsCl density gradient (fig.2,3). If necessary, rDNA can still be further purified on a second CsCl gradient.

The easy availability of rDNA from *Physarum polycephalum* will hopefully facilitate further investigations on its structure and function. Since our observations indicate that these large surface cultures retain a high degree of synchrony, they might also be of interest for other experimental applications in the study of this organism.

Acknowledgement

This work was supported by grant no. 3.312–0.78 from the Swiss National Science Foundation.

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