

ISOLATION OF NUCLEI FROM YEAST

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ABSTRACT

A method for isolation of nuclei from *Saccharomyces cerevisiae* in high yield is described. The DNA/protein ratio of the isolated nuclei is 10 times higher than that of whole cells. Examination of these nuclei in phase and electron microscopes has shown them to be round bodies having a double membrane, microtubules, and a dark crescent at one end. The optimum conditions for extraction and resolution of histones of these nuclei on acrylamide gels have been investigated. The nuclei have an active RNA polymerase (E.C. 2.7.7.6) and are able to synthesize RNA in vitro. They are also readily stainable with Giemsa's, Feulgen's, and acridine orange methods.

INTRODUCTION

It has become increasingly clear that to understand the nature of the replication and expression of the genome in yeast, it is essential to have a method for the preparation of nuclei at different stages of growth. From light microscope (13, 15) and electron microscope (13, 18, 19, 22) studies, a well-defined nucleus resembling that of higher organisms has been identified in yeast. Two recent reports on the partial fractionation of nuclei from *Saccharomyces carlsbergensis* (23) and *Schizosaccharomyces pombe* (6) have served as the basis of a method for preparing high yields of pure nuclei from the wild-type diploid *S. cerevisiae* Y 55. Some properties of these purified nuclei have been studied and are described.

METHODS

Growth Conditions and Preparation of Homogenates

S. cerevisiae Y 55 was subcultured at 30°C for 24 hr on YEP agar slants (1% yeast extract, 2% bacto-peptone, 2% glucose, and 2% agar). Cells from the slant were suspended in distilled water and inoculated into YEP medium (500 ml) and grown with continuous shaking at 30°C to an optical density (660 nm) of between 2 and 10. The culture was

harvested by centrifugation at 2°–4°C for 5 min at 3300 g. The cells were washed once by suspending the pellet in 100 vol of distilled water and recentrifuging. The cell pellet was then suspended in 5 vol of homogenizing medium (HM) (1 M sorbitol, 20% glycerol, and 5% polyvinylpyrrolidone [PVP obtained from Sigma Chemical Co., St. Louis, Mo., average molecular weight 40,000]). The suspension was then poured into a French press with its outlet tube replaced by a stainless steel tube about 2 cm long and with an internal diameter of 0.95 mm. The diameter of the tube was critical for maximum yield of intact nuclei to be obtained. The plunger was inserted and the press was placed into an equilibrated mixture of dry ice and ethanol and allowed to cool for about 7 min. The frozen mixture was then forced through the outlet at a pressure of 20,000 psi. When used in this way, the French press has properties similar to those of an Eaton press (7) which was also used for small-scale preparations. Leaving the harvested cell pellet in the freezer overnight, or leaving the cells (in YEP or HM) in the cold at 2°–6°C for 6 hr, leads to a marked reduction in the number of intact nuclei. Thus, it is important that harvested cells be processed immediately.

Isolation of Nuclei from the Homogenate

All of the following centrifugations were carried out at 0°–4°C in plastic centrifuge tubes. The dis-

rupted cells from the French press were thawed and centrifuged at 5500 *g* for 5 min. The supernatant was carefully withdrawn with a plastic pipette to avoid inclusion of any loosely packed layer. The supernatant was centrifuged at 10,600 *g* for 10 min to sediment the nuclei. Most of the mitochondria remain in the supernatant. The pellet was a crude preparation of nuclei which appeared as round bodies in the phase-contrast microscope. These nuclei could be purified further by suspension in 10 vol of HM and layering onto stepwise gradients consisting of 8 ml of 2.6 M sorbitol, 7 ml of 3.2 M sorbitol, all containing 20% glycerol, and 5% PVP-40. The gradients were centrifuged at 13,000 *g* (R_{average} for 20 min in an SW 25.3 rotor in a Beckman Model L ultracentrifuge (Beckman Instruments, Inc., Fullerton, Calif.). Mitochondria remain on top of the gradient, nuclei sediment to the top of the 3.2 M sorbitol layer, and the pellet consists of unbroken cells and debris. The nuclear layer was carefully withdrawn with a plastic pipette, diluted 1:4 with 5% PVP 40-20% glycerol, and centrifuged at 8000 rpm for 12 min. The pellet consists mainly of nuclei.

Some of the nuclei (about 10%) still have adhering cytoplasmic material. Attempts to remove it with 0.5-2% citric acid, 0.01-0.03% Triton X 100, or gentle homogenization by hand were unsuccessful.

Phase Microscopy and Counting of Nuclei

The Zeiss phase-contrast microscope was used. Nuclei were counted in Petroff-Hausser and Hebler's counting chamber.

Estimation of DNA, RNA, and Protein

The pellet of whole cells or nuclei was washed once with 10 vol of 70% ethanol and then with 10 vol of 0.1% perchloric acid (PCA) in 70% ethanol. The washed pellets were then extracted twice with 10 vol of 3:1 alcohol-ether at 65°C for 3 min and centrifuged. The sediment was washed with 10 vol of 0.2 M PCA, 10 vol of 1 M PCA, and was finally extracted twice with 2 vol of 1 M PCA for 30 min at 70°C. The combined supernatants from the two PCA extractions (70°C) were used for measurement of DNA and RNA, and the sediment was solubilized in 5 vol of 1 N NaOH at 60°C for 1 hr for protein determination. DNA was assayed colorimetrically with a diphenylamine reagent (4), with calf thymus DNA as a standard. RNA was estimated by the orcinol method (5), with yeast RNA as a standard. Colorimetric determination of protein content was performed with the Folin phenol reagent as described by Lowry et al. (12), with bovine serum albumin as a standard.

Staining with Giemsa's, Feulgen's, and Acridine Orange Methods

The nuclear suspension (in HM) was thinly smeared on microscope slides, dried at 37°C, and then fixed for 30 min in 3:1 alcohol-acetic acid. The fixing agents were removed by placing the slides into successively lower ethanol concentrations (95%, 75%, 50%, and 35%) for 2-3 min and finally into water.

For acridine orange staining, the slides were treated as described by Nash and Plaut (20) and examined with a fluorescence microscope.

For Feulgen staining, a hydrolysis was carried out as described by Itikawa and Ogura (9), followed by the staining and destaining procedures as described by Barka and Anderson (3).

For Giemsa staining, the slides were treated as described by Robinow (21).

Extraction and Acrylamide Gel Electrophoresis of Histones

The extraction of histones was carried out as described by Lee and Scherbaum (10). The nuclei were washed once with 0.14 M NaCl and extracted overnight at 6°C with 3 M NaCl, with gentle, continuous stirring. The suspension was then centrifuged at 40,000 *g* for 2 hr with a Type 50 Ti rotor in a Beckman Model L ultracentrifuge. The pH of the supernatant was carefully brought to 1.8 by the addition of 1 N HCl, and the resulting precipitate was removed by centrifuging at 2000 *g* for 30 min. The supernatant, consisting of acid-soluble nuclear proteins, was dialyzed against 1 mM HCl at 5°C for 24 hr. After lyophilization, the proteins were dissolved in 6 M urea and centrifuged at 10,000 *g* for 10 min. The resulting clear supernatant contains purified histones.

This purified histone preparation was fractionated by electrophoresis on polyacrylamide gels (14). For each gel tube of 4 mm (ID) × 90 mm (L), 3 ma current was applied and electrophoresis was carried out in the cold at 4°-6°C for 2 hr. The gels were removed from the tubes by rimming with a hypodermic needle containing 0.1% Triton, were stained with amido black (7% in acetic acid) for 1 hr, and the excess stain was removed electrophoretically by using 3 ma per tube and 7% acetic acid in both reservoirs.

Electron Microscopy of Isolated Nuclei

The isolated nuclei were fixed for 30 min in a solution containing 2% glutaraldehyde, 0.25 M sucrose and 50 mM potassium cacodylate, pH 7.5. The samples were washed once in the above solution without glutaraldehyde and stained for 60 min at 4°C in a solution containing 2% osmium tetroxide, 0.25 M sucrose, and 50 mM potassium cacodylate,

pH 7.5. Following this procedure, the samples were washed once in the above solution without the osmium tetroxide, stained for 15 min at 4°C with a uranyl acetate solution in 25% ethanol v/v, dehydrated in graded solutions of ethanol (50, 80, 95, and 100%), and washed two times with absolute propylene oxide and embedded in Epon. Light gold or silver sections were cut with a diamond knife on a Sorvall MT-2 ultramicrotome (Ivan Sorvall, Inc., Norwalk, Conn.), mounted on 400 mesh copper grids, "post" stained with lead citrate, and examined in a Philips 300 electron microscope operated at either 40 or 60 kv.

RNA Polymerase Assay

The reaction mixture (0.2 ml) contained 0.1 M Tris-HCl buffer of pH 8.0, 15 mM β -mercaptoethanol, 10 mM MgCl₂, 10 mM phosphoenolpyruvate, 7.5 μ g pyruvate kinase, 0.5 mM of unlabeled guanine triphosphate (GTP), cytidine triphosphate (CTP), and adenine triphosphate (ATP), 0.25 mM of unlabeled uridine triphosphate (UTP), and 0.025 mM (14.1 Ci/mmol) of UTP-H³. After incubation at 37°C for 10 min, the reaction was terminated by the addition of 3 ml of 5% trichloroacetic acid (TCA) containing 0.2 M sodium pyrophosphate. The precipitate was collected on a millipore filter (Millipore Corp., Bedford, Mass.), washed twice with cold 1% TCA, dried, and the radioactivity was measured in a Tri-Carb scintillation spectrometer (Packard Instrument Co., Downers Grove, Ill.) by using 4 g/liter 2,5 diphenyloxazole (PPO) and 50 mg/liter, 1,4 bis-[2-(5-phenyloxazolyl)]-benzene (POPOP) in toluene. 1 unit of enzyme is defined as that amount of enzyme protein which catalyzes the incorporation of 1 picomole of labeled UTP into acid-insoluble material at 37°C in 10 min.

RESULTS

Phase Microscopy

Under the phase microscope, the nuclei appear as round, clearly outlined, homogeneously brown bodies with a dark crescent at one end, and they are variable in size. For technical reasons it was not possible to obtain a uniform focal plane for all of the nuclei present in any field. Some broken nuclei were always observed in our preparations.

Chemical Composition of Nuclei

As can be seen from Table I, the DNA/protein ratio is at least 10-fold higher in isolated nuclei than in whole cells. The proportions of DNA/RNA per protein are 1:2.5:16 in nuclei, in contrast to 1:81:187 in whole cells. The proportions of DNA/

TABLE I
Chemical Composition of Isolated Nuclei as Compared to Whole Cells

	pg/nucleus	pg/cell
DNA	0.046	0.048*
RNA	0.121	3.93
Protein	0.717	9.05

* This includes 5% mitochondrial DNA.

The extraction and estimations of these macromolecules, and the counting of cells and nuclei were carried out as described in the text. Each number is the average of three experiments.

RNA, DNA/protein, and RNA/protein in our nuclear preparation from *S. cerevisiae* are much higher than those reported from *S. carlsbergensis* (23) or *Schizosaccharomyces pombe* (6). However, these values are very much lower when compared to those for nuclei isolated from higher organisms (2). From the data in Table I, the DNA content is 0.046 pg per nucleus and 0.048 pg per cell. Since about 5% of the cellular DNA of yeast *S. cerevisiae* grown under conditions described is localized in mitochondria, the nuclear DNA value would represent about 95% of total cellular DNA, which is in good agreement with the above findings. Leakage of DNA from unbroken nuclei and absorption of DNA in nuclei cannot be excluded.

On the basis of DNA recovery, the over-all yield of isolated nuclei is about 50% (Table II). Of the remainder, 20% of the nuclei are in cells not disrupted, and about 30% of the nuclei are lost during isolation.

Stained Nuclei

Giemsa's- and Feulgen-stained nuclei appear as pink, round bodies. In the former, the stain was more or less uniform, while in the latter there was a more densely stained portion. Duffus also observed that nuclei isolated from *Schizosaccharomyces pombe* (6) stained readily with Giemsa's but could not be stained with Feulgen's. This may be due to low DNA content in this nuclear preparation. After staining with acridin orange, nuclei appear as greenish-yellow, round bodies. Cytoplasmic contamination, about 10-15%, is seen as fluorescent material attached to nucleus.

Histones

The purpose of the 0.14 M NaCl washes was to remove contaminating nonhistone proteins (10).

TABLE II
Yield of DNA during Purification

Fraction	DNA	Recovery
	μg	%
Homogenate	9878	100
Supernatant (S_1)	7103	
Nuclear pellet (P_2)	6305	
First gradient on 2.5-4.0 M	4932	
Repeat gradient on 2.5-4.0 M	4617	46.7

The counting of nuclei and the DNA estimation were carried out as described in the text. S_1 refers to the supernatant obtained after the first centrifugation, and P_2 refers to the pellet obtained after the centrifugation of S_1 . For details see the text.

Starting from 10 mg of nuclear protein, it was possible to obtain about 2.31 mg of histones. Thus, about 23% of the nuclear proteins of yeast appear to be histones, whereas in rat liver one-third of the nuclear proteins are histones (2, 24).

Purified histones could be fractionated into at least eight distinct fractions by acrylamide gel electrophoresis (Fig. 1 a). The relative proportions of various histones can be ascertained from the densitometric scanning of the electrophoretic pattern (Fig. 1 b).

Electron Microscopy

The electron micrographs revealed that most of the nuclei were morphologically intact, are about 2μ in diameter, and are surrounded by double membranes. Aside from having a more condensed nucleoplasm, these fixed, isolated nuclei are very similar to those seen in sections of intact yeast cells (13). Many nuclei show bundles of microtubules of varying length (Fig. 2 b). In Fig. 2 a, a cross-section of a bundle of microtubules (30 in number) is visible.

RNA Polymerase

Isolated nuclei are able to incorporate labeled UTP- ^3H into acid-insoluble product (Table III). When either the extract or one of the substrates is omitted, there is negligible or no incorporation. Further, the product formed has been identified as a heteropolymer of polynucleotides, by employing double-labeling experiments with two labeled (^{14}C , ^3H) ribonucleotides or by omitting one of the bases. Considering the losses during nuclear

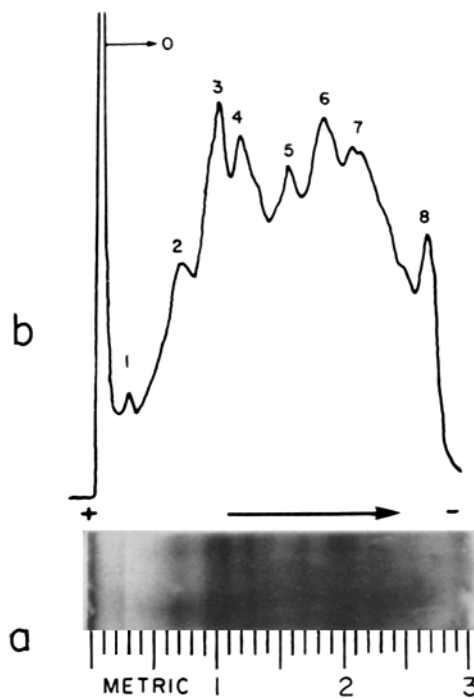


FIGURE 1 Acrylamide gel electrophoretogram of histones isolated from yeast nuclei. (a) shows resolution on gel, and (b) shows the densitometric tracing: + and - indicate the positive and negative electrodes; the arrow points in the direction of migration. For isolation of histones, nuclei were washed with 0.16 M NaCl. The deoxyribonucleoprotein was extracted in 2 M NaCl, and the histones were extracted by 0.25 N HCl. Electrophoresis was carried out for 2 hr at a constant current of 3 ma/tube of 0.6 cm \times 90 mm, using β -alanine buffer of pH 4.3. The removal of tubes from gels, staining in amido black, and destaining were carried out as described in the text.

isolation, the results in Table III indicate that over 90% of the cellular RNA polymerase activity is located in the nucleus.

DISCUSSION

Isolated nuclei are extremely fragile and are sensitive to mechanical pressure and changes in the medium. Thus, a successful isolation procedure requires that mild methods be used for cell rupture and that the isolation conditions protect the integrity of these organelles.

Of the several methods attempted for disrupting yeast cells while keeping the nuclei intact, a modified French press gave the best results. The

optimum period of cooling in dry ice-ethanol was between 7 and 11 min. If the sample was frozen too hard in the press, then the yield of nuclei was considerably reduced. A similar effect of excessive

freezing on the isolation of nuclei of *S. pombe* has been observed by Duffus (6).

The presence of PVP-40 throughout the isolation procedure was essential to maintain the

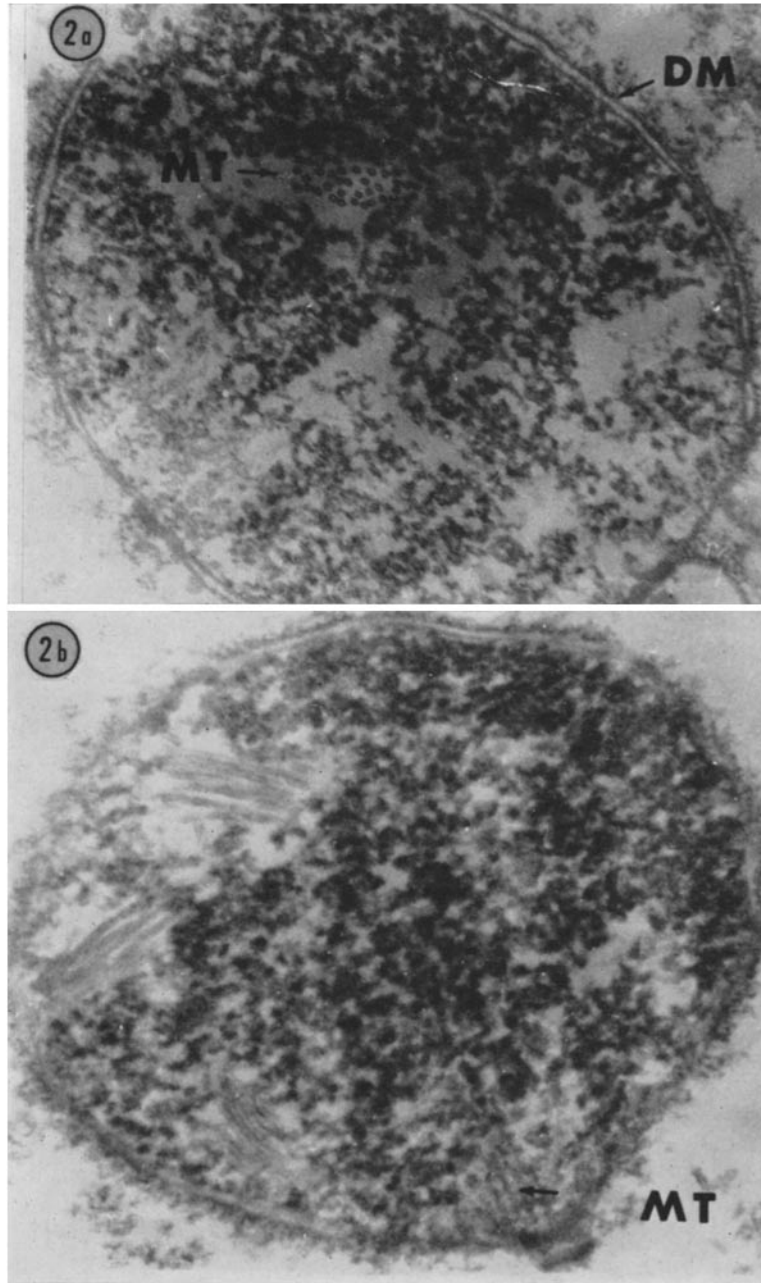


FIGURE 2 Electron micrographs of sections through a pellet of yeast nuclei. For preparation of the nuclei, see the text. (a) and (b) are thin sections of nucleus showing double membrane (DM) and microtubules (MT) in vertical and longitudinal sections, respectively. $\times 60,000$.

TABLE III
RNA Polymerase Activity Distribution

	Enzyme activity	Protein	Specific activity
	units	mg	units/mg protein
Whole homogenate	41.0	1600	0.025
Nuclear preparation	18.0	331	0.056

One unit of enzyme activity is defined as that amount of enzyme protein which catalyzes the incorporation of 1 pmole of labeled UTP into an acid-insoluble product at 37°C in 10 min.

morphological integrity of the nuclei and, in the later stages of purification, to prevent clumping of mitochondria. In the absence of PVP-40, there was a marked reduction in the number of nuclei when the disrupted cells were examined with the phase microscope. Above 8% PVP-40, the nuclei shrink into smaller and darker spheres. Such preservation of nuclear morphology by PVP-40 has also been observed for nuclei isolated from *Tetrahymena* (10) and *S. carlsbergensis* (23). The addition of Ca^{++} is not necessary during homogenization, which would suggest that enough bivalent cations were present in the homogenate, since the inclusion of 3 mM Ca^{++} in the homogenizing medium has been observed to be very desirable for the isolation of nuclei in tissues of higher organisms (1, 2). When yeast cells were broken in HM without glycerol and diluted immediately with an equal volume of HM with 40% glycerol, no nuclei were found in the homogenate. Lovelock (11) has reported a similar protective action for glycerol, which protects biological materials against damage caused by freezing and thawing. The optimum sorbitol concentration used was from 1 to 2.5 M. The HM used has a pH of 4; the use of HM at higher pH increases the number of broken nuclei even though it affords slightly better cell breakage. This observation is in accordance with the observations of Mirsky and Ris (16) on the effect of pH on the isolation of nuclei.

An electron micrograph of isolated nuclei of *S. carlsbergensis* has very recently been described by Molenaar et al. (17). These authors and others (12) have concluded that DNA is complexed with histones in nuclei in the form of 100-A thick

strands containing RNA in electron-opaque spots. They suggested that the dark crescent of the nucleus consists mainly of RNA and protein and may be equivalent to the nucleolus of higher organisms.

The availability of isolated nuclei has made possible an examination of the number and kind of histones in yeast. Tonino and Rozijn (25) isolated the histones from chromatin of baker's yeast by extraction with 0.25 N HCl and fractionated them on carboxyl methyl cellulose columns into two arginine-rich fractions. On acrylamide gel electrophoresis, these two fractions were found to be heterogeneous. Those authors noted the absence of lysine-rich histones in yeast and considered it to be responsible for the lack of condensation of chromosomes in metaphase. In the present experiments, seven histone fractions were identified in highly purified nuclei isolated from *S. cerevisiae*. These fractions comprise 23% of the total nuclear protein. Since possible degradation of histones during isolation cannot be ruled out, the number of them in yeast nuclei is as yet uncertain. Experiments are in progress to isolate and characterize these proteins.

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