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STUDIES ON THE YEAST NUCLEUS

I. THE ISOLATION OF NUCLEI

TH. H. ROZIJN AND G. J. M. TONINO

Laboratory for Physiological Chemistry, The State University, Utrecht (The Netherlands)

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SUMMARY

1. A method for isolating pure cell-nuclei from the yeast *Saccharomyces carlsbergensis* is described. The yield of nuclei ranged from 70 to 80 % as judged by the recovery of deoxyribonucleic acid.

2. The morphology of the nuclei was dependent upon the ionic conditions in the medium which was used for the isolation.

3. The isolated nuclei were characterized by high ratio of ribonucleic acid to deoxyribonucleic acid (3.7:1) and of protein to deoxyribonucleic acid (35:1).

4. It is suggested that the nuclei may contain a substantial amount of nucleolar material, not consolidated into a single nucleolus, but more or less dispersed throughout the nucleus.

INTRODUCTION

Several authors[1-14] have investigated the nucleus of yeasts (particularly of *Saccharomyces cerevisiae*), either by means of light microscopic techniques, using fixed and stained preparations of yeast cells, or by means of electron-microscopic techniques. The picture of the nucleus emerging from these studies is that of a spherical, homogeneous body, about 2 μ in diameter, situated outside the vacuole and surrounded by a double membrane in which "pores" are present. This nucleus divides by a simple process of elongation and constriction, during which the nuclear membrane appears to persist. Although some authors[1-5] have observed structures inside the nucleus which they thought to be chromosomes, the most favoured view today is that structural units comparable to the chromosomes found in higher organisms are lacking. MUNDKUR[6] considers the observed "chromosomes" to be aggregations of chromatin particles produced by the procedures involved in fixation, hydrolysis and staining. MUNDKUR[6] and HASHIMOTO[12] both postulated the existence in the yeast nucleus of uniformly dispersed submicroscopic units of chromatin, which function as chromosomes.

Not only chromosomes appear to be lacking, but most authors also report the

Abbreviation: PVP, medium consisting of 8 % polyvinylpyrrolidone (mol. wt. 40 000), 0.5 mM $MgCl_2$ and 0.02 M potassium phosphate (pH 6.5).

absence of nucleoli. As there is accumulating evidence[15,16] that the nucleoli found in higher organisms occupy a central role in the production of RNA and probably also in the production of components of the cytoplasmic ribosomes, it is rather surprising that nucleoli should be lacking in yeast cells which are characterized by a high rate of synthesis of RNA and a high content of cytoplasmic ribosomes.

Perhaps we may assume that in the case of the yeast nucleus we are dealing with a primitive condition, in which nucleolar material is present in the nucleus, not consolidated into a single structure, but dispersed throughout the nucleus.

Recent findings[17–19] indicate that in cell nuclei of higher organisms the synthesis of RNA at different sites on the chromosomes is inhibited by histones. This is in agreement with STEDMAN'S[20] speculation that histones function as gene suppressors in the process of tissue differentiation. MUNDKUR[6] suggested that in the yeast nucleus large quantities of RNA may be synthesized continuously (see also MITCHISON[21,22]), and at non-specific sites. If we assume this suggestion to be correct, it becomes interesting to look for the presence in the yeast nucleus of histone-like proteins which can act as regulators of the DNA-dependent synthesis of RNA.

To answer the questions concerning the presence or absence in the yeast nucleus of nucleolar material and of histone-like proteins, it seems imperative at this moment not to rely entirely upon cytological methods of investigation, but to apply besides a direct biochemical analysis of isolated yeast nuclei.

The present paper describes a method which permits the isolation of morphologically intact nuclei from the yeast *Saccharomyces carlsbergensis*.

EXPERIMENTAL

Preparation of protoplasts

The first step in any procedure for the isolation of intact nuclei from yeast cells will be the disruption of the cell for the purpose of liberating the cellular contents. To break open the tough wall of the yeast cell one needs rough methods which inevitably lead to severe damage of the nuclei. However, the cell wall can be dissolved completely with the aid of a hemicellulase from the gut juice of the snail *Helix pomatia*, when susceptible yeast strains in the logarithmic phase of growth are used. In this way it is possible, under suitable osmotic conditions, to convert the yeast cells into intact protoplasts. The procedure for the conversion into protoplasts of the yeast strain (*S. carlsbergensis*, No. 74 from the British National Collection of Yeast Cultures) used in the present investigation, has been described previously[23]. These protoplasts, which disintegrate very easily, are suitable starting material for the isolation of nuclei.

Homogenization of protoplasts

Protoplasts were lysed at 4° by suspending them in 5 volumes of a solution (0.02 %, w/v) of Triton X-100 in the following medium (designated "PVP medium"): 8 % polyvinylpyrrolidone (mol. wt. 40 000)–0.5 mM $MgCl_2$–0.02 M potassium phosphate (pH 6.5). The lysate was homogenized in a Potter–Elvehjem type homogenizer with close-fitting perspex pestle. One or two movements up and down with the tube along the rotating pestle (500 rev./min) sufficed to obtain a good dispersion of the cellular contents. The tube of the apparatus was always surrounded by a jacket with crushed ice during the homogenization.

Isolation of nuclei

The homogenates were diluted immediately after homogenization with an equal volume of a 0.6 M solution of sucrose in PVP medium and centrifuged in a refrigerated centrifuge at 3000 $\times g$ for 7 min to sediment the nuclei. The supernatant fluid, containing small particles, vacuoles and small membrane fragments, was discarded. The sediment contained the nuclei, contaminated with large fragments of membrane. The crude nuclear sediment was suspended in a small volume of 0.6 M sucrose in PVP medium. The nuclei were then freed from the contaminating membranes by high-speed gradient centrifugation on a layered sucrose-gradient using a swinging bucket rotor (Spinco SW 39) and a model L ultracentrifuge. The gradient consisted of the following layers from bottom to top: 1.5 ml 2.0 M, 1.0 ml 1.8 M, 1.0 ml 1.5 M and 0.5 ml 1.2 M sucrose, all in PVP medium. 1 ml of the crude nuclear suspension was layered on top of the gradient and centrifuged at 25 000 rev./min for 60 min. The purified nuclei were recovered as a small pellet at the bottom of the centrifuge tube, while the contaminants, which accumulated in 3 bands (Fig. 1) were easily discarded by drawing off all the supernatant material. The yield of nuclei ranged from 70 to 80 % as judged by the recovery of DNA.

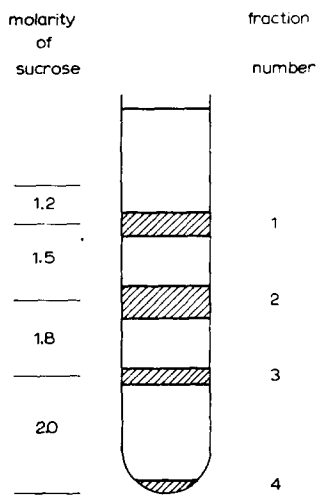

Fig. 1. Sucrose-gradient after centrifugation. 1 and 2, membrane fragments; 3, small membrane fragments and broken nuclei; 4, intact nuclei.

Phase-contrast microscopy of isolated nuclei

The fragility of the isolated nuclei caused them to rupture very easily under even slight mechanical pressure. They also had a strong tendency to adhere to glass surfaces. Owing to these properties it was difficult to achieve a uniform focal plane for the phase-contrast photomicrography of a large number of nuclei. Therefore some nuclei were always out of focus in the photomicrographs of our nuclear preparations.

The isolated nuclei were round and clearly outlined, and varied somewhat in size (Fig. 2). Contaminating non-nuclear material was present in small amount and consisted mainly of tiny shreds of cytoplasmic material adhering to some of the

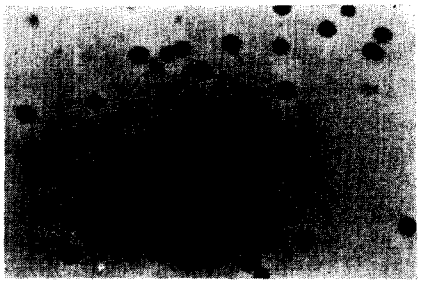

Fig. 2. Phase-contrast micrograph of yeast nuclei isolated by the method described in the text. Instrumental magnification 1060×.

nuclei. Occasionally an intact yeast cell was seen in specimens of the purified preparations. The total amount of contaminating material was estimated to be not more than 1 % of the total nuclear mass.

Comments on the isolation procedure

The presence of polyvinylpyrrolidone in the media used for isolation was necessary to maintain morphologic integrity of the yeast nuclei during the isolation procedure. It also facilitated the centrifugal separation of the nuclei from smaller cytoplasmic particles. Omission of polyvinylpyrrolidone led to swelling of the nuclei and to the appearance of large blebs on the nuclear surface. Concentrations of more than 10 % produced dense, pyknotic nuclei which were easily fragmented during homogenization, and caused agglutination of the smaller cytoplasmic particles. Such high concentrations also brought about an undesirable stabilization of the protoplast membrane, rendering the protoplasts markedly resistent to the disruptive forces of the homogenizer. A concentration of about 8 % was found to be suitable.

A pH of about 6.5 and a Mg^{2+} concentration of 0.5 mM were optimal. If the pH was higher than 7.0, or if Mg^{2+} was omitted, the nuclei swelled and eventually dissolved completely. A pH lower than 6.0 and Mg^{2+} concentrations higher than 1 mM both caused severe clumping of the nuclear contents against the nuclear membrane, giving the nuclei the appearance of empty spheres covered by a small "cap" of high refractivity. (Fig. 3). These "capped" nuclei were very susceptible to mechanical damage, thus making it almost impossible to isolate them intact.

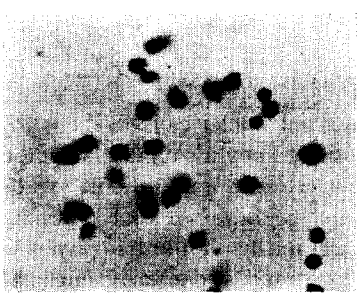

Fig. 3. Phase-contrast micrograph of yeast nuclei isolated at pH 5.6. Note the presence of "capped" and damaged nuclei. Instrumental magnification 1060×.

When protoplasts were suspended in PVP medium without Triton X-100 they swelled and eventually burst rather explosively. Many nuclei suffered mechanical damage during their extrusion from these bursting protoplasts. This damage could be largely prevented by the addition, to the PVP medium, of a low concentration (0.02 %) of the non-ionic detergent Triton X-100 (*i*-octylphenoxypolyethoxyethanol). The detergent produced a rapid and gentle lysis of the protoplasts, and also effected a better dispersion of the liberated cellular contents. Nuclear morphology was not altered by the presence of this low concentration of Triton X-100.

When nuclei were kept in PVP medium without sucrose they gradually showed some alteration of their morphology: some swelling occurred and their outlines became irregular. These alterations could be prevented by the addition of sucrose (0.3–0.6 M). However, if sucrose was added prior to the homogenization step it prevented the lysis of the protoplasts by osmotic stabilization, thus making it necessary to break open the protoplasts by vigourous homogenization with consequent severe damage of the fragile nuclei. Therefore sucrose was added immediately after homogenization.

The crude nuclear preparations obtained after centrifugation of the homogenates could be purified considerably by repeated washing with 0.6 M sucrose in PVP medium. However, even after numerous washings, the nuclear preparations were still contaminated with small amounts of membrane-like material. Besides, many nuclei were damaged by the repeated re-suspension and centrifugation, because of their fragile nature. Highly purified preparations could be obtained however by high-speed centrifugation of the crude nuclear preparations on a layered sucrose-gradient. The nuclei purified in this way appeared to be morphologically intact, although the possibility cannot be ruled out that they were depleted of low-molecular-weight constituents, as was found for nuclei which had been isolated from animal cells with the aid of hypertonic sucrose solutions[24].

The chemical composition of isolated nuclei

For the determination of DNA, RNA and protein, the nuclear pellets were washed three times with ice-cold 0.25 N $HClO_4$, and then digested in 0.3 N KOH at 37° for 1 h (ref. 25). The alkaline digest was fractionated by acidification with perchloric acid into an acid-soluble fraction containing the RNA, and an acid-insoluble fraction containing the DNA.

RNA was determined by the spectrophotometric method described by SPIRIN[26]. For the determination of DNA the procedure of BURTON[27] was used. Protein was determined in the alkaline digest by a modification[28] of the method of LOWRY *et al.*[29], using bovine serum albumin as a standard.

The chemical composition of the isolated nuclei is given in Table I. It will be clear that the figures in this table do not necessarily represent the true composition of yeast nuclei: some of the RNA and the protein found in our preparations might have been contributed by the small amount of contamination, while on the other hand some RNA and protein might have been lost from the nuclei during their isolation. Nevertheless, the conclusion that yeast nuclei are characterized by high ratios of RNA to DNA and of protein to DNA seems to be justified. The low concentration of DNA is in accordance with the low colour intensity of nuclei in yeast cells which have been stained by the Feulgen technique[7].

TABLE I

CHEMICAL COMPOSITION OF ISOLATED NUCLEI

For each experiment the sum of DNA, RNA and protein is set at 100. The results are expressed as percentages of this sum, with standard deviations. The figures for protoplasts are given for the sake of comparison.

	Number of experiments	*DNA (%)*	*RNA (%)*	*Protein (%)*
Nuclei	9	2.5 ±0.2	9.2±0.6	88.3±0.7
Protoplasts	6	0.20±0.04	21.7±1.6	78.1±1.6

DISCUSSION

Careful examination of the isolated nuclei by phase-contrast microscopy revealed the presence in many nuclei of a crescent-shaped segment which was slightly more opaque than the rest of the nucleus. In nuclear preparations that had been stained by the addition of a low concentration (about 0.001 %) of crystal violet, the same segment could be seen as an area coloured a little more darkly than the rest of the nucleus. We are tempted to believe that the opaque crescents of the isolated nuclei correspond to the peripheral clusters of particles which MUNDKUR[7] observed by means of electron-microscopy in nuclei of frozen-dried yeast cells.

When the Mg^{2+} concentration in the medium was lowered, or when the pH was raised, the nuclei swelled and the opaque segment was no longer discernable. On the other hand, an increase of the Mg^{2+} concentration and a lowering of the pH both caused a condensation of the nuclear contents upon the opaque segment, which under these conditions appeared as a "cap" of high refractivity, covering an optically empty nucleus. When such "capped" nuclei were stained with low concentrations of crystal violet, the "cap" took on a deep purple colour, while the rest of the nucleus was coloured pale blue. This suggests that the greater part of the nucleoproteins of these "capped" nuclei was located in the region of the "cap". "Capped" nuclear bodies have been described earlier by EDDY[30], who could release them from yeast protoplasts by ultrasonic treatment at pH 5 in the presence of relatively high concentrations of Ca^{2+} and Mg^{2+}. In the absence of both Ca^{2+} and Mg^{2+}, he observed swollen, homogeneous bodies which dissolved rapidly.

The observed dependence of the nuclear morphology on the composition of the medium is not peculiar to yeast nuclei. Similar phenomena have been observed in nuclei isolated from animal cells[31,32].

Some authors[1,10] who have studied the yeast nucleus in fixed and stained yeast cells, hold the belief that not the entire nucleus, but only a crescent-shaped segment is Feulgen positive. MUNDKUR[6] however, using frozen-dried yeast preparations, reported that the entire nucleus is Feulgen positive. The same author also assumed that the Feulgen positive crescents, observed by others, were artifacts caused by the fixation procedure. Our observations on the striking alterations in the morphology of isolated nuclei, brought about by changes in the chemical composition of the medium, suggest that MUNDKUR is right. In this connection it may be important to mention the effect of basic dyes upon the morphology of isolated nuclei. When for instance crystal violet was added in relatively high concentrations (about 0.01 % and more) to nuclear suspensions, the nuclei were transformed into "capped" nuclei.

Most probably, the commonly used fixation and staining procedures cause a clumping of the nuclear contents against the nuclear membrane, thus giving rise to structures comparable with the "capped" nuclei described by us. It is conceivable that in these "capped" nuclei all Feulgen-positive material is concentrated in the region of the "cap".

The interpretation of observations on yeast nuclei, whether in fixed and stained yeast cells, or isolated, evidently is not an easy task. Such observations reflect to a large extent the techniques used, and they do not necessarily give information on the intracellular condition of the nucleus.

From the figures in Table I it follows that the ratio of RNA to DNA (3.7:1) and of protein to DNA (35:1) in the isolated nuclei are much higher than those reported for nuclei isolated from animal cells[33]. The nucleoli found in higher organisms are rich in RNA and protein[15]. Such nucleoli cannot be recognized inside the yeast nucleus. The high RNA and protein content of the yeast nucleus suggests however that a substantial amount of nucleolar material may in fact be present, perhaps more or less dispersed throughout the nucleus.

Preliminary experiments on the metabolic activity of the isolated nuclei have demonstrated the presence of RNA polymerase (EC 2.7.7.6)[34].

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