AN IMPROVED METHOD FOR THE ISOLATION OF YEAST NUCLEI ACTIVE IN RNA SYNTHESIS IN VITRO

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Received December 3, 1973

SUMMARY

An improved method has been developed for the isolation of nuclei from Saccharomyces cerevisiae for the study of RNA synthesis in vitro. Utilization of Ficoll in the isolation procedure greatly increases the activity of RNA polymerase in isolated nuclei. Nuclei prepared by this procedure are essentially free of mitochondrial DNA.

INTRODUCTION

Isolation of nuclei from yeast presents a difficult problem. A successful procedure must take into account the problem of efficient disruption of the thick cell walls of yeast while keeping the nuclei intact. Isolated nuclei are extremely fragile and sensitive to the composition of medium and changes in mechanical pressure. Recently, we reported a method for isolation of nuclei from Saccharomyces cerevisiae using a modified French press (1). Nuclei prepared by the above method have been successfully used for isolation of high molecular weight DNA (2-4) ,chromatin and histones and localization of nuclear enzymes (5). However, when RNA polymerase activity was assayed in isolated nuclei it was observed that polyvinylpyrrolidone (PVP) used in the isolation medium had an inhibitory effect on the incorporation of nucleotides into RNA. Furthermore, when PVP was added to purified enzyme preparations the RNA synthesis was inhibited almost completely. Elimination of PVP, however, resulted in nuclear lysis. Recent report on the use of Ficoll in isolation of yeast nuclei from spheroplasts by Wintersberger et al.(6) has prompted us to study its effect on isolating yeast nuclei from whole cells. Results show that Ficoll protects the structure of yeast nuclei without interferring with RNA synthesis in vitro. Nuclei prepared by this method are free of mitochondrial

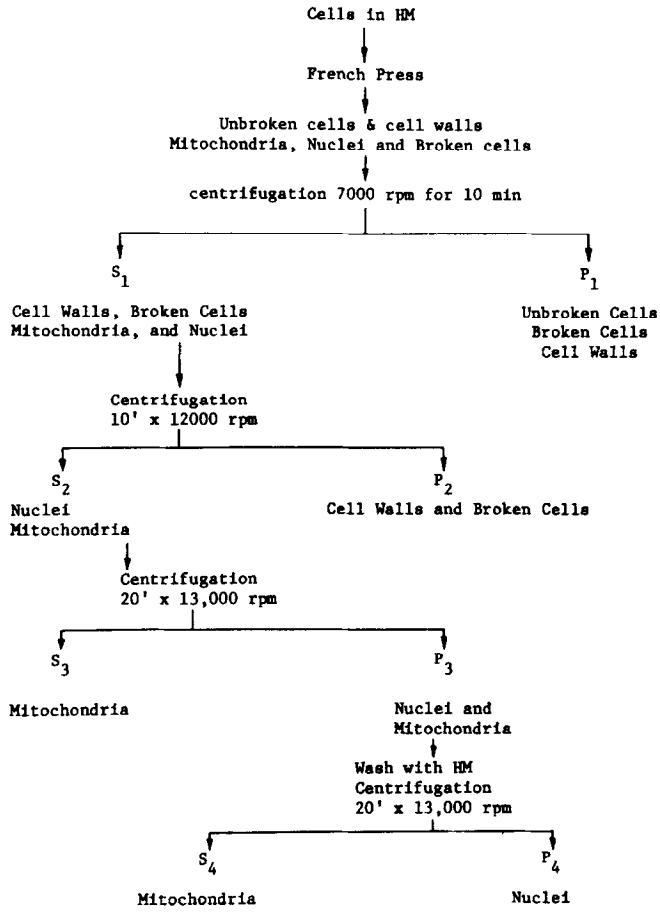

Figure 1. Scheme for Isolation of Nuclei From Yeast Cells. Washed yeast cells were suspended in homogenizing medium and frozen suspension was forced through modified French press as described earlier. All centrifugations were carried out with rotor SS-34 in a Sorvall RC2B centrifuge at 4°C.

contamination as judged by CsCl gradient analysis of DNA. Under phase microscope they appear indistinguishable from PVP prepared nuclei. In addition, this method permits the rapid large scale isolation of pure nuclei.

MATERIALS AND METHODS

Unlabelled nucleoside triphosphates, Ficoll and PVP-40 were purchased from Sigma Chemical Company. UTP ^{3}H (specific activity 26.9 C/mmole) was obtained from New England Nuclear. Glass fiber paper GF/C were purchased from Whatman.

Saccharomyces cerevisiae Y55 was grown, harvested and washed as previously described (1). The washed cell pellet was suspended in 2-5 volumes of isolation medium consisting of: 1 M Sorbitol, 20% glycerol and 7% Ficoll. Cell suspension was broken in a modified French press (1).

The disrupted cells from the French press were thawed and made 1:5 (w/v) with homogenizing medium. The centrifugation scheme of isolation procedure is shown in Figure 1. All centrifugations were carried out in Sorvall RC-2B centrifuge, rotor SS-34 at 4°C. Examinations under phase contrast microscope were made at every step to ascertain the intactness and recovery of nuclei during purification. The homogenate was centrifuged at 7000 rpm for 10 min. to remove unbroken cells and cell walls. Centrifugation of the resulting supernatant at 12000 rpm for 10 min accomplished further removal of cell walls. The supernatant which contained nuclei and mitochondria was centrifuged at 13000 rpm for 20 min to pellet the nuclei. The resulting nuclear pellet was resuspended in homogenizing medium and washed twice with the same medium at the same speed to remove contaminating mitochondria. Alternatively, the nuclei could also be freed from mitochondria by centrifugation of nuclear suspension over 2 M Sorbitol containing 30% glycerol and 7% Ficoll at 18000 rpm for 20 min at 4°C in SW 27 rotor in Model L ultracentrifuge. Following centrifugations through the gradient, mitochondria remain in 1 M Sorbitol layer while nuclei sediment as a pellet at the bottom of the centrifuge tubes.

Nuclei were isolated from cells harvested at optical densities ranging from 5 to 13 with similar yields, however, for RNA polymerase determinations cells were harvested at optical densities of 4-6.

Assay for RNA polymerase activity in isolated nuclei were carried out in duplicate under low ionic strength conditions by modifications of methods described earlier (7).

Reaction mixture of 100 µl contained 5-10 µg nuclear DNA, 5 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.9 and unlabelled nucleoside triphosphate (GTP, ATP,

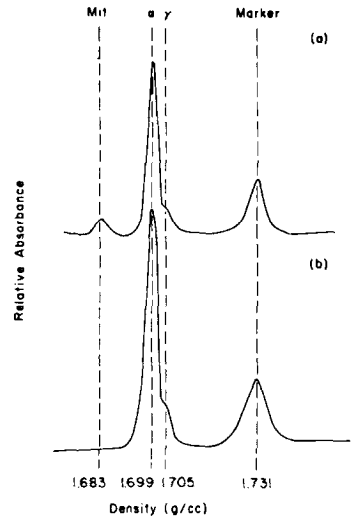

Figure 2. DNA Isolated From Whole Yeast Cells and Purified Nuclei. DNA was isolated from whole cells (a) and purified nuclei (b) and centrifuged to equilibrium in analytical CsCl density gradients as described earlier in the text. The density increases from left to right.

CTP) at 1.0 mM each and 0.5 mM UTP. UTP ^{3}H was used at concentrations of 0.02 mM (25 curies/mM). The incubation was carried out at 37°C for 20 min. The reaction was terminated by addition of 1 ml of 5% TCA containing 0.2 M sodium pyrophosphate. The precipitate was collected on GF/C paper, washed 3 times with 15 ml of cold 5% TCA, dried and counted in a Beckman LS-225 Liquid Scintillation Counter. DNA was measured by diphenylamine method (6) and the polymerase activity was expressed on the basis of DNA.

Purification and assay of purified RNA polymerases was carried out as described previously (8).

DNA extraction from whole cells and nuclei and CsCl gradient analysis of DNA to determine mitochondrial contamination was carried out as previously described (2).

RESULTS AND DISCUSSION

In early experiments on RNA synthesis by isolated nuclei, it was observed that the polymerase activity declined with increasing amount of PVP isolated nuclei employed in the assay mixture. This observation led to an analysis of

Table I: Effect of in vitro addition of various components of isolation medium on RNA polymerase activity of isolated nuclei in vitro

Substance Added (25 µl)	UTP ^{3}H Incorporated cpm
None	17471
1 M Sorbitol	18430
5% PVP	10885

Nuclei were suspended in 0.25 M sucrose and 25 µl of nuclear suspension was used per assay. (See text for details)

the effect of various components of the isolation medium on the RNA polymerase activity in isolated nuclei. As shown in Table 1, 5% PVP inhibited by 38% RNA polymerase activity in isolated nuclei whereas 1 M Sorbitol had no effect. The inhibitory effect on RNA polymerase activity was further confirmed by studies with purified nuclear enzymes. As can be seen from Table 2, PVP at the concentration used in isolation procedure inhibited over 85% of the activities of RNA polymerase I and II. However, elimination of PVP from the homogenizing medium resulted in complete lysis of nuclei.

These findings have led to a reexamination of the medium used for the isolation of yeast nuclei. Based on the report of Wintersberger et al. (6) it was found that when a combination of 1 M Sorbitol, 20% glycerol and 7% Ficoll were used in the nuclear isolation procedures, a preparation of nuclei were obtained indistinguishable under phase contrast microscopy from those prepared by the PVP method previously reported (1). It was also observed that in the present method using Ficoll medium the breakage of whole cells and yield of nuclei (40-50%), both on wet weight and DNA bases was essentially similar to the method reported earlier (1).

Table II: Effect of in vitro addition of isolation medium on activity of purified RNA polymerase I and II

Enzyme	Medium Added	UTP ^{3}H incorporated cpm	% Inhibition
I	-	1650	
I	PVP	219	85
I	Ficoll	1550	<10
II	-	5054	
II	PVP	645	79
II	Ficoll	5302	0

RNA polymerase I and II were purified as described in text and the activity was measured by counting the radioactivity in the TCA precipitable fraction in a Tricarb Scintillation Counter after incubating the reaction mixture with ^{3}H-UTP for 20 min. at 33°C.

In all following RNA polymerase assays it has been ascertained as before (8) that H^3 UTP is being incorporated into RNase sensitive product, the synthesis of which requires all four nucleotide triphosphates. RNA polymerase activity was tested in Ficoll prepared by nuclei and compared on DNA basis with PVP prepared nuclei. The specific activity of RNA polymerase activity, as shown in Table III, was 55% lower in the PVP prepared nuclei. Since the nuclei were resuspended in 0.25 M sucrose before subjecting it to polymerase assay it can be concluded that PVP used in isolation medium binds to nuclei and interferes with RNA synthesis. Furthermore, when the purified enzyme activity was tested it was observed (Table 2) that Ficoll had almost no inhibitory effect on either RNA polymerase I or II.

To further characterize the nuclei prepared by the Sorbitol, glycerol and Ficoll isolation procedure, they were examined for possible contamination by mitochondria. For this purpose, DNA was isolated from Ficoll prepared nuclei and analyzed on CsCl density gradient centrifugation in the Model E analytical centrifuge. As can be seen in Figure 2, such DNA preparations contain typical nuclear DNA bands but are lacking (less than 1%) in detectable bands of mitochondrial DNA.

Table III: Effect of the composition of homogenizing medium on the RNA polymerase activity of isolated nuclei in vitro

Homogenizing medium	picomoles of UTP-^{3}H incorporated/µg DNA
Sorbitol + PVP	62.44
Sorbitol + Ficoll	139.68

Nuclei were suspended in 0.25M sucrose. Nuclear suspension containing 5-10 µg of DNA was used per assay.

With the increasing interest in using isolated nuclei for nucleic acid synthesis, we believe that the procedure described here provides an improved method for the study of synthesis and degradation of RNA.

ACKNOWLEDGEMENTS

This work was supported by Grant No. VC106B from the American Cancer Society and Grant No. 5ROI-A110610-2 from the National Institutes of Health.

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