Nuclear Buoyant Density Determination and the Purification and Characterization of Wild-Type Neurospora Nuclei Using Percoll Density Gradients

KATRIN J. TALBOT AND PETER J. RUSSELL
Biology Department, Reed College, Portland, Oregon 97202

ABSTRACT

A procedure has been developed using Percoll density gradients for the isolation and purification of nuclei from germinated conidia of wild-type Neurospora crassa St. Lawrence strain 74A. Crude nuclei were purified isopycnically in gradients of Percoll, which is silica coated with polyvinylpyrrolidone. A DNA:RNA:protein ratio of 1:3.5:6.5 was found in purified nuclei. Cytoplasmic contamination was found to be negligible in the nuclear preparations, as determined by electron microscopy and by following a radioactively-labeled ribosome tag during the isolation procedure. A small amount of endogenous ribonuclease activity was detected in the crude nuclear preparations, but not in suspensions of nuclei purified in the Percoll gradients. Ribosomal RNA was extracted from the nuclei in good yields, and electrophoretic analysis indicated the presence of precursor rRNA molecules, as well as the mature 17S and 25S rRNA species. Using the Percoll gradient system, the buoyant density of purified Neurospora nuclei was determined to be 1.08 grams per milliliter based on refractive index measurements.

The availability of purified nuclei greatly facilitates such studies as the synthesis and processing of precursor ribosomal RNA (pre-rRNA) and of precursor nucleoprotein particles. Although few problems are encountered with nuclei extraction in animal systems, the thick cell wall and prevalent endogenous nucleases in plants and fungi (3, 5), which are released upon cell disruption, make it a much more difficult task in these systems. In the fungal system Neurospora crassa, there has been only one report of the successful isolation of nuclei (3). This paper describes a procedure for the isolation and purification of Neurospora nuclei which uses Percoll as the gradient medium. Using this method, we have been able to determine accurately the buoyant density of the nuclei, since Percoll consists of colloidal silica particles coated with polyvinylpyrrolidone, to which the nuclear membrane is impermeable. The pure nuclei obtained with this procedure are essentially devoid of RNase activity, while crude nuclei show some RNase activity. High-mol-wt precursor rRNA and mature rRNAs can be isolated readily from the pure nuclei.

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MATERIALS AND METHODS

Strains and Culture Techniques. The wild-type strain of N. crassa used was the St. Lawrence strain 74A. The growth media (Vogel's minimal and Vogel's complete) and culture techniques used were all as described previously (4). Conidia (asexual spores) were harvested from 7-d-old cultures of the wild-type strain grown in 125-ml flasks containing 30 ml of solid complete medium. The flasks were incubated at 37°C for 48 h and then at 25°C for the remainder of the growth period.

Growth Conditions for Nuclei Extraction. To obtain mycelia from which to extract the nuclei, 300 ml Delong flasks containing 125 ml of liquid Vogel's minimal medium were inoculated with conidia under sterile conditions to a final concentration of 2 x 10^7 conidia/ml. The cultures were then incubated at 25°C with shaking for 8 h at which time they were in the mid-log phase of growth. The resultant mycelial growth was collected by vacuum filtration and washed with distilled H2O. The wet weights of the mycelial mats of each culture were determined and the cell masses were used for subsequent nuclei extraction.

Isolation and Purification of Nuclei. The isolation of crude nuclei was accomplished with a modified version of the technique of Hautala et al. (3). The mycelial mat was vigorously resuspended in three volumes of ice-cold buffer A (1 M sorbitol, 7% [v/v] Ficoll 400, 20% [v/v] glycerol, 5 mM MgCl2, 10 mM CaCl2, 1% [v/v] Triton X-100, adjusted to pH 7.5). The resultant homogeneous slurry was poured into a French-pressure cell (precooled at −70°C overnight), and placed at −70°C for 45 min. A maximum pressure of 20,000 p.s.i. was applied to disrupt the cells. Three volumes of ice-cold buffer A were added to the frozen eluant, and the mixture was vortexed. The solution was homogenized in a sterilized Omnimixer bucket at a setting of 55 for 20 min at 4°C.

To remove the bulk cellular debris, the homogenate was centrifuged at 2,400g for 10 min at 4°C in a Du Pont-Sorval RC5 centrifuge, SS34 rotor. A 10-cm 14-gauge cannula was used to remove the supernatant liquid. The pellets were resuspended in the same volume of buffer A as removed, and were centrifuged above. Two repetitions of these steps generated three supernatants. The pooled supernatants were centrifuged for 50 min at 9,000g and the crude nuclear pellets were obtained after careful removal of the supernatant liquid. The crude nuclei were used either immediately in further purification steps, or in RNA extractions, or they were stored at −70°C.

Further purification entailed resuspension of the crude nuclear pellet in 10 volumes of ice-cold buffer B (1 M sucrose, 50 mM Tris-HCl [pH 7.5]; 5 mM MgCl2; 10 mM CaCl2; 1% [v/v] Triton X-100); the pellet was repeatedly expelled through a 14-gauge cannula. The nuclear suspension was then ground in a 40-ml capacity Potter-Elvehjem tissue grinder. Ten to 15 strokes were adequate for effective homogeneity of the solution.
The density gradient material, Percoll (Pharmacia Fine Chemicals), was used in subsequent purification steps. An isomotic stock solution of Percoll was made by adding 9 parts (v/v) of Percoll to 1 part (v/v) of 2.5 M sucrose. Lower starting densities of Percoll were obtained by adding the appropriate amount of 0.25 M sucrose. The nuclear suspensions from the grinding were mixed with the appropriate amounts of the Percoll-sucrose stock solution in Beckman 1.6 X 7.62 cm, 10.4 ml polycarbonate bottle assemblies. These were centrifuged at 4°C for 45 min at 58,300g (ω90- = 6.66 cm) using a DuPont-Sorval T865.1 angle rotor in a DuPont-Sorval OTD-2 ultracentrifuge. The nuclei were collected from the gradients by centrifuging fractions containing the nuclear band for 2 h at 100,000g in a Beckman SW50.1 swinging-bucket rotor in a Beckman Model L ultracentrifuge. Under these conditions, the Percoll particles pellet and the nuclei remain above the gel formed. The nuclei were pelleted from the supernatant liquid by centrifugation for 20 min at 5,000g at 4°C in a DuPont-Sorval RC5 centrifuge, SS34 rotor.

Preparation of Nuclear Pellets for Electron Microscopy. Crude or purified nuclear pellets were centrifuged for 15 min at 10,000 g at 4°C in the SS34 rotor. One ml of mixed aldehyde fixative (from 2.4% [v/v] glutaraldehyde, 1.2% [v/v] freshly prepared paraformaldehyde, 80 mM sodium cacodylate [pH 7.3–7.4], 2 mM CaCl2, 37 mM sucrose) was added to each tube of nuclei. To facilitate pellet formation in the fixation process, the tubes were centrifuged at 9,220g for 30 min.

After approximately 14 h of fixation at 4°C, the pellets were centrifuged for 10 min at 20,200g and the fixative was removed. Each pellet was washed with 1 ml of washing buffer (80 mM sodium cacodylate [pH 7.3–7.4], 2 mM CaCl2, 37 mM sucrose) and washed overnight at 4°C. The pellets from a 10 min, 20,200g centrifugation were drained and specimens postfixed with 2% (w/v) OsO4 for 1.5 h.

Increasing concentrations of ethanol were used successively to dehydrate the nuclear pellets. Prior to embedding, the pellets were infiltrated with propylene oxide and Epon, after which the specimens were embedded in BEEM embedding capsules. Thick sections of the trimmed pellets (0.5 μm sections) were taken on microtomes and stained with toluidine blue. Thin sections (0.1 μm in thickness) were made, mounted on copper grids, and stained with various stains as described in “Results.”

Ribosome Isolation. Flasks of 100 ml of Vogel’s minimal medium were inoculated with 107 conidia/ml and the cultures were labeled with 2.0 μCi/ml of [5-3H]uridine (30.0 Ci/mmol). The cultures were incubated at 25°C for 18 h with shaking. The isolation of cytoplasmic ribosomes followed those procedures described by Russell et al. (8). The ribosomes were stored in 0.1 ml aliquots at −70°C.

Assessment of Ribosome Contamination. To determine the relative amount of ribosomal contamination in the crude and purified nuclear pellets, radioactively labeled ribosomes were prepared as described above. A culture of 100 ml of Vogel’s minimal medium was inoculated with 2 × 107 conidia/ml and incubated for 8.5 h at 25°C with shaking. After that time, an 0.1 ml aliquot of the 3H-labeled ribosomes (containing about 2.5 × 107 cpm) was added and the nuclei preparation procedures began. Throughout the isolation and purification procedures, ribosomal “contamination” of each fraction with radioactive ribosomes was determined by removing 0.1-ml samples of the cellular preparations and measuring the radioactivity. The total volume of the culture or slurry was measured at each sample time so that total radioactivity also could be determined.

Preparation of Total Cell RNA. RNA was extracted from mid-logarithmic phase cultures as described by Russell et al. (8) with the following modifications: (a) one-half volume of chloroform was added after each phenol extraction and the mixture was shaken for 1 min just prior to centrifugation; (b) after the aqueous phase showed no protein precipitate following successive phenol extractions, it was extracted with 2 volumes of ether; (c) the nucleic acids were precipitated from the aqueous phase at −70°C rather than −20°C with the addition of two volumes of 95% ethanol containing 0.2 M sodium acetate.

RNA Analysis by Acrylamide Gel Electrophoresis. RNA was subjected to electrophoresis in 2.4% (w/v) acrylamide and 0.11% (w/v) N,N’-methylene bisacrylamide gels containing 0.5% (w/v) agarose essentially following the procedures outlined by Russell et al. (9). After 7.5 h of electrophoresis at 3 mamp/gel, the gels were removed from the tubes and scanned at 260 nm in a spectrophotometer to locate the position of the 25S and 17S RNA species.

...A Mickle gel slicer was used to fractionate the gels into 1-mm slices. To hydrolyze the RNA in each slice, 125 μl 24% (v/v) HClO4, 18% (v/v) H2O2, was added to each gel slice. After incubation at 37°C for at least 8 h, 65 μl 10 N NaOH, 0.1 ml H2O, and 5 ml PBD-100 (25% [v/v] Triton X-100, 75% [v/v] xylenes, 0.8% [w/v] 2-phenyl-5-[4'-biphenyl]-2,3,4-oxadiazole [PBD], 0.05% [w/v] 2-[4'-biphenyl]-6-phenyl-benzoxazole [PBO]) were added to each vial and the radioactivity was determined in a Beckman scintillation spectrometer.

DNA, RNA, and Protein Analyses. For the extraction of DNA, RNA, and protein, the methods of Sturani et al. (13) was used. Cold 1 N TCA was used to suspend mycelial pads or nuclei. After two washings with cold 1 N TCA, the pellets were resuspended in 4 ml of 3% (w/v) HClO4, and heated for 30 min at 90°C. After centrifugation, the DNA and RNA contents of the supernatant were determined using the diphenylamine (2) and orcinol (11) assays, respectively.

The pellet was resuspended in 3 volumes 1 N NaOH and left overnight at room temperature. After centrifugation, the protein content was determined by the Bio-Rad method using a BSA standard. This uses the dye Coomassie Blue G-250 as an indicator and is patterned after the method described by Bradford (1).

Endogenous Ribonuclease Activity. The assay for endogenous ribonuclease activity in nuclear preparations involved incubation of the nuclei with 3H-labeled RNA from Neurospora. The assay was a modified version of that used by Luthe and Quatrano (5), with which they tested the effect of endogenous nuclease activity from wheat nuclear preparations on the size distribution of H3-labeled RNA. The nuclei were incubated for 30 min. Some reaction mixtures contained 5 mM ethyleneglycol-bis-(β-aminoethyl ether)-N,N'-tetraacetic acid (EGTA), 100 mM KCl, and 125 mM (NH4)2SO4. At the end of the incubation period, the mixtures were centrifuged and the nuclei pelleted. The supernatants containing the RNA were layered onto 24% acrylamide gels and subjected to electrophoresis. The gels were scanned at 260 mm to determine the positions of mature 25S and 17S rRNAs, fractionated into 1.0-mm slices, and the radioactivity in each fraction was determined as already described.

Extraction of Nuclear Ribosomal RNA. Following the suggestions of Poulson (7), the nuclei were suspended in 1 mM potassium phosphate buffer, 2 mM MgCl2, and 0.32 M sucrose with 0.3% (v/v) Triton X-100. The solution was shaken at 20°C until the solution cleared. The nuclei were also treated with 50 μg/ml DNase I to eliminate any nucleohistone gel which would trap some of the nuclear RNA.

RESULTS

Isolation and Gradient Purification of Neurospora Nuclei. Eight to 9-h cultures of germinated conidia were found to give the optimal yields of nuclei, since least resistance was encountered in cell wall breakage at that growth stage. The mode of freezing the mycelial preparation was also varied to find the optimum conditions for cell lysis. The best crude nuclear yield was obtained
were employed for more efficient pellet formation in the initial stages of the isolation procedure.

A range of starting densities of Percoll from 1.05 to 1.12 g/ml was tested in separate experiments to determine the most useful for isopycnic banding of Neurospora nuclei in gradients formed by centrifugation in an angle rotor. The starting densities were determined from known amounts of Percoll diluted in 0.25 M sucrose. Inasmuch as a linear relationship exists between refractive index and density of the diluted Percoll, the densities of the nuclear bands were easily determined.

After each experiment, the tube contents were fractionated into 12 fractions of 50 drops each, using a peristaltic pump, and Brix readings were taken with an A/O refractometer for the determination of refractive indices. The best resolutions of nuclei into discrete bands were found in tubes of starting densities between 1.06 and 1.09 g/ml. Subsequent determinations with these values as starting densities in Percoll revealed that the best banding of nuclei was obtained with starting densities in Percoll solutions of 1.08 g/ml. Under those conditions, the nuclei banded in one region of the gradient, and could be collected upon gradient fractionation and subsequent high-speed centrifugation.

Tests were made to determine the optimum centrifugal forces for gradient generation from Percoll solutions. We found that 1.5 \times 10^6 to 1.7 \times 10^6 total revolutions (corresponding to 60–70 min of centrifugation time at 60,000g of the T865.1 rotor in the DuPont-Sorvall OTD-2 ultracentrifuge) generated the best gradients and maximum resolution of the purified nuclei band. Centrifugations for longer than 70 min resulted in a slight pelleting of the Percoll colloidal silica particles, visualized as transparent pellets after centrifugation. Figure 1 shows the density gradient for a typical Percoll nuclei purification. Under the optimum conditions established, the density of purified N. crassa nuclei was determined to be 1.078 g/ml based on refractive index measurements.

The removal of Percoll from the nuclei suspension after fractionation proved to be relatively simple: clear pellets of sedimented silica particles were obtained after 2 h of high-speed centrifugation. The nuclei remained above the pellet. Final centrifugation of the nuclear fraction resulted in pale, off-white pellets. These pellets could be frozen at −70°C without degradation upon subsequent thawing.

Electron Microscopy. To judge the relative purity of nuclear preparations, electron microscopy studies were undertaken to check for cytoplasmic contaminants. In the preparation of the pellets for Epon embedding, the nuclei were either fixed in pellet form with periodic centrifugations to maintain the pellet, or were fixed in suspension. No significant differences were noted in the final copper-grid examinations, although a slightly greater amount of scattered presumptive chromatin material was observed in the pellets which had been fixed in suspension.

To find optimum staining conditions, four different stains were applied to the grids. The nuclei appeared as darkly stained circular bodies most clearly when a combination of uranyl acetate and a lead stain were applied to the specimens. Other stains tested were Reynold’s lead nitrate, alcohol uranyl stain, and aqueous uranyl acetate. The microscopic studies indicated that the crude and purified nuclear preparations were both relatively free of large cytoplasmic contaminants and mitochondria, with the latter “cleaner” than the former.

Contamination of Nuclei with Cytoplasmic Ribosomes. We assessed the contamination of the nuclei with free cytoplasmic ribosomes either by copurifying with the nuclei or by associating with the outer nuclear membrane. To do this, we radioactively tagged the nuclear isolation procedure with labeled, purified,

| Table 1. Macromolecular Content of Nuclei from N. crassa Cultures |
|-----------------------------|------------------|------------------|
| Preparation Stage          | DNA:RNA:Protein  |
| Whole cells from mycelial  | 1:16:63          |
| Crude nuclear pellet       | 1:7.5:36         |
| Purified nuclear pellet    | 1:3.6:5          |

Fig. 1. Percoll density gradient used for the purification of nuclei. Tubes were prepared for analysis using 1.08 g/ml Percoll solution in Buffer B as the starting density. After centrifugation at 58,300g \( (\text{rpm} = 6.66) \) for 45 min in a DuPont-Sorvall T865.1 rotor, the samples were fractionated and Brix readings were taken for density determination.

Fig. 2. Electrophoretic analysis of \(^{14}C\)-labeled Neurospora RNA that had been incubated with Percoll-purified nuclei. For the preparation of labeled RNA, a culture of wild type was initiated with \( 10^7 \) conidia/ml and labeled with \([2-{ }^{14}C\]uracil (ICN, Irvine, CA, 40 mCi/mmol) to a final concentration of 0.1 \( \mu \)Ci/ml. After 16 h at 25°C, the mid-logarithmic phase culture was harvested and the RNA was extracted. Purified nuclei were isolated from Percoll gradients as described in “Materials and Methods.” The assay for endogenous ribonuclease activity involving the incubation of \(^{14}C\)-labeled RNA with nuclei was as described in “Materials and Methods.”
cytoplasmic ribosomes. In the preparations of crude and purified nuclei, 1.5% and 0.53% of the initial radioactivity were found, respectively. Almost 80% of the ribosomal ‘contamination’ was eliminated following homogenization of the broken cells after French pressing. The results suggest that contamination of the crude and purified nuclear pellets by free cytoplasmic ribosomes is minimal.

**DNA:RNA:Protein Ratios.** Table 1 shows the DNA, RNA, and protein content of whole cells, crude, and purified nuclear pellets, as determined by diphenylamine, orcinol, and the Bio-Rad protein assays on TCA and HClO₄ extracts. We found a 1:6.5 DNA:protein ratio in the Percoll-purified nuclei and this is slightly lower than the 1:7.0 ratio found for Ludox-purified Neurospora nuclei (3).

**Endogenous Ribonuclease Activity.** In the tests for endogenous RNase activity in nuclear preparations, degradation of purified Neurospora ¹⁴C-labeled cytoplasmic RNA was assessed in the presence of various nuclear preparations. In these experiments, the RNA was incubated with the nuclei and then the RNA was analyzed by acrylamide gel electrophoresis. Tubes of RNA without nuclei were also incubated and subjected to electrophoresis as a control for possible degradation not related to nucleases. Comparison of the radioactivity profiles of Neurospora cytoplasmic RNA and RNA incubated and centrifuged showed little difference in the relative radioactivity maxima. Incubation of the RNA with Percoll-purified nuclei did not appear to degrade the test RNA at all (Fig. 2). However, when the RNA was incubated in the presence of crude nuclei, there was evidence of a slight degradation, most visible in the 17S rRNA peak (Fig. 3). This degradation was alleviated by the addition of 5 mM EGTA, 100 mM KCl, and 125 mM (NH₄)₂SO₄, components known to eliminate nuclease activity in other systems. Addition of these “stabilizing” materials in the test of pure nuclei made no difference in the radioactivity profiles of the RNA.

**Nuclear Ribosomal RNA.** Attempts at the extraction of rRNA from crude and purified *N. crassa* nuclei in the past have met with considerable failure in resolving the presumptive rRNA into discrete peaks of radioactivity on acrylamide gels. The present studies, however, consistently produced RNA species which were clearly separated by their mol wt through gel electrophoresis.

Using the techniques described in “Materials and Methods,” much greater yields of rRNA with good optical densities ratios at

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**DISCUSSION**

The isolation of crude nuclei from germinated conidia of *N. crassa* was undertaken to produce nuclei for use in studies of precursor-rRNA synthesis and processing, and of ribonuclease-particle maturation. The only previous report of the isolation of nuclei from mycelial cultures of *N. crassa* was by Hautala et al. (3). Their work was motivated by a desire to study the synthesis of specific mRNA and no studies were done to determine whether precursor-rRNA and mature rRNA molecules could be isolated from the nuclei they obtained. In this paper we report a nuclear isolation procedure based on that of Hautala et al. (3), but modified to maximize the yield and to maintain the integrity of the RNA of our particular interest. The modifications include changes in growth conditions, buffer and gradient compositions and centrifugal forces.

In the work of Hautala et al. (3), nuclei were extracted from cultures of *Neurospora* conidia in liquid Vogel’s minimal medium grown at 25°C for 16 h. We found optimal yields with 8- to 9-h cultures. With our method, nuclear preparations of higher DNA content relative to previous ratios (3) were recovered, and lower relative protein content was found in both crude and purified nuclei, indicating a greater degree of purity.

In our attempts to use the method of Hautala et al. (3), we encountered problems in purifying nuclei when LUDOX gradients were used. Specifically, the colloidal silica particles of LUDOX precipitate at low temperatures, high salt concentrations, and in the presence of Triton X-100. The latter is a component of the extraction buffer used in the nuclei isolation steps immediately prior to the use of gradients. Our results with Percoll gradients indicate that Percoll is a very effective alternative to LUDOX for the purification of *Neurospora* nuclei from crude nuclear preparations. The problems of gradient precipitation, viscosity, and toxicity affecting subcellular particle integrity are eliminated by the coating of colloidal silica with PVP, as in the Percoll medium (6). The absence of large osmotic effects as is observed with other gradient materials has allowed us to determine the density of wild-type *N. crassa* nuclei to be 1.08 g/ml.

Cytoplasmic contamination of crude and purified nuclear prep-
arations was monitored by marking the nuclear isolation procedure with a radioactively labeled ribosome preparation by electron microscopy studies, through assays for DNA, RNA, and protein content, and with an assay for the presence of active extranuclear enzymes. In all tests, Percoll-purified nuclei had less contamination than crude nuclei. As shown by the ribosome-labeling studies, Percoll-purified nuclei were essentially uncontaminated with free cytoplasmic ribosomes that may have attached to the nuclear membrane during purification.

Major problems with endonuclease activity have been encountered in nuclear preparations in other systems (4, 5, 12, 14). In those cases, most of the RNA detected had S values between 4 and 10. Although discrete species of 17S and 25S rRNA have been found even in crude Neurospora nuclei, a check for nuclease activity was made in case precursor rRNA was degraded consistently. Some endogenous nuclease activity was detected when crude Neurospora nuclei were incubated with 14C-labeled Neurospora RNA, but essentially no nuclease activity was found in Percoll-purified nuclei. Finally, although extracted RNA from crude nuclei includes high-mol-wt species that are presumptive precursors to mature rRNA, studies of pre-rRNA processing in the nucleus will be greatly facilitated now that pure nuclei free of cytoplasmic and gradient-generated contaminants can be obtained.

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LITERATURE CITED