Isolation of Yeast Nuclear Pore Complexes and Nuclear Envelopes

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I. Introduction

Here we describe two isolation procedures for yeast, one producing nuclear pore complexes (NPCs), the other nuclear envelopes (NEs) and nuclear membranes, starting from highly enriched yeast nuclei. Both result in material of sufficiently high yield and degree of enrichment to be potentially useful in a variety of preparative and analytical studies, including the identification of NPC components and their localization in the NE.

II. Materials and Instrumentation

All catalogue numbers are indicated in parenthesis. Nycodenz (Accudenz, AN 7050) was obtained from Accurate Chemical and Scientific Corporation, Bradford's assay stock solution (Bio-Rad protein assay, 500-0006) from Bio-Rad, heparin (sodium salt, grade 1-A; H-3393) and sodium taurodeoxycholate (T-0875) from Sigma, dithiothreitol (DTT, Cleland's reagent, 233155) from Calbiochem, formaldehyde (~37% stock solution in 10% methanol, 47629) from Fluka, glutaraldehyde (~25% stock solution, 360802F) from BDH, Tween-20 (Surfact-Amps-20, 10% stock solution, 28320) from Pierce, pancreatic RNase (RNase A, 109169) from Boehringer, and centrifuge tubes from Beckman (SW55 Ultraclear, 344057) or from Sorvall (10 ml Ultra bottles, 03020, and screw caps, 03613). Carbon / formvar coated 300 mesh copper electron microscope grids (01821) and uranyl acetate (19481) were from Ted Pella, Inc. All other materials were obtained as described (Rout and Kilmartin, 1997; see "Preparation of Yeast Nuclei and Spindle Pole Bodies" method).

III. Procedures

As discussed, both procedures require the same starting material, highly enriched yeast nuclei (Rozijn and Tonino, 1964; Kilmartin and Fogg, 1982; Rout and Kilmartin, 1990). The method for the production of this nuclear fraction is described in the preceding chapter (Rout and Kilmartin, 1997). Best results are obtained with nuclei from S. uvarum, but both procedures have been successfully applied to S. cerevisiae strains with the inclusion of the appropriate modifications where necessary.

A. Preparation of a highly enriched nuclear pore complex fraction

Solutions

All solutions other than those listed below were prepared as described (Rout and Kilmartin, 1997). The following solutions can all be stored for long periods at either 4°C or -20°C.

1. NPC Extraction Solution: 10 mM bis-Tris-HCl, 0.1 mM MgCl2, 1.0% sodium taurodeoxycholate, 10 µg / ml RNase A, 0.5 mM DTT, pH 6.50.

2. Sucrose solutions in bt buffer: Prepare the 1.45 and 1.85 M sucrose-bt solutions (refractive indexes of 1.4032 and 1.4225 respectively) by dilution of stock 2.50 M sucrose-bt solution with bt buffer plus 0.02 volumes of 10% Triton X-100. It is very important that refractive index (measured at room temperature), rather than weight or volume, is used to measure the molarity of the sucrose solutions.

3. Sucrose solutions in bt-DMSO buffer: Prepare 1.75 M sucrose-bt-DMSO solution by dissolving 120 g sucrose in 20 ml of 0.1 M bis-Tris-HCl, pH 6.50, 20 µl 1 M MgCl2, 0.2 ml 10% Tween-20 and distilled water to a total volume of 150 ml. When the sucrose has dissolved, add 40 ml of DMSO and make up to a total volume of 200 ml with distilled water. Check the refractive index is close to 1.450. Prepare 1.00 sucrose-bt-DMSO solution by dissolving 342.3 g and sucrose in 100 ml of 0.1 M bis-Tris-HCl, pH 6.50, 100 µl 1 M MgCl2, 1 ml 10% Tween-20 and distilled water to a total volume of 700 ml. When the sucrose has dissolved, add 200 ml of DMSO and make up to a total volume of ~1000 ml with distilled water to a refractive index of 1.4120. Prepare 1.20 M sucrose-bt-DMSO solution similarly, but by starting with 408.9 g sucrose and making to a refractive index of 1.4220.

4. Solution P2: Dissolve 90 mg 4-(2-aminoethyl)benzenesulfonyl fluoride and 2 mg pepstatin A in 5 ml dry absolute ethanol.

Steps

1. All solutions contain a 1:1000 dilution of Solution P (freshly added) and are kept on ice, unless otherwise stated. Solution P can also be substituted with the more stable (and less toxic) Solution P2. Nuclear lysis and the separation of the lysate over a stepped sucrose gradient are exactly as described, with the crude NPC fraction being recovered from the S / 1.75 M interface. Usually ~5 ml of the fraction is recovered per Beckman SW28 tube, resulting in a total of 15 - 20 ml per 36-liter culture (Rout and Kilmartin, 1990, 1997).

2. The S / 1.75 M fraction must be assayed in order to determine the correct amount of heparin to add for the next enrichment step. Three assays can be used to arrive at a consensus.

Assay 1. To 100 µl aliquots of the S / 1.75 M fraction are added 20 µl of NPC Extraction Solution and varying amounts of 10 mg / ml and 100 mg / ml heparin stock solutions, covering a range of 0 - 0.5 mg / ml final heparin concentrations. These are incubated for 1 hr on ice and then their absorbance (A600nm) measured and compared with a 100 µl aliquot of the S / 1.75 M fraction containing no additions. The correct amount of heparin (together with the NPC Extraction Solution) usually results in a reduction of turbidity to 40% of the value of the untreated fraction.

Assay 2. 5 µl aliquots of the Assay 1 incubations are placed onto glow-discharged electron microscope grids and allowed to sit for 5 min at room temperature. Each grid is then rinsed with two drops of bt-DMSO and then inverted (sample side down) onto a drop of bt-DMSO containing 4% formaldehyde and 0.5% glutaraldehyde sitting on a sheet of Parafilm. After 10 min at room temperature, the grid is rinsed once more with several drops of bt-DMSO and then stained with 6 - 10 drops of 4% uranyl acetate in distilled water. The excess staining solution is wicked away with a sliver of filter paper, and after air drying the grid is ready to examine in the electron microscope. NPCs appear as donuts containing a plug in the middle; too much heparin causes eventual disintegration of the plug and the round donuts become distorted and oval. If there is too little heparin, on the other hand, contaminating material appears as aggregates of the same size or larger than the NPCs; enough heparin must be added to reduce the particulate contaminants to a size significantly smaller than that of the NPCs.

Assay 3. The protein concentration of the S / 1.75 crude NPC fraction is determined by using the Bradford's protein assay from BioRad following the manufacturer's instructions. The correct amount of heparin (from a 100 mg / ml stock) to add to the NPC extraction reaction (step 4, below) is then 0.045 mg per 1.0 mg of S / 1.75 M fraction protein. This assay is the most reliable and the one used routinely.

3. Before starting, make up the required number of gradient tubes: one Beckman Ultraclear SW55 tube per 1 ml of S / 1.75 M fraction to be processed, each containing 0.5 ml of the 2.50 M sucrose-bt solution, 1.5 ml of the 1.85 M sucrose-bt solution, and 1.5 ml of the 1.45 M sucrose-bt

solution (layered from bottom to top in that order). Also prepare a beaker full of distilled water pre-chilled by the addition of ice to a temperature of 10°C.

4. To each 1 ml of the S / 1.75 M fraction in a 50 ml Falcon tube, quickly add 0.2 ml of NPC Extraction Solution, 5 µl Solution P, and the correct amount of heparin (usually 0.045 mg heparin per mg of fraction protein, as in Assay 3). Start a timer immediately and thoroughly vortex the mixture for 30 sec before placing the tube in the chilled beaker of water.

5. Incubate for a total of 15 min, vortexing the tube for 5 sec every 3 min.

6. Centrifuge the tube to remove the froth (700 g, 4 min, 4°C) and then load the resulting extracted fraction onto the gradient tubes, 1.2 ml per tube. This sample layer is overlaid with ~0.4 ml of bt-DMSO (for balance) and the tubes centrifuged in a Beckman SW55 Ti rotor at 50,000 rpm for 5 hr at 4°C.

7. After the spin is complete, hold the tubes against a black background with a strong light illuminating them from above. A strong sharp white band should be visible at the S / 1.45 M interface whilst beneath it at the 1.45 M / 1.85 M interface should be a weaker diffuse white band. Occasionally a faint sharp white band can be found immediately above the diffuse band, but this has not seemed to be a cause for concern. A very faint band is also often discernible at the 1.85 M / 2.50 M interface, particularly if the tubes are allowed to warm slightly. The tubes are unloaded by volume from the top, at the walls of the tube. The top fraction from every tube is collected and pooled before starting to collect the second fraction from any tube, and so on for each fraction. The first 1.5 ml from each tube is collected, and the resulting pool from all tubes is termed the S fraction. The second 1.5 ml pool (containing the strong sharp white band) is called the S / 1.45 fraction. The enriched NPC fraction is recovered in 1.3 ml from the 1.45 M / 1.85 M interface (containing the weaker diffuse white band), and termed the 1.45 / 1.85 fraction. The final 0.8 ml containing the 1.85 M / 2.50 M interface (installed for diagnostic purposes; insufficient extraction will cause NPC material to begin to accumulate in this fraction) and is called the 1.85 / 2.50 fraction. The fractions can be stored at this stage by freezing at -70°C. For most analytical purposes the enriched NPC fraction (in which the NPCs represent 20 - 30% of the total protein) is clean enough, and has the advantage over the highly enriched NPC fraction of being significantly more concentrated. Note the presence of a significant amount of particulate contaminants.

8. If the highly enriched NPC fraction is required, then immediately beforehand make up one gradient tube per 2 ml of the 1.45 / 1.85 enriched NPC fraction to be processed, each being a Beckman Ultraclear SW28 tube containing a 5 ml cushion of the 1.75 M sucrose-bt-DMSO solution overlaid with a continuous linear gradient formed from 14.5 ml of the 1.20 M sucrose-bt-DMSO and 14.5 ml of the 1.00 sucrose-bt-DMSO solution. These gradients are delicate and so must be handled carefully.

9. The enriched NPC fraction is diluted with an equal volume of bt-DMSO buffer containing a 1:500 dilution of solution P.

10. 4 ml aliquots of this are gently overlaid onto each gradient tube, and then this sample layer is gently overlaid with a little bt-DMSO for balance if necessary. All the tubes are then centrifuged in a Beckman SW28 rotor at 28,000 rpm for 24 hr at 4°C.

11. After centrifugation, remove each tube from the rotor and hold it against a black background with a strong light illuminating it from above. There should be two diffuse bands visible; an extremely faint white band about one-third of the way down the tube, and a stronger band just above the 1.20 M / 1.75 M interface; it is this latter band that contains the NPCs.

12. As the DMSO also contributes to the refractive indexes it is difficult to completely prevent batch-to-batch variations in the sucrose-bt-DMSO solutions. Therefore the desired separation is sometimes not seen. The 24 hr centrifuge run seems to be a minimum, so any such variation can

usually be dealt with by centrifuging the tubes for longer times. Normally the tubes should be replaced in the rotor, spun again at 28,000 rpm for 2 hr at 4°C, and the position of the lower band noted again at the end of the run. Repeat this step until the required band separation is achieved.

13. Each gradient tube is unloaded from the top, at the tube wall. Usually a total of 5 fractions are collected from each gradient tube. The first 9 ml is termed fraction #1 and the second 9 ml, fraction #2. The third 9 ml, fraction #3, is collected to within about 5 mm above the lower diffuse white band. Fraction #4 is the 10 ml above and containing the 1.20 M / 1.75 M interface. It is this fraction that contains the NPCs and is referred to as the highly enriched NPC fraction. Fraction #5 was the final 1 ml or so collected after vortexing to resuspend any pellet. A more cautious approach is to collect more fractions, each containing less volume; for example, if the position of the NPCs is not certain, then 13 fractions may be collected, representing 3 ml per fraction from each gradient tube. The fractions are stored at -70°C. Assay 2, or SDS-polyacrylamide gel analysis (below), can be used to determine the position of the NPCs, and the NPC-containing fractions then pooled. Note the removal of almost all of the particulate contaminants.

An important method of quality control is to run SDS-polyacrylamide gels of aliquots from each fraction. An aliquot of each fraction proportional in volume to the total volume of that fraction should be taken. Hence, if 4.0 ml of S / 1.75 M fraction were processed to yield 6.0 ml of S, 6.0 ml of S / 1.45, 5.2 ml of 1.45 / 1.85, and 0.8 ml of 1.85 / 2.50 fractions, then aliquots of (say) 12 µl of the S, 12 µl of the S / 1.45, 10.4 µl of the 1.45 / 1.85, and 1.6 µl of the 1.85 / 2.50 fractions should be taken.

B. Preparation of a highly enrich Nuclear Envelope fraction

Solutions

All solutions other than those listed below were prepared as described for the NPC preparation (Section A) or in the preceding chapter (Rout and Kilmartin, 1997).

1. Sucrose / Nycodenz solution: Warm 20 ml of H20 in the microwave until it reaches close to boiling temperature. Add 20 g of Nycodenz and dissolve it by vigorous stirring on a heating plate. When the Nycodenz is dissolved add 10.0 ml of 100 mM bis-Tris-HCl, pH 6.50, 10 µl of 1.0 M MgCl2 and 71.88 g of sucrose and H20 to just below 100 ml. After the sucrose has dissolved let the solution cool before bringing the volume to 100 ml.

2. Sucrose solutions in bt buffer: Prepare 1.50 M (refractive index 1.4057), 2.00 M (refractive index 1.4295) and 2.25 M (refractive index 1.4414) sucrose-bt solutions by dilution of the stock 2.50 M solution with bt buffer. It is very important that refractive index (measured at room temperature), rather than weight or volume, is used to measure the molarity of the sucrose solutions.

Steps

1. Nuclei pellets are prepared as described in step 1 of the “Preparation of Spindle Pole Bodies” section from the preceding chapter (Rout and Kilmartin, 1997). The tubes containing the nuclei pellets are placed on ice.

2. The following procedure is carried out in the cold room and one tube at a time. Add to the first tube the following precooled solutions: 1.0 ml bt-DMSO, 1.0 µl 2.0% DNase I and 10.0 µl Solution P per 100 OD260 units. Immediately resuspend the pellet by vortexing at maximum setting. Note that is extremely important that the solution swirls around the sides of the tube, such that the shear force needed to lyse the nuclei efficiently is developed. This step can take up to 10 min. It is important to start vortexing the tubes immediately after adding the nuclear lysis solution to the nuclei pellet. Typically all the solutions are prepared in advance, precooled on ice and

transported to the cold room where a vortexer is already in place. The solutions are added at the last minute and the vortexing is started and continued with the minimum number of interruptions until the pellet has dissolved. When the pellet is completely resuspended transfer the tube to room temperature, warm it to approximately 25° C by holding it in the hand and then incubate it for 5-10 min at room temperature. The nuclear lysate must be properly warmed up before incubation at room temperature. If the lysate has not reached room temperature, then the DNA digestion may not be complete at the end of the incubation and the nuclear envelopes will not float up properly in the ensuing gradient. After this incubation, replace the tube on ice. Repeat the procedure described in this step for the other tubes. Successful nuclear lysis may be checked by light microscopy. Under a 100x phase-contrast oil immersion objective the NEs appear as short black lines, many in the shape of a "C" (retaining the original curvature of the NE), and there should be little other large particulate debris in the lysate though many small particles.

3. 1.0 volume of Sucrose / Nycodenz solution is then added to each tube, the samples are thoroughly mixed and the tubes are centrifuged in a Beckman 70 Ti rotor or equivalent (i.e. Beckman 50.2 Ti or Sorvall T865 rotor) at 6,000 rpm for 6 min at 4° C. The supernatants are removed without disturbing the pellet and pooled in a fresh tube. 2.0 times the initial volume of Sucrose / Nycodenz solution are added and the samples are mixed thoroughly.

4. The sample is divided in the appropriate number of Beckman SW28 Ultraclear tubes by adding 13 ml of it to each tube. A 36-liter nuclei preparation usually yields enough nuclear lysate to fill 6 Beckman SW28 Ultraclear tubes. Each tube is overlaid with 12 ml 2.00 M sucrose-bt and 12 ml 1.50 M sucrose-bt, and centrifuged in a Beckman SW28 rotor at 28,000 rpm for 24 hrs at 4°C.

5. The tubes are unloaded from the top. A faint white band at the top of the tube contains a few vesicular remnants and is completely removed (collect ~6.0 ml per tube). The NEs are found at the 1.50 M / 2.00 M interface, appearing as a broad, white, slightly flocculent band (collect ~12.0 ml per tube). The protein concentration of this fraction is typically ~0.5-1.0 mg/ml, i.e. ~35-70 mg total are obtained from a 36-liter nuclei preparation. Next is a dense, sharp yellowish / white band containing a few nuclear envelopes and dead cell remnants (collect ~12.0 ml per tube). The final ~6.0 ml, including a dense brownish / white pellet, contains soluble and particulate matter mainly derived from chromatin and cell wall remnants that contaminated the nuclei preparation. The NE-containing fraction may be stored at -70°C. When examined by electron microscopy using the procedure described above (Section A, Step 2, Assay 2) the NEs appear as sheets studded with small white circles (the ribosomes on the outer nuclear membrane) and dark structured larger circles (the NPCs). Microtubules from SPBs can sometimes be seen projecting from the NEs, as can occasional isolated SPBs torn out of their NEs.

C. Preparation of an enriched nuclear membranes fraction

Solutions

1. Heparin Extraction solution (HES): Prepare a 100 mg / ml Heparin stock by dissolving 5 g of Heparin in distilled water and making up to 50 ml (store at -20° C). To prepare 50 ml of HES (prepared fresh every time) add 5 ml 100 mg / ml Heparin, 5 ml 100 mM bis-Tris-HCl, pH 6.5, 5 ml 1 M MgCl2, 5 ml 1M DTT and 0.25 µl Solution P to 39.74 ml of distilled water.

2. RNase stock: Dissolve 100 mg of RNase A in 10 ml 0.01 M Na acetate, pH 5.2. Heat to 100° C for 15 min. After cooling slowly to room temperature adjust the pH by adding 0.1 volumes of 1 M Tris-HCl, pH 7.4. Store aliquots at -20°C.

3. Sucrose solutions in bt buffer: Prepare 1.00 M (refractive index 1.3815) and 2.00 M (refractive index 1.4295) sucrose-bt solutions by dilution of the stock 2.50 M solution with bt buffer, as above.

4. KCl extraction solution (KES): To prepare 50 ml of KES (prepared fresh every time) mix 12.5 ml 2.0 M KCl, 5 ml 1 M DTT and 0.25 µl Solution P with 37.25 ml of distilled water.

Steps

1. The 1.5 / 2.0 nuclear envelope fraction is mixed with 5 volumes of HES on ice. Typically 0.2 volumes (i.e. ~14 ml) of the total amount obtained from 3 x 1011 spheroplasts are used to prepare nuclear membranes and mixed with 70 ml of HES. The mixture is vortexed twice for 10 sec each time at maximum setting and incubated 1 hr on ice. At the end of the incubation, the RNase stock solution is added to a final concentration of 10 mg / ml and the incubation is continued for 15 min at 10°C in a plastic beaker containing water and ice (as with Section A, Step 5 above).

2. Sucrose step gradients are prepared in the appropriate number of Beckman Ultraclear SW28 tubes, keeping in mind that ~20 ml of extracted nuclear envelope sample is loaded over each gradient. To prepare the gradients, 9 ml of 1.00 M sucrose-bt are overlaid onto 9 ml of 2.00 M sucrose-bt in each centrifuge tube. The heparin-extracted nuclear envelopes sample is loaded over the sucrose-bt step gradients (~20 ml per gradient). The tubes are centrifuged in a Beckman SW28 rotor at 28,000 RPM for 1 hr and 15 min at 4°C.

3. The fractions are collected from the top of the tube using a hand-held pipette. The first fraction (~18 ml per tube) is collected by aspirating until just above the 1.00 M interface. This fraction is clear and contains the bulk of the soluble proteins. The next fraction (~9 ml per tube) is collected from just above the first interface to just above the 1.00 M / 2.00 M interface. This fraction is also clear and contained some of the soluble proteins together with a few of the nuclear envelope membranes. The bulk of heparin extracted nuclear membranes is recovered at the 1.00 M / 2.00 M interface and appears as a rather tight white band (~5 ml per tube). The last fraction (~6 ml per tube) consists of the remainder of the gradient including the pellet. It is clear and some times contains small amounts of nuclear membranes. The enriched nuclear membrane fraction can be stored at -70°C.

4. If necessary, traces of heparin can be removed from the enriched nuclear membranes fraction by extracting it with 0.5 M KCl. The nuclear membrane fraction is thoroughly mixed with 3 volumes of KES and incubated for 1 hr on ice. After the incubation, the mixture is loaded over a 5 ml 1.00 M sucrose-bt cushion in the appropriate number of Beckman SW28 Ultraclear tubes and the nuclear membranes are decanted by centrifugation at 28,000 RPM for 1 hr and 15 min at 4° C. The pellet of nuclear membranes is resuspended in 6.5 ml of 1.50 M sucrose-bt per tube. The KCl washed nuclear membranes fraction can be stored at -70° C. When examined by electron microscopy using the procedure described above (Section A, Step 2, Assay 2) the H-NEs have lost the ribosomes, giving them a smooth appearance, and the exposed NPC proteins have also been removed, leaving only the empty NPC pores. Often the H-NEs have a smooth curvature, likely retaining the original curvature of the NEs.

IV. Modifications

Often NEs are only required in analytical amounts from S. cerevisiae strains. They may be prepared by the following modifications to the numbered steps in the original NE preparation protocol (Section B):

1. Load ~50 OD260 units of nuclei in a Sorvall 10 ml Ultra bottle, add 0.2 volumes of 8% PVP solution and 0.01 volumes of Solution P, and mix well by vortexing. Allow to settle on ice, overlay to the top of the tube with 8% PVP solution, and centrifuge in a Beckman 70.1 Ti rotor or Sorvall T-875 rotor, 40,000 rpm, 1 hr, 4°C.

2. Aspirate the supernatant thoroughly and resuspend by vortexing the nuclei into 0.5 ml of bt-buffer (not bt-DMSO buffer) plus 10μM CaCl2 and 10μM ZnCl2, 0.5 μl 2.0% DNase I, and 5 μl Solution P. Allow to stand for 5 min at room temperature.

3. Centrifuge for 1 min at 2000 g. Add 2.0 ml of Sucrose / Nycodenz solution to the resulting supernatant and vortex for 1 min.

4. Place in a Beckman SW55 centrifuge tube. Overlay this with 1.5 ml of 2.25 M sucrose-bt containing 0.005 volumes of Solution P and then overlay this to within 2 mm of the top of the tube with 1.50 M sucrose-bt plus 0.005 volumes of Solution P. Centrifuge in a Beckman SW55 rotor at 50,000 rpm for 24 hr at 4°C.

5. After the spin, a dense flocculent white band should be visible at the 1.50 M / 2.25 M interface, and this is where the NEs may be found. The gradient can then be unloaded from the top, in whatever volume fractions seem appropriate (depending on the size of the NE band).

V. Pitfalls

One should note that the procedures for preparing the highly enriched NPC fraction and NE fraction described in this chapter do not work well when nuclei prepared using techniques other than the one described (Rout and Kilmartin, 1997) are used as a starting material. This may be related to the fact that the conditions used to pack the nuclear pellet cannot be varied much. If the pellet is too loose, the nuclei resuspend without lysing properly; whilst if too firm, the vortexing will not resuspend them at all. Also, variations in available equipment may necessitate some experimentation to determine the correct lysis conditions for each laboratory. These points cannot be stressed too hard because they have been the source of much frustration for people trying to reproduce this technique.

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