Isolation and Characterization of Nuclear Envelopes from the Yeast *Saccharomyces*

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Abstract. We have developed a large scale enrichment procedure to prepare yeast nuclear envelopes (NEs). These NEs can be stripped of peripheral proteins to produce a heparin-extracted NE (H-NE) fraction highly enriched in integral membrane proteins. Extraction of H-NEs with detergents revealed previously uncharacterized ring structures associated with the NE that apparently stabilize the grommets of the nuclear pore complexes (NPCs). The high yields obtained throughout the fractionation procedure allowed balance-sheet tabulation of the subcellular distribution of various NE and non-NE proteins. Thus we found that 20% of endoplasmic reticulum (ER) marker proteins are localized at the NE. Using a novel monospecific mAb made against proteins in the H-NE fraction and found to be directed against the pore membrane protein POM152, we showed that while the majority of POM152 is localized in the NE at the NPC, a proportion of this protein is also present in the ER. This ER pool of POM152 is likely to be involved in the duplication of nuclear pores and NPCs during S-phase. Both the NEs and H-NEs were found to be competent for the in vitro posttranslational translocation of prepro-α-factor. They may also be suitable to investigate other ER- and NE-associated functions in cell-free systems.

T HE nuclear envelope (NE)[1] defines the boundary of the nucleus in eukaryotic cells and is composed of two distinct membranes enclosing a lumenal (perinuclear) space. Facing the nucleoplasm is the inner nuclear membrane, which in higher eukaryotes is often lined by a filamentous network called the nuclear lamina. Towards the cytoplasm is the outer nuclear membrane which is continuous with the ER membranes and is thought to perform rough ER functions. The inner and the outer nuclear membranes join to form specialized circular apertures containing the nuclear pore complexes (NPCs), which regulate the exchange of material between the nucleus and cytoplasm. It is widely assumed that the NE plays a role in the control of nuclear architecture both during interphase and at mitosis. More specifically, it has been proposed that the NE could contribute in defining the spatial distribution of specific segments of the genome such as the telomeres inside the nucleus, thereby facilitating the regulation of DNA transcription and replication. Some enzymatic activities may also be restricted to the NE; for example it is likely that proteins involved in the nucleus-specific phosphoinositide metabolism are localized to the inner nuclear membrane. Despite considerable progress in the past few years, the molecular details of many important functions of the NE still remain poorly defined (for reviews see Hurt et al., 1992; Gilson et al., 1993; Rout and Wente, 1994; Moore and Blobel, 1994; Kilmartin, 1994).

In the yeast *Saccharomyces*, morphometric studies indicate that the NE represents roughly 30% of the functional rough ER (Preuss et al., 1991). Thus, the isolation of NE from yeast would be useful for the study of both NE and rough ER functions. Furthermore, yeast present numerous advantages over higher eukaryotes as a system to study these functions. They have neither the complications of developmental regulation of nuclear processes, nor of nuclear disassembly, having a closed mitosis; in addition, the genetics and molecular biology of yeast are better understood than in any other eukaryote, and a large program is under way to complete the entire yeast genome sequence by the end of the decade (Maddox, 1992). Unfortunately the cell biological and biochemical characterization of cellular membranes and compartments in budding yeast remains incomplete, and would benefit from the development of rigorous cellular fractionation techniques comparable to the ones available for higher eukaryotes.

We describe here a procedure for the preparation of a highly enriched NE fraction from the yeast *Saccharomyces*. NEs were prepared from yeast nuclei on a large scale and in high yield. To understand the relationship between peripheral and integral membrane components that define

1. *Abbreviations used in this paper.* CM, crude microsome; gpαF, glycosylated pro-α-factor; H-NE, heparin-extracted NE; MT, microtubule; NE, nuclear envelope; NPC, nuclear pore complex; ppαF, prepro-α-factor; PVP, polyvinylpyrrolidone; SPB, spindle pole body.

the various functions of the NE, we prepared a highly enriched nuclear membrane fraction by stripping the NE fraction with heparin. The fractionation pattern of representative markers throughout the procedure was used to tabulate the distribution of various cellular organelles and functions within the cell. Heparin-extracted NEs (H-NEs) were used to raise a panel of mAbs, one of which is described in this paper. Both the NE and H-NE fractions were shown to be functional in an ER protein translocation assay and thus retain one of numerous potentially testable functions that are associated with the NE in vivo. Detergent extraction of H-NEs showed that ring structures associated with the NE are likely to be responsible for anchoring the NPCs to their grommets and stabilizing the pore membrane domain.

Materials and Methods

Yeast Subcellular Fractionation: Preparation of Enriched Nuclei and Highly Enriched NPCs

The yeast strain *Saccharomyces uvarum* (NCYC 74, ATCC 9080; American Type Culture Collection, Rockville, MD), considered a strain of *Saccharomyces cerevisiae* (Mortimer and Johnson, 1986), was used throughout the procedure. Enriched nuclei were prepared as previously described (Rout and Kilmartin, 1990, 1994). Briefly, 70–90 g of mid-log phase cells were obtained from a 36 liter yeast culture. Cells were harvested and converted to spheroplasts in 1.1 M sorbitol (Rout and Kilmartin, 1994). Spheroplasts were harvested by centrifugation and then lysed in 300 ml of polyvinylpyrrolidone (PVP) solution (8% PVP, 20 mM K-phosphate, pH 6.5, 0.75 mM $MgCl_2$). The cell lysate (fraction 1) was separated by centrifugation (15 min at 10,000 g) into crude cytosol (fraction 2) and a crude nuclei pellet (fraction 3). The nuclei were resuspended in 144 ml of 1.7 M sucrose in PVP solution and this suspension was divided into 12 equal aliquots. Each aliquot was overlayered over a three step sucrose/PVP gradient (8 ml each of 2.01 M sucrose, 2.10 M sucrose, and 2.30 M sucrose in PVP solution) in a SW28 tube (Beckman Instruments, Palo Alto, CA). The gradients were centrifuged in a rotor (Beckman SW28) at 28,000 rpm (103,000 g) for 4 h at 4°C. After centrifugation fractions were collected from the top. The load zone, including a thick layer at the top of the tube (fraction 4), and a thick yellowish band at the load/2.01 M interface (fraction 5), both contained intact mitochondria, vesicles and microsomes (as judged by EM). Very little material was present in the third gradient fraction at the 2.01/2.10 M interface (fraction 6). A dense white band at the 2.10 M/2.30 M interface contained the bulk of the nuclei (fraction 7). The bottom of the gradient (fraction 8) included a pellet composed mainly of cells remnants.

Highly enriched NPCs were prepared from nuclei (fraction 7) exactly as described in Rout and Blobel (1993).

Yeast Nuclear Envelope Preparation

NEs were prepared from the enriched nuclear fraction (fraction 7). The OD at 260 nm of the nuclear fraction was measured after 1 in 100 dilution in 1.0% SDS; approximately 1,000–2,000 OD_{260} nms were obtained from a 36 liter preparation. The nuclear fraction was adjusted to a refractive index of 1.4340 with PVP solution and centrifuged at 145,000 g for 1 h at 4°C. The supernatant was carefully but thoroughly removed by aspiration, and the tubes placed on ice. Typically, 20 ml of freshly prepared, ice cold bt-DMSO (bt buffer [10 mM *bis*Tris-Cl, pH 6.5; 0.1 mM $MgCl_2$] containing 20% [vol/vol] DMSO) in the presence of 20 μg/ml DNase I-EP (Sigma Chemical Co., St. Louis, MO) and 1% (vol/vol) solution P (18 mg/ml PMSF, 0.3 mg/ml pepstatin A in absolute ethanol), were added to 2,000 OD_{260} nm of nuclei. This was followed immediately by vigorous vortexing at 4°C until the pellet was completely resuspended. The suspension was then incubated at room temperature (~25°C) for 5–10 min. After incubation, the lysed nuclei were placed back on ice and 60 ml of 2.1 M sucrose, 20% Nycodenz (Accudenz; Accurate Chemical and Scientific, Westbury, NY) in bt buffer in the presence of 0.1% (vol/vol) solution P, were added, and thoroughly mixed. The suspension was divided into six tubes (Beckman SW28) and overlayered with 12 ml of 2.0 M sucrose (R.I. = 1.4295)

and 12 ml of 1.5 M sucrose (R.I. = 1.4055) in bt buffer containing 0.1% (vol/vol) solution P. The tubes were centrifuged in a rotor (Beckman SW28) at 28,000 rpm (103,000 g) for 24 h at 4°C. The tubes were unloaded from the top using a hand-held pipette. A faint white band at the top of the tube was completely removed (~6.0 ml collected per tube; fraction 9). The NEs were found at the 1.5 M/2.0 M interface, appearing as a broad, white, slightly flocculent band (~12.0 ml collected per tube; fraction 10). Next was a dense, sharp yellowish/white band containing a few NEs, chromatin, and cell remnants (~12.0 ml collected per tube; fraction 11). The final ~7.0 ml collected (fraction 12), including a dense brownish/white pellet, contained soluble and particulate matter mainly derived from chromatin.

Extraction of Yeast Nuclear Envelopes

For heparin extraction, 0.6 ml (~0.4 mg of protein) of the yeast NE fraction were mixed with 2.4 ml of a solution containing 10 mg/ml heparin (Sigma Chemical Co.), 0.1 mM DTT and 0.5% (vol/vol) solution P in bt buffer. After 1 h on ice, 50 μg/ml RNase A was added and the incubation was continued for 15 min at 10°C. The sample was over-layered onto two 1-ml layers of 1.0 and 2.0 M sucrose in bt buffer containing 0.1% (vol/vol) solution P, and centrifuged in a rotor (Beckman SW55) at 45,000 rpm (~192,000 g) for 30 min at 4°C. The tube was unloaded from the top using a hand-held pipette. The first fraction (~2 ml; fraction 13) contained the bulk of the solubilized proteins. The next fraction (~1.8 ml; fraction 14) contained some of the soluble proteins together with a few of the NE membranes. The bulk of H-NE membranes was recovered at the 1.0 M/2.0 M sucrose interface and appeared as a tight white band (~0.4 ml; fraction 15). The last fraction (~0.8 ml; fraction 16) sometimes contained small amounts of H-NEs.

The yeast NE fraction was extracted with sodium carbonate using a previously described method (Wozniak et al., 1994).

Posttranslational Translocation Assay

The procedures for the preparation of yeast nuclei and NEs described above were modified to maintain the ER-translocation activity throughout the fractionation procedure. Firstly, yeast spheroplasts were allowed to recover in YPD medium (1% yeast extract, 2% peptone, 2% dextrose) containing 1.0 M sorbitol for 30 min at room temperature before lysis. Secondly, all the solutions starting from the lysis buffer and including all the gradient solutions were supplemented with 2 mM DTT. Finally, the $MgCl_2$ concentration in the nuclear lysis buffer and in the solutions used for the NE flotation gradient (fractions 9–12) was raised from 0.1 to 0.5 mM. The degree of enrichment of "active" NEs was shown to be similar to that obtained with the original method (data not shown). Just before the in vitro protein translocation reaction the "active" NE fraction was concentrated 20-fold by pelleting at 70,000 g for 30 min, and gently resuspending in solution A (20 mM Hepes-KOH, pH 7.4, 100 mM KOAc, 2 mM Mg[OAc]$_2$, 2 mM DTT) containing 0.25 M sucrose. The heparin extraction of the "active" NE fraction was carried out as described above except that 2 mM DTT was added to all solutions and gradients, the RNase A digestion step was omitted and the H-NEs were pelleted through a 1.0 M sucrose cushion (1.0 M sucrose, 2 mM DTT, 0.5 mM $MgCl_2$, 0.5% [vol/vol] solution P in bt buffer) instead of being recovered over 2.0 M sucrose. Heparin traces were removed by resuspending the "active" H-NEs pellet obtained from 2.4 ml of NEs, in 2.4 ml of 0.5 M KCl, 2 mM DTT, 0.25 M sucrose, 0.5% (vol/vol) solution P and incubating the suspension for 1 h on ice. The membranes were recovered by centrifugation through a 0.5 ml, 0.6 M sucrose cushion (0.6 M sucrose; 10 mM *bis*Tris-Cl, pH 6.5; 0.5 mM $MgCl_2$; 0.1% [vol/vol] solution P), at 39,000 rpm (~100,000 g) in a TLS-55 Beckman rotor for 1 h at 4°C. The supernatant from the 0.5 M KCl wash was shown not to contain significant amounts of extracted proteins (data not shown). Finally, the sample was resuspended in a volume of 0.25 M sucrose in solution A equal to roughly 2.5% of the initial NEs volume.

Yeast crude microsomes (CMs), used as a positive control for the ER translocation reaction, and yeast crude cytosol were prepared as described (Waters et al., 1986; Waters and Blobel, 1986). [^{35}S]Methionine labeled prepro-α-factor (ppαF) was synthesized using a wheat germ in vitro translation kit (Promega Biotec, Madison, WI) following the specifications of the manufacturer. Immediately before use, the translation mixture containing ppαF was diluted with 3 vol of 8 M urea and incubated for 10 min at 20°C. The translocation reaction and the protease protection assays were performed as described (Waters and Blobel, 1986; Chirico et al., 1988). Typically, the translocation mix (total volume 150 μl) consisted of the following: 43.6 μl of "master mix" (14.4 mM Hepes-KOH, pH 7.4; 276

mM KOAc; 1.0 mM Mg[OAc]$_2$; 1.0 mM DTT; 1.7 mM ATP; 86 mM creatine phosphate; 0.7 mg/ml creatine kinase; 0.07 mM GDP-mannose; 0.07 mM UDP-glucose; 0.07 mM UDP-N-acetylglucosamine; 1.4% glycerol), 90 µl of yeast crude cytosol in solution A containing 1.0 mM Mg-ATP, and 14 µl of CM, NE, or H-NE membranes in solution A containing 0.25 M sucrose. This mixture was pre-incubated at 20°C for 5 min and the import reaction was then started by the addition of 2.4 µl of urea-denatured translation product. At the end of the reaction the sample was divided in three equal aliquots. 30 µl of water were added to the first aliquot. 10 µl of 8 mM CaCl$_2$, 10 µl of water and 10 µl of 800 µg/ml trypsin were added to the second aliquot. The third aliquot was treated as the second except that 10 µl of water were substituted with 10 µl of 8% (vol/vol) Triton X-100. All aliquots were incubated on ice for 30 min and the reactions were stopped by the addition of 10 µl of 50 mM PMSF. After an additional 10 min on ice, the samples were TCA precipitated and analyzed by SDS-PAGE and fluorography.

Miscellaneous

Protein concentrations were measured by either the modified Bradford assay of Pierce (Coomassie Plus; Pierce, Rockford, IL), or the Amido-black assay (Rexach et al., 1994), using BSA as a standard. SDS-PAGE, immunoblotting, and EM were performed essentially as described (Rout and Blobel, 1993). Detergent extracted H-NEs were produced by a pretreatment of the sample on the EM grid in 1.5% Triton X-100, 2.0% Digitonin in bt buffer for 15 min at 25°C immediately prior to fixation. Highly enriched NPCs (Rout and Blobel, 1993) were extracted with heparin by mixing 5 µl of the sample with 20 µl of 10 mg/ml heparin in bt-DMSO and incubating on ice for 30 min. After the extraction, the heparin-resistant material was sedimented over an EM grid and negatively stained as previously described (Rout and Blobel, 1993). The intensity of bands on immunoblots was quantified using the ImageQuant software in the Phosphor-Imager system (Molecular Dynamics, Sunnyvale, CA). Mice were immunized with native H-NEs using standard methods and mAbs were raised, identified, and isolated as previously described (Rout and Kilmartin, 1990). Cells were prepared for indirect immunofluorescence microscopy using the procedure of Kilmartin and Adams (Kilmartin and Adams, 1982) with the modifications of Wente et al. (1992) and Kilmartin et al. (1993). Double labeling with the mouse MAb118C3 and a polyclonal rabbit anti–Sec61p antibody (Stirling et al., 1992) was visualized using Cy3-labeled polyclonal donkey anti–mouse IgG (cross absorbed against rabbit IgG) and FITC-labeled polyclonal donkey anti–rabbit IgG (cross absorbed against mouse IgG) (Jackson ImmunoResearch Laboratories, West Grove, PA). The staining and photomicrographic recording conditions were as described (Wente et al., 1992)

Results

Comments on the Procedure

An excellent starting point for the preparation of NE fractions was provided by the yeast nuclear isolation method described by Kilmartin. Thus, fractions 1 to 8 of the procedure described here (Fig. 1), which included the nuclei (fraction 7), were prepared as reported (Kilmartin and Fogg, 1982; Rout and Kilmartin, 1990). To be useful as a more general assay for subfractionation, as well as a preparative method for NEs, the NE enrichment procedure needed to have a considerably higher yield and degree of enrichment than previous techniques (Mann and Mecke, 1982a,b; Kilmartin and Fogg, 1982). It was also of primary importance to retain, when possible, the morphological (and potentially functional) characteristics of intact NEs.

The mild buffer conditions previously determined to be favorable for nuclear fractionation were retained for the preparation of NEs (Kilmartin and Fogg, 1982; Rout and Kilmartin, 1990; Rout and Blobel, 1993). Nuclei were lysed in bt-DMSO in the presence of DNase. The presence of DMSO appeared to lessen the osmotic shock to the NEs during nuclear lysis in addition to its stabilizing effect on
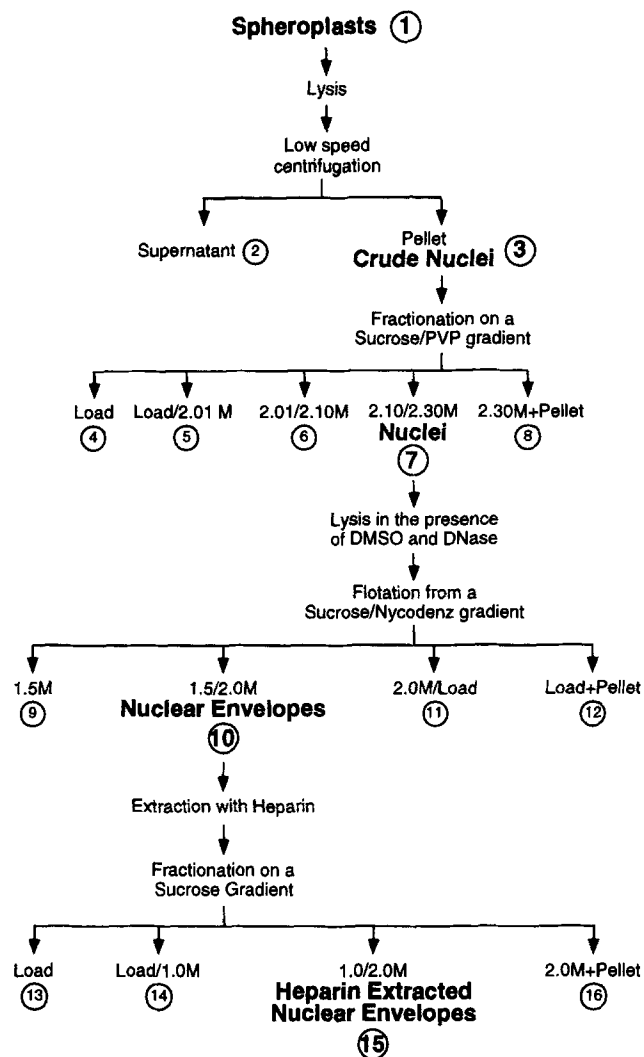

Figure 1. Schematic diagram of the yeast NE enrichment and NE heparin extraction procedures. Numbers enclosed in circles represent the fraction numbers as described in Materials and Methods and in the text. The NE-containing fractions are indicated in bold type.

spindles and NPCs (Rout and Kilmartin, 1990; Rout and Blobel, 1993). By increasing the density of the nuclear lysate with sucrose and Nycodenz, the NEs could be made to float to their buoyant density on an equilibrium sedimentation gradient, away from denser protein and nucleoprotein contaminants. The presence of Nycodenz in the adjusting solution reduced the viscosity of the resulting adjusted lysate, increasing the yield by ensuring the rapid egress of even the smaller NE fragments from the lysate. It also allowed a lower osmolarity, which reduced the osmotic shock to the NEs, especially when floating from the lysate into the lighter layer above. An appropriate step gradient was chosen to concentrate the NEs at a single step interface. The relatively narrow range of NE densities is evidenced by the high yield of NEs recovered in this layer (see below).

For the removal of the peripheral NE proteins and nucleoproteins, a heparin treatment was chosen instead of the more usually employed high pH treatments (Fujiki et

al., 1982) for two main reasons. First, the heparin treatment was performed in mild buffer conditions similar to those of the NE isolation procedure, lessening the chances of compromising membrane integrity or damaging membrane-bound complexes. Second, it proved especially efficient at removing the most significant peripheral NE "contaminants": chromatin (Courvalin et al., 1982; Rout and Blobel, 1993) and ribosomes. Indeed, purified yeast ribosomes were reduced from their normal sedimentation coefficient of 80 to <6 S by this treatment (data not shown). It was also known that high heparin concentrations would disassemble NPCs (Rout and Blobel, 1993; see below). The heparin-extracted material was run on a 10-40% (wt/vol) sucrose gradient and the sedimentation profile of the nucleoporins recognized by MAb414 and MAb350 was analyzed (Davis and Fink, 1990; Rout and Blobel, 1993). As expected, all of the detectable extracted nucleoporins displayed a behavior consistent with a sedimentation coefficient of <6 S (data not shown). A treatment with RNase was performed because although it did not have a significant effect on the protein composition of H-NEs, it was shown to effectively remove contaminating ribosomal RNA from this fraction (data not shown). This indicates that RNase digestion can be eliminated without significantly altering the efficiency of the heparin extraction procedure. The great disparity in size between the extracted material and the remaining H-NE membranes allowed the latter to be sedimented away from the former by a rapid centrifugation step.

Electron Microscopy of the NE Fractions

The NE and H-NE fractions were examined in detail by transmission EM of both negatively stained spreads and thin sections of pelleted material (Fig. 2). The NE fraction (Fig. 2, A and C) consisted mainly of large sheets of double membranes. Significant regions of these had blebbed and ballooned (which is not generally seen in the NEs of thin sectioned whole cell preparations), probably as a result of osmotic shock during nuclear lysis. The sheets were interrupted by numerous grommets, and the holes formed by these contained thin disks of relatively dense material. These structures were morphologically recognizable as NPCs in both transverse and tangential sections and negatively stained preparations. No clear examples of ordered filamentous structures could be found on either side of the transversely sectioned NPCs. The NPCs were present at $\sim$30/μm^2 in the negatively stained NEs (Fig. 2 C), two to three times the figure estimated for intact nuclei (Mutvei et al., 1992; Rout and Blobel, 1993). This considerable increase in density could be due to contraction of NEs no longer kept under elastic tension by underlying chromatin and associated structures, which might be exacerbated by osmotically induced swelling of the cisternal spaces between the NPCs. SPBs could also be found, still inserted in the NE and retaining many of their nuclear microtubules (MTs), attesting to the mild isolation conditions used (Fig. 2, A and C). The presence of these MTs indicates that the NE fraction would almost certainly be active in a MT nucleation assay (Kilmartin and Fogg, 1982; Rout and Kilmartin, 1990). The alignment of the asymmetric SPBs within the envelope unequivocally established the cellular

orientation of the two membranes (reviewed in Kilmartin, 1994). Many of the NEs retained their normal nuclear direction of curvature; concave on the nuclear (inner) side, convex on the cytoplasmic (outer) side. The exposed surfaces of the inner membranes were largely devoid of any material, including chromatin and any recognizable lamina (Fig. 2 A). In sharp contrast, the outer membranes often had exposed surfaces densely studded with ribosomes (Fig. 2 A). These were present at $\sim$800/μm^2 in the negatively stained NEs (Fig. 2 C), giving roughly 25 ribosomes per NPC (this latter figure should be independent of shrinkage or swelling of the NE and thus represent the in vivo figure more closely). The perinuclear cisternae generally contained low amounts of electron dense material, though some contained considerably more than others. The fraction contained no other recognizable organelles, except for occasional small remnants of undigested cell walls.

Many of the H-NEs were also recovered as large double-membraned sheets (Fig. 2, B and D). They appeared more ballooned and fragmented than the NE but their cisternae still contained electron dense material in thin sections (Fig. 2 B). However, they lacked any trace of ribosomes, by either thin section or negative stain (Fig. 2, B and D). The SPBs had apparently been removed from the envelopes, although by thin section occasional examples of heparin-extracted SPBs could be found free of membranes (Rout and Kilmartin, 1990). Thus, although many of the H-NEs were curved, there was no morphological marker left to tell whether they retained the native direction of curvature, like the NEs. Strikingly, the NPC grommets still remained, with the resulting holes being of approximately the same size and frequency as those found in the NEs, although the dense material comprising the morphologically recognizable NPC structure had been removed (Fig. 2, B and D). This indicates that despite the removal of the peripheral NPC components (see also below), integral and probably lumenal components of the NE that maintained the original circular architecture of the NPCs had been retained. The only recognizable contaminants were occasional cell wall remnants, carried through from the NE fraction.

SDS-PAGE Analysis and Immunoblots

To determine the protein composition and purity of the NE and H-NE fractions, the enrichment procedure was subjected to biochemical and immunological analyses. Protein samples obtained from each of the fractions collected during the preparation of isolated NEs and H-NEs (Fig. 1) were resolved by SDS-PAGE. To compare the novel heparin stripping procedure with the standard carbonate extraction method (Fujiki et al., 1982), yeast isolated NEs (fraction 10) were treated with sodium carbonate and the carbonate-resistant material (Fig. 3, *Carbonate Extracted NEs*) was run side by side with H-NEs on a protein gel.

Inspection of Fig. 3 reveals that the overall complexity and abundance of the proteins present in each of the enrichment steps decreased during fractionation. Furthermore, the fractionation behavior of specific supermolecular structures was followed by virtue of the characteristic
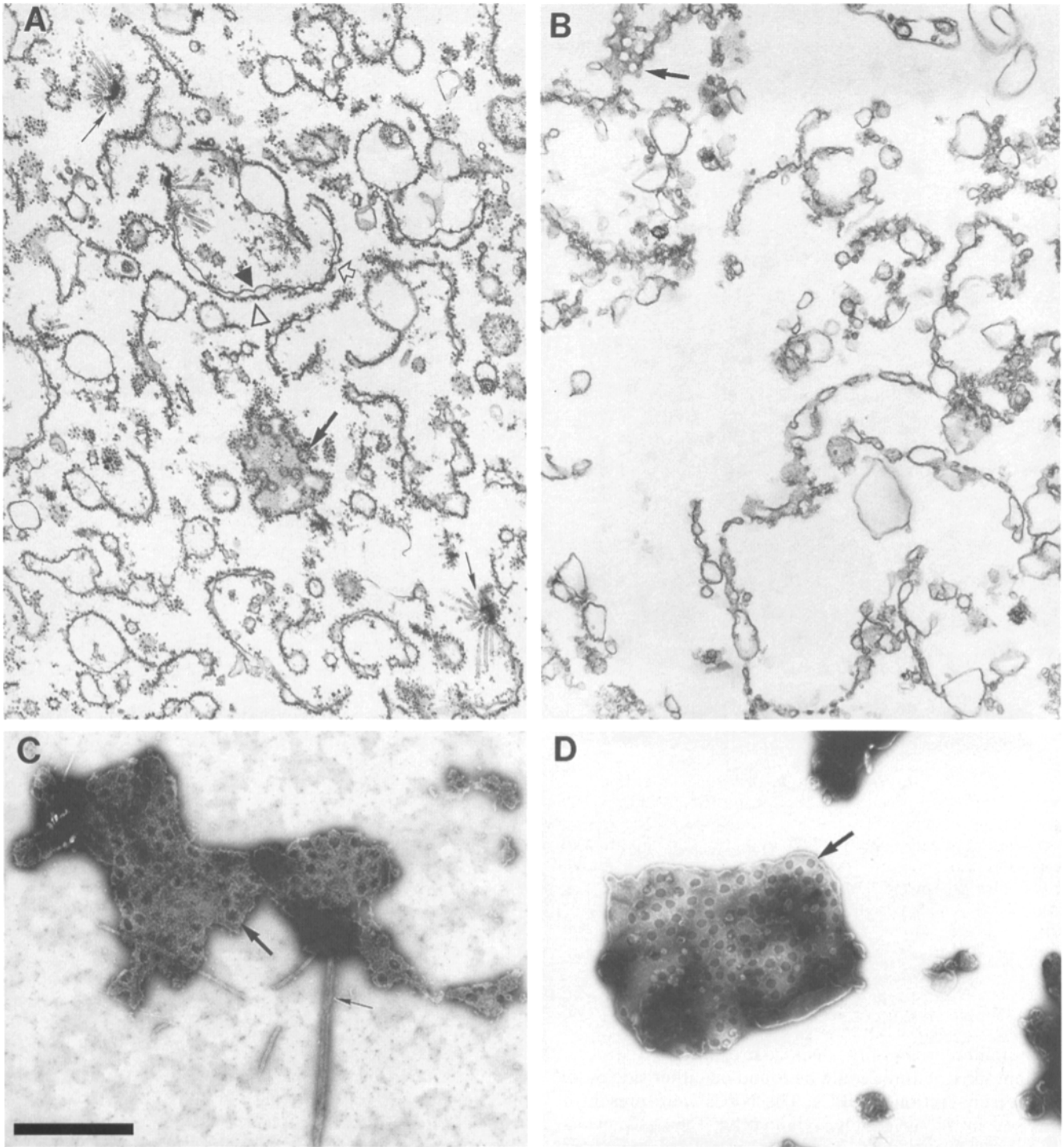
Figure 2. Morphological analysis of the NE and H-NE fractions. (*A* and *B*) Transmission electron micrographs of pelleted, thin sectioned NEs (*A*) and H-NEs (*B*). (*C* and *D*) Transmission electron micrographs of negatively stained NE (*C*) and H-NE (*D*) fractions. (*C*) The following structures are indicated: outer nuclear membrane (*open arrowhead*); inner nuclear membrane (*closed arrowhead*); longitudinal (*large open arrow*) and tangential (*large closed arrow*) sections of NPCs; SPBs and attached MTs (*small arrows*). (*B* and *D*) Circular apertures for the NPCs are indicated (*large closed arrows*). Bar, 1 μm.

banding pattern of certain of their components on SDS gels. Chromatin is represented by the four yeast histones (Fig. 3, *dots*) which were mainly lost after nuclear lysis and totally removed by heparin extraction. Characteristic ribosomal bands (Fig. 3, *asterisks*) were lost throughout the enrichment procedure and their complex behavior will be discussed below. Three bands are known to contain known NPC and pore membrane proteins (Fig. 3, *arrows*); all co-enriched with the NEs but only one, containing POM152 (a pore membrane specific integral membrane protein) was found in the H-NEs (Wozniak et al., 1994; Aitchison et al., 1995). The comparison of carbonate-extracted NEs
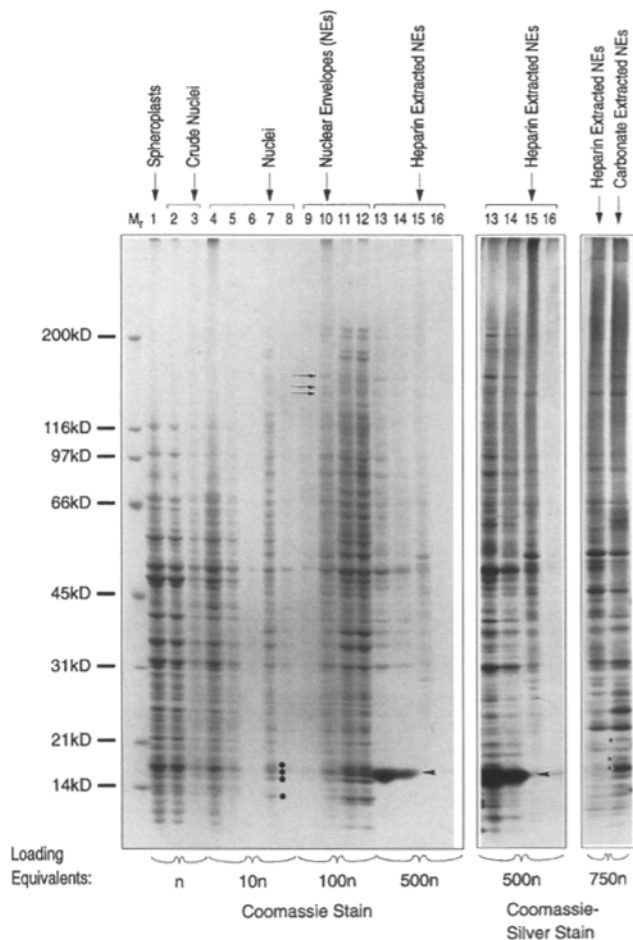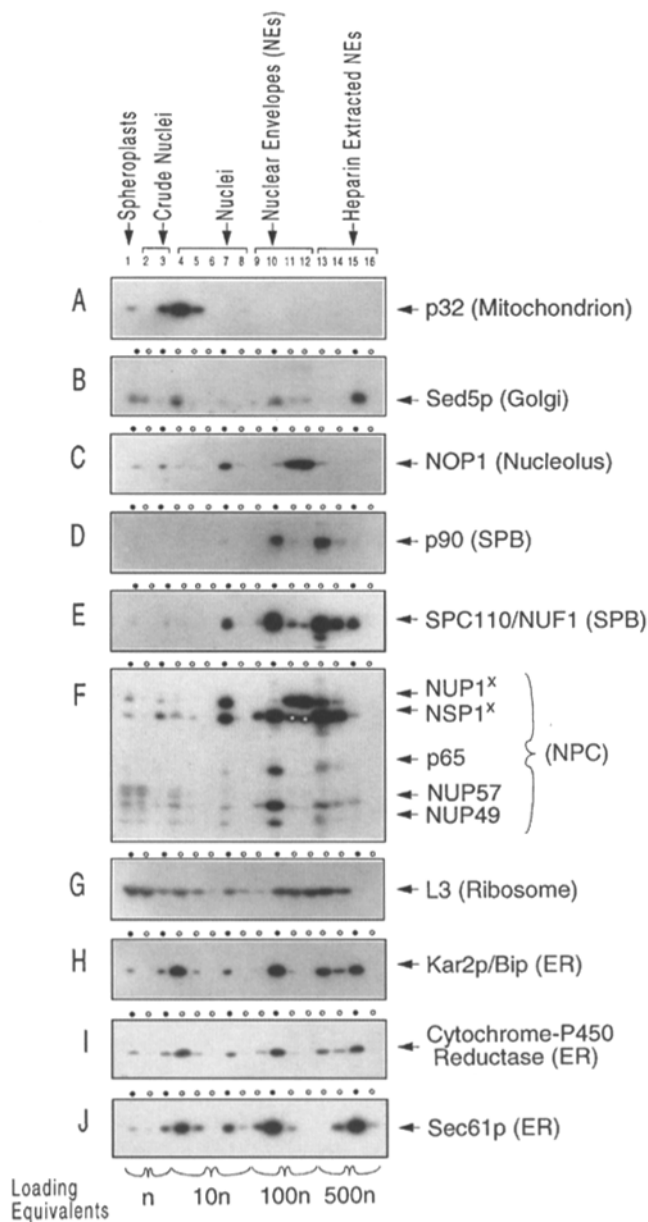
Figure 3. SDS-PAGE profile of proteins in subcellular fractions obtained during the preparation of NEs and H-NEs showing the loss of a large amount of contaminating proteins and concomitant coenrichment of representative NE proteins. (*Left* and *Middle*) Yeast cells were subjected to subcellular fractionation as described in Fig. 1 and in the text. (*Right*) NEs were treated with carbonate to remove peripheral membrane proteins and the carbonate-resistant material (*Carbonate Extracted NEs*) was directly compared with similar amounts of H-NEs (*Heparin Extracted NEs*). Proteins present in each of these fractions were resolved on a 5–20% polyacrylamide SDS gel. The gel was first stained with Coomassie brilliant blue (*Coomassie stain*) and then stained with silver (*Coomassie-Silver stain*). The lane number at the top of the gels reflects the fraction number (Fig. 1). Total cell lysate (*Spheroplasts*) and subsequent fractions containing NEs are indicated. Fractions that belong to each of the four enrichment steps are grouped as indicated by brackets at the top and bottom of the gels. The figures below the bottom brackets (*Loading equivalents*) represent the number of cell equivalents (*n*) that were used as a starting material to prepare each of the fractions. This number had to be increased from left to right to allow the detection of single proteins in the final lanes. Histones (*dots*), three characteristic bands containing known nuclear pore proteins (*arrows*: the lowest band is POM152, a pore membrane protein) and three representative ribosomal markers (*asterisks, right*) are indicated. Arrowheads point to a band that is believed to be the RNase A introduced in the course of heparin extraction. Numbers at the side of the left panel indicated the position of the molecular weight standards.

with H-NEs (Fig. 3) revealed that the protein composition of these two fractions was similar, suggesting that heparin is at least as efficient as carbonate in the removal of peripheral proteins from the nuclear membranes. On the other hand, certain specific proteins that were quantitatively removed by carbonate were retained after heparin extraction and vice versa. For example, the ribosomal markers appeared to be stripped by heparin with greater efficacy than by carbonate.

To assess the degree of enrichment of isolated NEs and H-NEs, the percentage yields of cytoplasmic, nucleoplasmic, NE specific (peripheral and membrane-bound), ER-specific, and ribosomal proteins were estimated by quantitative immunoblotting. The yields of the NE components were used together with measurements of the total amount of protein in each fraction to generate fold-enrichments for NE-containing fractions. These data allowed the construction of a balance sheet of the distributions of various cellular proteins and their associated organelles in the different steps of the fractionation procedure (Figs. 4, 6, and 8).

Analysis of three non-NE proteins from the mitochondria, the Golgi apparatus, and nucleolus demonstrated that potential cytoplasmic and nucleoplasmic contaminants were efficiently removed from the NE fractions. Mitochondria were followed by use of the integral membrane protein p32 (Pain et al., 1990). As expected virtually all of the signal fractionated away from NE-specific markers early in the procedure; most of this protein remained in the top two fractions of the nuclear gradient (Fig. 4 *A*, fractions *4* and *5*). Quantitative immunoblotting showed that less than 0.04% of the total cellular amount of p32 remained associated with isolated NEs and that less than 0.01% was associated with the H-NE fraction (data not shown). The integral membrane protein of the Golgi, Sed5p (Hardwick and Pelham, 1992), was mainly found in the crude cytosol fraction (Figs. 4 *B* and 8 *D*, fraction *2*; 79% of the total cellular signal). Approximately 90% of the crude nuclei pool of Sed5p (19% of the total) remained at the top of the nuclear gradient (Figs. 4 *B* and 8 *D*, fractions *4* and *5*). The small remaining amount fractionated with the nuclei, NEs, and H-NEs (Figs. 4 *B* and 8 *D*, fractions *7*, *10*, and *15*, respectively), consistent with this being an integral membrane protein that is involved in ER to Golgi transport and which therefore can be expected to be present, at least in small quantities, in the ER (Hardwick and Pelham, 1992; Hopkins, C., personal communication). The nucleolar protein NOP1 coenriches with the nuclei but was rapidly removed from the NE after nuclear lysis (Fig. 4 *C*; Aris and Blobel, 1988). Quantification of the immunoblot presented in Fig. 4 *C* demonstrated that only approximately 1.4% of NOP1 fractionated with the NEs and that NOP1 was undetectable in the H-NE fraction (data not shown).

Peripheral NE proteins, represented here by two SPB proteins p90 and SPC110/NUF1, and various known NPC proteins (nucleoporins) detected by MAb350 and MAb414, cofractionated with the NE until they were lost after treatment with heparin (Figs. 4, *D–F* and 8 *A*; Rout and Kilmartin, 1990; Kilmartin et al., 1993). An exception was represented by the nucleoporin NUP2 (Fig. 4 *F*, indicated by *white dots*), which falls off after DNase digestion of the nuclear fraction as it does in the NPC isolation procedure

(Rout and Blobel, 1993; Loeb et al., 1993). SPC110/NUF1 was partially retained ($\sim$10%) in the heparin stripped NE fraction. This could be a consequence of its localization within a heparin-resistant substructure of the SPB that may be removed from the NE but not solubilized during the extraction procedure (Rout and Kilmartin, 1990, 1991), a hypothesis supported by the EM data (above). The intensities of the signals generated by p90(SPB) (Fig. 4 *D*), SPC110/NUF1 (Fig. 4 *E*), and the 65-kD fragment of NUP145 (*p65[NPC]*, Fig. 4 *F*; Wente and Blobel, 1994) were measured and averaged to estimate the overall percentage yield of peripheral membrane proteins of the NE (Fig. 8 *A*). Approximately 80% of the total peripheral NE components was recovered in fraction 10 (isolated NEs), representing a >90% yield as compared to the nuclear fraction (fraction 7). The great majority of this signal was removed after heparin treatment (94% of the NE signal, totaling 95% of the original cellular signal). This may in fact underestimate the efficiency of the extraction of many proteins due to the somewhat unrepresentative behavior of SPC110/NUF1 (discussed above); for example, p65(NPC) was extracted with an efficiency greater than 98% (data not shown).

Since a NE specific membrane marker was not available, one was made by raising mAbs against the H-NE fraction. One of these was found to recognize a single band of $\sim$150 kD in isolated NEs, H-NEs, and highly enriched NPCs (Rout and Blobel, 1993). We used two approaches to demonstrate that this antibody, MAb118C3, specifically recognizes POM152 (Fig. 5). First, MAb118C3 recognizes a single band of the expected mobility in both isolated nuclei and NEs prepared from *wt* yeast cells and it fails to do so in NEs similarly prepared from a POM152 knock-out strain (PM7AB; a diploid *S. cerevisiae* strain homozygous for *pom152-2::HIS3*; generous gift from R. W. Wozniak). Second, this same antibody reacts with a single band of 150 kD present in chromatographic fractions highly enriched for POM152 (SDS–hydroxylapatite fraction 36 and HPLC fraction 69; Wozniak et al., 1994). MAb118C3 was used to monitor the fractionation pattern of the *wt* POM152 protein throughout the various steps of the NE, H-NE and NPC preparations (Figs. 6 and 8 *B*). As expected, POM152 was shown to coenrich with both the NE and H-NE as well as the highly enriched NPC fraction. Surprisingly, a significant amount (29%) of the signal frac-

Figure 4. Immunoblot analysis of the enrichment procedure showing that the fractionation behavior of various cellular markers is consistent with high yields and low levels of contamination in the NE and H-NE fractions. Fractions were prepared as described in Fig. 1 and in the text. Gels were exactly as described in Fig. 3. Blots were incubated in the presence of the various antibodies which were detected by incubation with a secondary rabbit anti-mouse antibody (in the case of the mAbs) and subsequently with ^{125}I-conjugated protein A. (*A*) The integral membrane mitochondrial protein, p32, detected with a polyclonal rabbit serum (Pain et al., 1990). (*B*) The integral membrane protein of the Golgi, Sed5p, detected with an affinity purified polyclonal rabbit serum (Sogaard et al., 1994). (*C*) The nucleolar protein NOP1, detected with the mAb D77 (Aris and Blobel, 1988). (*D*) The SPB peripheral membrane protein, p90, detected by the use of the mAbs, 35B5 and 48B6 (Rout and Kilmartin, 1990). (*E*) The SPB component SPC110/NUF1 detected with a mix of the mAbs, 3D2, 45D10 and 35A11 (Rout and Kilmartin, 1990). (*F*) Various peripheral nuclear pore proteins revealed by utilizing MAb414 and MAb350. NUP1^X indicates the overlapping signal of NUP1 (Davis and Fink, 1990) and NUP116 (Wente et al., 1992). Simi-

larly, NSP1^X indicates NSP1 (Nehrbass et al., 1990) and NUP100 (Wente et al., 1992). These mAbs also recognize NUP2 (*white dots*), p65 (a 65-kD breakdown product of NUP145), NUP57 (Grandi et al., 1995), and NUP49 (Wente et al., 1992). Other non-specifically cross-reacting proteins are detected by this antibody and they were described elsewhere (Davis and Fink, 1990; Rout and Blobel, 1993). (*G*) The mAb, TCM1, was used to follow the ribosomal marker, L3 (generous gift of S. P. Johnson and J. R. Warner). (*H*) The lumenal heat-shock protein of the ER, Kar2p/Bip was detected using the mAb 2E7 (Napier et al., 1992). (*I*) A rabbit antiserum was used to recognize the ER membrane associated protein, Cytochrome P450 reductase (Sutter and Loper, 1989). (*J*) The integral membrane protein of the ER, Sec61p, was detected with a rabbit anti-peptide serum (Stirling et al., 1992). The lanes are numbered as in Fig. 3. The NE containing fractions are indicated above the blots. *Loading Equivalents*, see legend of Fig. 3.
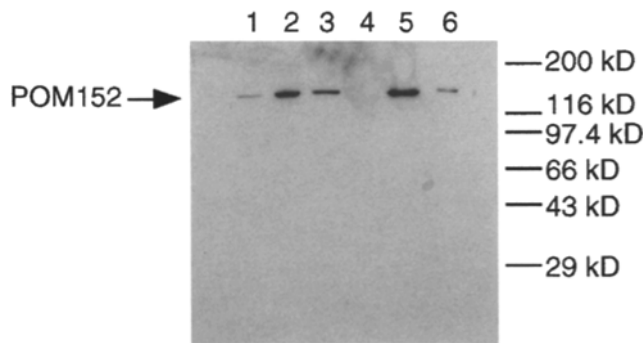
Figure 5. The mAb, MAb118C3, specifically recognizes the pore membrane protein, POM152. Yeast nuclei (lane *1*) and NEs (lane *2*) were prepared following the procedure presented in Fig. 1 and in the text. NEs were also prepared from wild-type *S. cerevisiae* yeast cells (W303; lane *3*) and from a POM152 knock-out strain (PM7AB; lane *4*), using the same method. *S. uvarum* cells were fractionated as described (Wozniak et al., 1994), to produce chromatographic fractions highly enriched for POM152 (SDS-hydroxylapatite fraction number 36 [lane *5*] and HPLC fraction number 69 [lane *6*]). Equal protein amounts from the above mentioned fractions were resolved on SDS-PAGE and transferred to a nitrocellulose filter. The blot was incubated with MAb118C3 and bound immunoglobulin was detected by chemiluminescence (ECL; Amersham Life Science, Arlington Heights, IL), following the instructions of the manufacturer. The position of POM152 is indicated. Numbers on the right of the gel denote the position of molecular weight standards.

tionates together with rough ER markers (fractions *4* and *5*). The majority (82%) of the nuclear associated POM152 (42% of the total cellular amount) was recovered in the NE fraction similar to the behavior of the peripheral
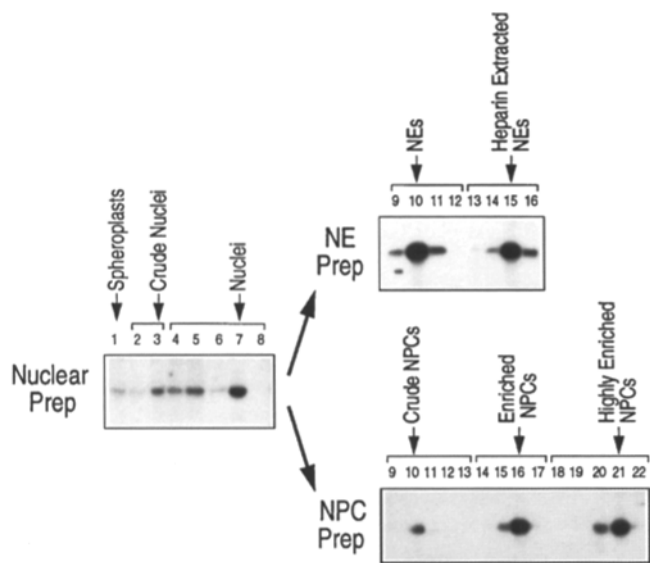
Figure 6. The pore membrane protein, POM152, coenriches with both a highly-enriched NPC fraction and with nuclear membranes. Yeast nuclei (*Nuclear Prep*; Rout and Kilmartin, 1990) were used as the starting point for the preparation of either the NE and H-NE fractions (*NE Prep*) or of a highly enriched NPC fraction (*NPC Prep*; Rout and Blobel, 1993). Blots similar to the ones used in Fig. 4 and the ones described by Rout and Blobel (1993) were probed with MAb118C3 that reacts against POM152.
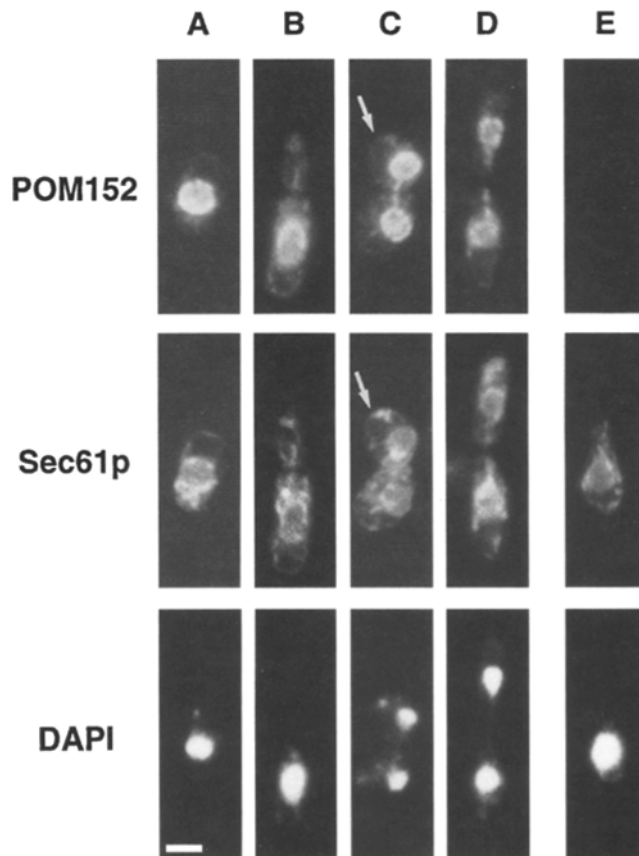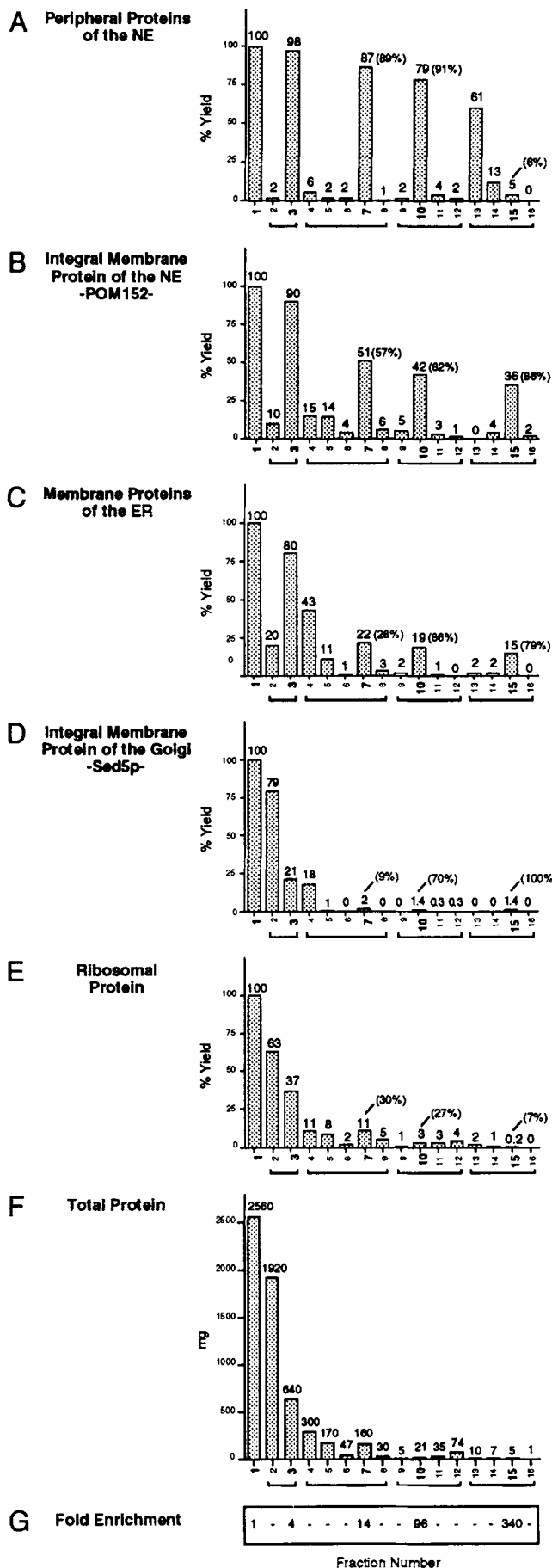

Figure 7. Double immunofluorescence staining of *wt* yeast cells showing in vivo POM152 localization at the NE and at the ER. Logarithmically growing *wt* yeast cells were harvested, fixed and incubated with MAb118C3 (anti-POM152) followed by a rabbit anti-Sec61p antibody (*A–D*) or with the rabbit serum against Sec61p alone (*E*). All slides were incubated with a mixture of FITC-conjugated donkey anti–rabbit and Cy3-conjugated donkey anti–mouse IgGs and they were subsequently photographed on a fluorescent microscope. Cells at various stages of the cell-cycle starting from interphase (*A*) all the way to cytokinesis (*D*) can be observed. ER peripheral cisternae are indicated by arrows. The absence of any signal in *POM152, E* demonstrates that there was no bleed-through from the FITC-channel. Bar, 2 μm.

markers. Furthermore, nearly 90% of this signal was resistant to heparin extraction in contrast with the peripheral NE components. To confirm that this result reflects the in vivo subcellular localization of POM152, logarithmically growing cells were stained with both MAb118C3 and a rabbit anti-Sec61p polyclonal antibody and viewed by immunofluorescence microscopy (Fig. 7). Indeed, this shows that whilst the majority of POM152 was localized at the NE, a certain amount of this protein was found at the peripheral ER. A POM152 knock-out strain (PM152-75; a haploid *S. cerevisiae* strain carrying *pom152-2::HIS3*; Aitchison et al., 1995) was examined by immunofluorescence under conditions similar to the ones used for the experiment presented in Fig. 7. As predicted no signal was detected, either at the nuclear rim or at the cellular periphery where ER staining would have been expected (data not shown).

The behavior of ER proteins during the enrichment pro-

A Peripheral Proteins of the NE

B Integral Membrane Protein of the NE -POM152-

C Membrane Proteins of the ER

D Integral Membrane Protein of the Golgi -Sed5p-

E Ribosomal Protein

F Total Protein

G Fold Enrichment

| 1 | - | 4 | - | - | - | 14 | - | - | 96 | - | - | - | - | 340 | - |

Fraction Number

cedure was monitored using the ER markers Sec61p, Cytochrome P450 reductase and Kar2p/Bip (Figs. 4, *H–J* and 8 *C*; Yabusaki et al., 1988; Rothblatt et al., 1989; Rose et al., 1989; Normington et al., 1989). As expected the majority of each ER marker was found in fractions 4 and 5. However, approximately 20% of each ER marker was found associated with the nuclei (fraction 7). Subfractionation of the nuclei showed that all three proteins coenriched absolutely with the NEs. The extent to which each of the ER markers cofractionated with the H-NE fraction correlated with their degree of association with the ER membrane. Hence, the integral membrane protein Sec61p coenriched absolutely with H-NEs (Fig. 4 *J*). Cytochrome P-450 reductase was more easily extracted by heparin, consistent with previous observations (Black and Coon, 1982; Yabusaki et al., 1988; Fig. 4 *I*). Roughly half of the rough ER lumenal protein Kar2p/Bip (55%; data not shown) was resistant to heparin extraction (Fig. 4 *H*). This could indicate that a substantial proportion of this protein is protected from heparin because it is enclosed in intact membranous compartments (i.e., perinuclear cisternae). This explanation is unlikely since approximately 80% of Kar2p/Bip present in both NE fractions is sensitive to trypsin (data not shown), suggesting that the majority of the perinuclear cisternae is accessible to heparin but not efficiently extracted by it.

Ribosomes were followed using a mAb against the large subunit protein L3 (Fried and Warner, 1981; Figs. 4 *G* and 8 *E*). The fractionation pattern was complex but as expected this ribosome marker was observed to associate with cytosolic ribosomes (fraction 2) and ER-bound ribosomes (fractions 4 and 5). The L3 cofractionating with the nucleus (fraction 7) appeared to be associated with two different nuclear compartments, which were separated during the preparation of isolated NEs. 27% of the nuclear L3 was found in the ribosomes bound to the outer surface of the NE (fraction 10). The remaining 73% (fractions 11 and 12, 7% of the total) was found in fractions containing the nuclear and nucleolar remnants. As this antibody stains the cytoplasm and the nucleolus but not the rest of the nucleus by immunofluorescence microscopy (data not shown), the remaining signal is believed to represent the

Figure 8. Quantitative analysis of the NE enrichment procedure. (*A–E*) The immunoblots presented in Figs. 4 and 6 or similar ones were subjected to quantitative analysis. Fractions are numbered as in Fig. 1 and grouped with brackets as in Figs. 3 and 4. An estimate of the amount of a given marker present in each fraction is expressed here as a percentage of the total cellular amount calculated from the sum of the quantity found in fractions 2 (crude cytosol) and 3 (crude nuclei). The figures in parentheses represent the percentage yield of each of the markers relative to the NE containing fraction from the preceding fractionation step. The SPB proteins p90 and SPC110/NUF1, and the NPC protein p65 were used to construct the histogram of *A*. Similarly, the data presented in panel C reflect the results of the quantification of cytochrome P450 reductase and Sec61p. (*F*) Total amount of protein present in each fraction. (*G*) The percentage yields of the peripheral NE markers (*A*, fractions *1–12*) and of POM152 (*B*, fractions *13–16*) were used together with the numbers expressing the total amount of protein of each NE containing fraction to determine the fold-enrichment of nuclear membranes throughout the enrichment procedure.

ribosomal proteins associated with nucleolar pre-ribosomal structures. Greater than 93% of the NE-bound protein was removed upon heparin extraction of the NE fraction, leaving only 0.2% of the total cellular ribosomal protein bound to the nuclear membrane after heparin treatment.

The removal of contaminating proteins was dramatically demonstrated in Fig. 8 *F*, where less than 1% of the total cellular protein was present in the NE fraction (fraction *10*) while less than 0.2% was associated with the H-NE (fraction *15*). These data were used in combination with the numbers representing the percentage yield of nuclear membranes in fractions 1, 3, 7, 10, and 15 (generated from the yields of peripheral NE proteins [fractions *1–12*] and POM152 [fractions *13–16*]) to calculate the approximate degree of enrichment of NEs throughout the fractionation procedure (Fig. 8 *G*). As can be seen the enrichment of the NE and H-NE fractions was roughly 100- and 340-fold respectively, which represent a 68% overall recovery of nuclear membranes. The degree of enrichment of the NE and H-NE fractions described here was highly reproducible between preparations (data not shown). Similar preparations have been successfully made, with minor technical modifications, from numerous other *S. cerevisiae* strains (see for example Fig. 5).

Protein Translocation Activity

Having determined the protein composition of the NE and the H-NE fractions it was necessary to evaluate their functional integrity. Cell-free systems have been developed to study various NE functions including nuclear transport, MTs nucleation, nuclear fusion, budding of ER to Golgi transport vesicles, and translocation of proteins across the rough ER membrane (i.e., outer nuclear membrane). The latter assay was chosen as it is comparatively straightforward and since both the NE and the H-NE fractions would be expected to have ER protein translocation activity. "Active" NE and H-NEs fractions (see Materials and Methods for details) were mixed with radiolabeled, urea-denatured ppαF in the presence of yeast cytosol and an energy source (Chirico et al., 1988). The presence of translocated pro-α–factor was demonstrated using two standard criteria: (*a*) the acquisition of protease-resistance; and (*b*) the appearance of core-glycosylated forms of pro-α-factor. Moreover, both the absolute and the specific translocation activity were determined for each sample studied (Fig. 9).

When ppαF was incubated with either NEs or H-NEs (Fig. 9, *NEs* and *heparin-extracted NEs*, respectively), a significant fraction of it was translocated into the ER lumen (i.e., perinuclear space) similar to that observed with the CM fraction (Fig. 9, *crude microsomes*). Interestingly, the specific translocation activity of the NE and H-NE fractions appeared to be significantly higher (three- and fivefold, respectively) as compared with the CM fraction (Fig. 9). All of the glycosylated pro-α–factor (Fig. 9, *gpαF*) was protease resistant, unlike much of Kar2p/Bip (see previous section). Thus, either only sealed membranes are translocation-competent or translocated gpαF is associated with lumenal proteins such that it is resistant to trypsin even in unsealed membranes. Detergent would dis-
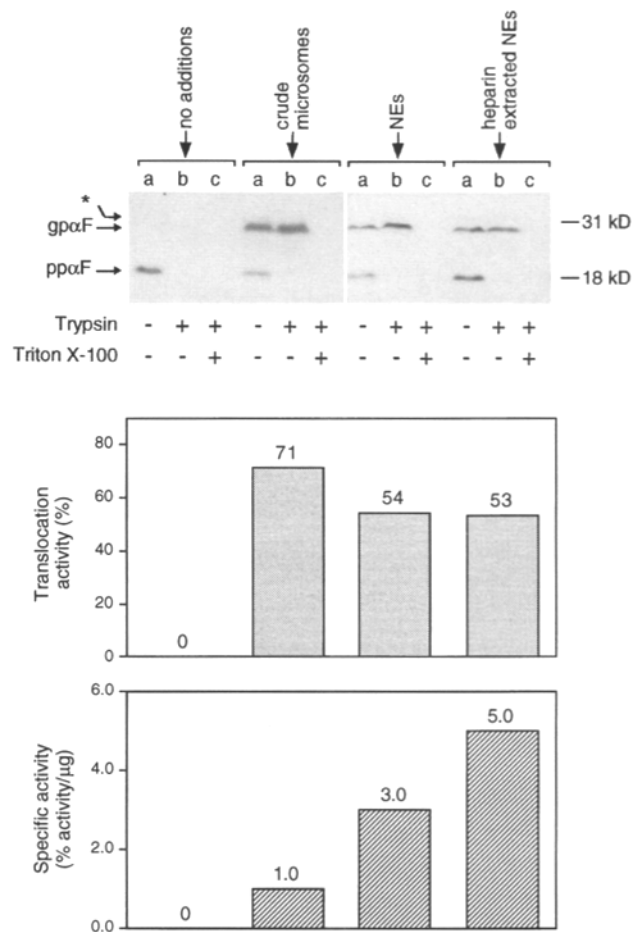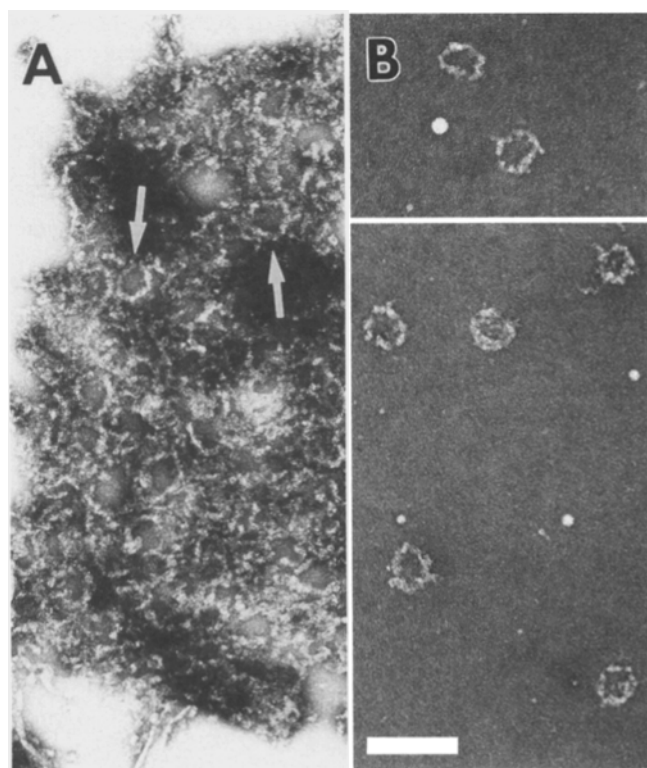

Figure 9. Both the isolated NEs and H-NE fraction are active in a cell-free protein translocation assay. "Active" NEs and H-NEs and CMs were prepared as described in Materials and Methods. (*Top*) [^{35}S]Methionine labeled, in vitro synthesized ppαF was denatured with urea immediately before adding it to a reaction mixture containing either buffer (*no additions*), NEs, H-NEs (*heparin extracted NEs*), or CMs (*crude microsomes*) in the presence of ATP and crude cytosol. After 1 h at 20°C the reaction was stopped and each sample was divided in three equal aliquots. The first set of aliquots was incubated on ice without further treatments (lanes *a*). The second set was digested with trypsin (lanes *b*). The third set was treated with Triton X-100 before trypsin digestion (lanes *c*). All samples were analyzed by SDS-PAGE and fluorography. The position of ppαF and of the tri-glycosylated form of the protein (glycosylated pro-α-factor [*gpαF*]) is indicated. An α-factor specific band migrating slower than the fully glycosylated product is indicated by an *asterisk*. This previously reported product presumably corresponds to gpαF prior to mannose and glucose trimming (Waters et al., 1988). The position of the molecular weight standards is indicated at the right of the gel. (*Middle*) The intensity of the bands present in lanes *a* (no treatment) was measured with the Phosphorimager system and the translocation activity was determined in each case by calculating the percentage of the total radioactivity that corresponded to translocated material. (*Bottom*) The percentage translocation activity in successive twofold dilutions of each fraction was determined and a graph of activity versus volume of sample was constructed. The slope of the graph in the linear range (% activity/μl) was divided by the total amount of protein present in each fraction to yield the specific activity (*Specific activity [% activity/μg]*).

rupt this association and make gpαF sensitive to proteolysis. These results demonstrate that both the translocation apparatus and the glycosylating enzymes are active in our highly enriched NE and H-NE fractions. This provides evidence that the outer nuclear membrane and the perinuclear space not only share ER components but also its functions. That both of the NE fractions are functionally well-preserved suggests that they could be used to develop cell-free systems to investigate other NE and ER functions.

Detergent Extraction of H-NEs

The retention of the NPC grommets after heparin extraction of the isolated NEs suggested that they were stabilized by a heparin-resistant NPC substructure associated with the membrane, most likely in the perinuclear space. H-NEs were therefore extracted with detergents after attachment to an EM grid in order to reveal any substructures underlying the grommets (Fig. 10). Negative staining of these samples revealed the presence of numerous rings of approximately the same internal diameter (~100 nm) and distribution as the grommets (Fig. 10 A, arrows), with an extensive filamentous network lying between them. To

investigate this finding further, a highly enriched fraction of NPCs (Rout and Blobel, 1993) was treated with heparin under conditions similar to the ones used to extract isolated NEs. Heparin-resistant material was then sedimented onto an EM grid and negatively stained, as for the extracted H-NEs (above). This also produced ring-like structures, strongly resembling those seen in the extracted H-NEs but without any other associated material (Fig. 10 B). Preliminary experiments indicated that, when the highly enriched NPCs were similarly extracted in solution and sedimented over a velocity gradient, the rings thus isolated contained the pore membrane protein POM152 as a major constituent; likewise, the detergent extracted H-NEs were also found to retain POM152 (data not shown). The rings must therefore be derived from integral pore membrane proteins possibly associated with peripheral proteins present in the perinuclear space. Attempts to separate the rings directly from the H-NEs in solution have so far failed.

Discussion

This paper presents a method for the large scale isolation of yeast NEs and for extraction of isolated NEs with heparin. EM analysis showed that both of these preparations are devoid of gross contaminants and are morphologically well-preserved. Moreover, negative staining of H-NEs showed that heparin extraction removes the main structure of the NPCs leaving open pores in the membrane. Using biochemical criteria it was demonstrated that key cytoplasmic and nucleoplasmic contaminants are absent from both NE fractions and that NE-associated proteins were recovered with yields ranging between 80–90%. The NE and H-NE fractions are respectively 100- and 340-fold enriched based on the yields of NE specific markers. Both the NEs and H-NEs were shown to be active in a cell-free ER translocation assay, each having a higher specific activity than that of previously published crude ER-derived membranes (Waters and Blobel, 1986; Rothblatt and Meyer, 1986; Hansen et al., 1986). Finally, extraction of H-NEs with detergents showed that the grommets left in the NE after heparin treatment are apparently stabilized by previously uncharacterized ring structures of approximately the same internal diameter as the grommets themselves.

The high yields of the NE fractions allowed the construction of a balance-sheet that tallies the distribution of representative markers in various nuclear and non-nuclear compartments. For example, this strategy was used to determine the quantitative localization of ribosomal proteins within the cell. Using the same approach it was established that while the majority of the Golgi integral membrane protein Sed5p (98% of the total cellular amount) is indeed found in low-density membranes that characterize this organelle, a small pool of this protein is associated with the ER consistent with its role in ER to Golgi transport and with previous observations (Hopkins, C., personal communication). It was also possible to show that the NE represents approximately 20% of the ER, similar to what was previously reported (Preuss et al., 1991). A possible caveat of the results presented here is that cells are subjected to exhaustive cell-wall digestion (3 h at 30°C) before lysis.

Figure 10. Detergent extraction of H-NEs suggests that ring structures associated with the NE may be involved in stabilizing the grommets of the NPCs. (A) Isolated H-NEs were immobilized on EM grids, extracted with detergents and negatively stained as described in Materials and Methods. Arrows point to heparin-resistant ring structures that have the same internal diameter as the NPC grommets seen in Fig. 2 D. (B) Highly enriched NPCs (Rout and Blobel, 1993) were extracted with heparin as described in Materials and Methods; following extraction, the heparin-resistant material was sedimented onto EM grids and negatively stained as above. Bar, 0.2 μm.

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This treatment stops cell division and could alter the amount and composition of various organelles. However, the qualitative subcellular localization of each of the markers used here has been previously ascertained. In all cases (with the exception of NUP2), this localization corresponds with the quantitative distribution of the marker, and the organelle(s) with which it is associated, in the enrichment procedure presented here. This indicates that the quantitative data obtained from the subcellular fractionation of a given marker accurately reflect the subcellular distribution of that marker in vivo. Hence, quantitative analysis of the fractionation behavior predicted that a proportion of POM152 would be associated with the ER, which was confirmed by immunofluorescence staining of cells. This suggests that the balance-sheet approach could be used to predict the subcellular localization and proportional distribution of other cellular components.

As mentioned above, NEs and H-NEs are competent in the translocation of ppαF to the perinuclear space. These fractions therefore provide a viable source of yeast rough ER membranes that could be used to develop cell-free systems for the study of various rough ER functions and to further purify the molecular components involved in these functions. For example, systems for the reconstitution of protein translocation into proteoliposomes have been developed and used successfully in yeast (Brodsky and Schekman, 1993; Brodsky et al., 1993). The utilization of the highly enriched NE fractions described here instead of cruder fractions may enhance the potentiality of these systems. In addition, the NE and H-NE fractions could be used to develop cell-free systems that will allow the molecular dissection of other important NE related functions. These include: nuclear transport, at least in the aspect of specific docking and undocking of the substrate to the NPC (Moore and Blobel, 1993, 1994; Gorlich et al., 1994; Adam and Adam, 1994; Radu et al., 1995); chromatin–NE interactions, such as the binding of telomeric structures to the inner nuclear membrane (Dresser and Giroux, 1988; Klein et al., 1992; Palladino et al., 1993); assembly of the mitotic spindle (Kilmartin and Fogg, 1982; Rout and Kilmartin, 1990; Masuda et al., 1992); regulation of events involving the nucleus such as karyokinesis and karyogamy (Latterich and Schekman, 1994); and regulation of gene expression (Mori et al., 1993; Cox et al., 1993).

The ring structures underlying the grommets in H-NEs, as revealed by detergent extraction, appear to be the same as those produced by heparin treatment of highly enriched NPCs (which were extracted with detergents in the course of their isolation). These rings are derived from the periphery of the NPC disks, contain the pore membrane protein POM152 as a major component, and apparently serve to support the membrane grommets in the H-NEs. These rings may be the structural and functional analogs of the lumenal spoke domains/radial arms found in vertebrate NPCs (Akey and Radermacher, 1993), where they could both anchor the NPC within the nuclear membrane and stabilize the reflexed membrane of the pore grommet.

There is evidence to suggest that some NE-associated structures have not been preserved during the enrichment procedure. In particular, the NPC protein NUP2 was lost at nuclear lysis, as it is when NPCs are isolated (Rout and Blobel, 1993). This protein may be part of a fragile, peripheral structure that is sheared off during NE preparation. Other such structures may be similarly lost. No structures resembling the nuclear lamina or major components that could be lamins were found in the NE preparations, except for the unidentified filamentous material remaining after detergent extraction of the H-NEs. Antibodies that were previously reported to cross-react with lamins A and B analogues in yeast (Georgatos et al., 1989), did not detect any co-enriching bands of the expected molecular weight either in the NE or in the H-NE fractions (data not shown). Further studies and better reagents are needed to assess whether a nuclear lamina is present in yeast and to what degree it cofractionates with the NE fractions.

The high degree of enrichment of the NE and H-NE fractions makes them excellent material for raising mAbs against NE and ER specific components; this has been demonstrated by the isolation of the anti-POM152 mAb, MAb118C3. A second mAb, raised by immunizing mice with isolated NEs, specifically recognizes the novel nucleoporin NUP159 and was essential in the isolation and immunolocalization of this protein (Kraemer et al., 1995). An additional 170 monoclonal lines have been isolated that recognize at least 10–15 different antigens present in the NE fractions. These are still in the early phases of characterization. MAb118C3 has been used to gain new insights into the in vivo behavior of POM152. The unexpected localization of POM152 could have important implications for the understanding of the mechanisms that lead to the assembly of new NPCs in actively growing yeast cultures. This result could only have been obtained by use of a mAb that allows the detection of the unaltered protein at normal levels of expression. The NE and H-NE fractions can therefore provide the source material to generate the reagents necessary to study the activities associated with the NE, and at the same time be the substrates with which these activities can be studied.

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