Nuclear Pore Complex Assembly Studied with a Biochemical Assay for Annulate Lamellae Formation

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Abstract. Formation of the nuclear pore is an intricate process involving membrane fusion and the ordered assembly of up to 1,000 pore proteins. As such, the study of pore assembly is not a simple one. Interestingly, annulate lamellae, a cytoplasmic organelle consisting of stacks of flattened membrane cisternae perforated by numerous pore complexes, have been found to form spontaneously in a reconstitution system derived from *Xenopus* egg extracts, as determined by electron microscopy (Dabauvalle et al., 1991). In this work, a biochemical assay for annulate lamellae (AL) formation was developed and used to study the mechanism of AL assembly in general and the assembly of individual nucleoporins into pore complexes in particular. Upon incubation of *Xenopus* egg cytosol and membrane vesicles, the nucleoporins nup58, nup60, nup97, nup153, and nup200 initially present in a disassembled form in the cytosol became associated with membranes and were pelletable. The association was time and temperature dependent and could be measured by immunoblotting. Thin-section electron microscopy as well as negative staining confirmed that annulate lamellae were forming coincident with the incorporation of pore proteins into membranes. Homogenization and subsequent flotation of the membrane fraction allowed us to separate a population of dense membranes, containing the integral membrane pore protein gp210 and all other nucleoporins tested, from the bulk of cellular membranes. Electron microscopy indicated that annulate lamellae were enriched in this dense, pore protein–containing fraction. GTPγS prevented incorporation of the soluble pore proteins into membranes. To address whether AL form in the absence of *N*-acetylglucosaminylated pore proteins, AL assembly was carried out in WGA-sepharose–depleted cytosol. Under these conditions, annulate lamellae formed but were altered in appearance. When the membrane fraction containing this altered AL was homogenized and subjected to flotation, the pore protein–containing membranes still sedimented in a distinct peak but were less dense than control annulate lamellae.

THE nuclear pore is responsible for establishing the distinct nuclear and cytoplasmic compartments of the eukaryotic cell. The pore is a highly selective channel through which macromolecular traffic must pass to enter or exit the nucleus. Although ions and other small molecules freely diffuse through the pore, larger molecules require a specific targeting signal for transit (for reviews, see Goldfarb and Michaud, 1991; Forbes, 1992; Gerace, 1992; Osborne and Silver, 1993; Powers and Forbes, 1994).

The nuclear pore complex is a large and elaborate structure of ~120 million daltons and is comprised of ~1,000 proteins. Structurally, it appears to consist of three rings stacked one upon the other, with the central ring composed of eight spokes surrounding a central hub or transporter. Short fibers extend into the cytoplasm from the cytoplasmic ring of the pore, while an unusual basket structure extends from the nucleoplasmic ring (see Pante and Aebi, 1993, for a review). Of the estimated 60–100 different polypeptides that comprise the nuclear pore, only a handful have been identified. These include a component of the basket (nup153), proteins of the central region of the pore (p62/nup60, nup58, and nup54), components of the cytoplasmic filaments (nup180, nup200/CAN, and TPR), and integral membrane pore proteins (gp210 and POM121), among others (Davis and Blobel, 1986; Starr et al., 1990; Cordes et al., 1991; Finlay et al., 1991; Hallberg et al., 1993; Radu et al., 1993; Sukegawa and Blobel, 1993; Wilken et al., 1993; Byrd et al., 1994; see Rout and Wente, 1994, for a review). A family of pore glycoproteins that contain *N*-acetylglucosamine residues and consequently bind the lectin WGA are among these pore proteins (for reviews,

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see Hart et al., 1989; Forbes, 1992; Rout and Wente, 1994). A number of pore proteins have also been characterized in yeast, and a subset have homologues in vertebrate pores (for reviews, see Fabre and Hurt, 1994; Rout and Wente, 1994). Nonetheless, the dozen or so vertebrate nucleoporins identified to date are estimated to constitute only 5–10% of the total mass of the pore (Snow et al., 1987; Finlay et al., 1991; Loeb et al., 1993).

While relatively little is known about the molecular composition of the pore, even less is known about the mechanism of pore assembly and disassembly. Studies of pore formation were hampered for many years by the lack of in vitro systems that would allow for biochemical analyses. More recently, it has been possible to examine nuclear envelope and pore assembly using nuclear reconstitution systems. A CHO cell extract derived from mitotic cells has been used to study lamin and nuclear envelope assembly (Burke and Gerace, 1986; Foisner and Gerace, 1993). Many groups have used a different cell-free extract derived from *Xenopus* eggs to study various nuclear processes (for review, see Leno and Laskey, 1990; Smythe and Newport, 1991; Almouzni and Wolffe, 1993). The *Xenopus* extract, which contains large stockpiles of disassembled nuclear components, readily reconstitutes nuclei in vitro.

Using the *Xenopus* system, reconstituted nuclei were observed to import nuclear proteins, indicating that nuclear pores were also being reconstituted in vitro (Newmeyer et al., 1986*a*, *b*). Biochemically altered nuclear pores were created by carrying out nuclear assembly in an egg extract depleted of the family of *N*-acetylglucosamine–modified nucleoporins. It was found that pores continued to form but were less dense in appearance and failed to import, indicating that this family of pore proteins is not absolutely required for nuclear pore assembly but is required for pore function (Finlay and Forbes, 1990). The effect of removing single pore proteins from the nuclear reconstitution system has also been examined and has proved enlightening on the topics of nuclear function and the function of specific nucleoporins (Dabauvalle et al., 1990, 1991; Finlay et al., 1991; Miller and Hanover, 1994; Powers et al., 1995, see Discussion). Focusing on the assembly of nuclear membranes, Wilson and Newport (1988) have shown that the egg contains a specific class of nuclear membrane vesicles, present in a disassembled state. Vigers and Lohka (1991, 1992), analyzing the membranes of the egg, identified two particulate fractions (NEP-A and NEP-B) which, in addition to cytosol, are required for proper nuclear envelope assembly. The molecular identity of these membrane-containing fractions has yet to be determined but should prove interesting for understanding the mechanism of nuclear envelope assembly. Interestingly, Sheehan et al. (1988), also using the egg extract, observed a putative intermediate in the pathway of pore assembly which occurs specifically at the end of mitosis (see Discussion).

An inherent complication of studying pore assembly using nuclear reconstitution assays is that the assembly of the pore cannot be examined independent of other nuclear events, such as chromatin decondensation and lamin assembly. One way to circumvent these constraints would be to study pore formation in annulate lamellae (Chen and Merisko, 1988; for review, see Kessel, 1992). Annulate lamellae (AL)[1] are found in the cytoplasm and consist of stacks of flattened membrane cisternae perforated by numerous and densely packed pore complexes lacking both chromatin and a lamina. AL are frequently observed in rapidly growing or differentiating cells, such as male and female gametes, tumor cells, and virally infected cells. Although they may have an additional functional role, AL are believed to store excess nuclear envelope and pore components for later use (Kessel, 1983, 1992; Stafstrom and Staehelin, 1984; Merisko, 1989).

Nuclear and annulate lamellae pores appear morphologically indistinguishable in their basic structure when viewed by a number of different electron microscopy techniques (Smith and Berlin, 1977; for review, see Kessel, 1992; see Discussion for exceptions). Several nucleoporin-specific antibodies as well as gold-labeled WGA recognize AL pores (Chen and Merisko, 1988; Allen, 1990; Dabauvalle et al., 1991). Moreover, nucleoplasmin-coated gold particles associate with the pores of both the nuclear envelope and AL (Feldherr et al., 1984), mimicking the binding step of nuclear import (Newmeyer and Forbes, 1988; Richardson et al., 1988). It is not yet known whether AL pores are capable of translocating nuclear signal proteins.

Annulate lamellae disassemble, together with the oocyte nucleus, upon meiotic maturation of an oocyte into an egg (Stafstrom and Staehelin, 1984; Bement and Capco, 1989). The disassembled nuclear membrane and pore components derived from these events are present in the mature egg in amounts equal to that found in >4000 somatic nuclei. It is these disassembled components that provide the basis of the nuclear assembly extract described previously. When DNA or chromatin is added to the extract, nuclei assemble (Lohka and Masui, 1983, 1984; Forbes et al., 1985; Newport, 1987). Interestingly, if no DNA or chromatin is added, Dabauvalle et al. (1991) found that AL assemble spontaneously in an extract. Specifically, employing immunofluorescence and electron microscopy, they observed the formation of stacks of AL replete with pore complexes. These findings suggest that if nuclear formation is prevented from proceeding normally around chromatin templates, the default pathway is to assemble pore and nuclear membrane components into AL.

Thus, the in vitro formation of annulate lamellae provides an attractive model system for studying pore assembly and disassembly, independent of the concomitant processes involved in nuclear assembly. Previously, pore assembly in both nuclei and AL could only be studied by using microscopy or, in the case of nuclei, by using functional assays. Here we describe the development of a biochemical assay that allowed us to study the assembly of nuclear pore proteins into the pore complexes of annulate lamellae. We used this assay to study the conditions necessary for AL assembly, the inhibitors of AL assembly, and an initial characterization of the nucleoporins present in annulate lamellae pores. In addition, we found that AL, once formed, can easily be recovered from a *Xenopus* assembly extract by pelleting. They can then be separated from a

1. *Abbreviations used in this paper*: AL, annulate lamellae; Con A–HRP, Concanavalin A–horseradish peroxidase; GSB, gel sample buffer; WGABP, WGA-binding protein.

large fraction of the other membranes in the *Xenopus* extract by homogenization and flotation, which potentially allows isolation of the vertebrate pore. A study of whether the *N*-acetylglucosaminylated nucleoporin family is necessary for AL assembly has also been pursued and characteristics of such modified AL examined.

Materials and Methods

Antibodies

The antibodies to the *Xenopus* nucleoporins nup60, nup97, and nup200 were raised against the appropriate gel-purified protein. Before use, they were affinity purified against their respective antigens immobilized on nitrocellulose strips (Macaulay et al., 1995; Powers et al., 1995). The antibody to rat nup58 has been previously described (Finlay et al., 1991). The integral membrane nucleoporin gp210 was detected with either Concanavalin A–horseradish peroxidase (Con A-HRP; E.Y. Laboratories, Inc., San Mateo, CA) or an affinity-purified rabbit antiserum raised against an *E. coli*–expressed glutathione-S-transferase fusion protein corresponding to amino acids 28–222 of rat gp210 (Wozniak et al., 1989). Nup153 was detected by mAb414 (Davis and Blobel, 1986; obtained from BAbCO). The mAb recognizing ribophorin was the generous gift of Dr. David Meyer (UCLA). Rabbit anti-HSA was obtained from Calbiochem-Novabiochem Corp. (La Jolla, CA).

Preparation of Xenopus Egg Extracts

Both crude and fractionated *Xenopus* egg extracts were prepared in egg lysis buffer (ELB) (10 mM Hepes-NaOH, pH 7.4, 3 mM $MgCl_2$, 50 mM KCl, 250 mM sucrose, 1 mM DTT, 100 μg/ml cycloheximide) as previously described (Newmeyer and Wilson, 1991), except for the following modifications. The cytosol was clarified by a second centrifugation at 200,000 *g* for 30 min, and 50-μl aliquots were stored at −70°C until use. Membranes were washed by dilution in at least 20 vol of membrane wash buffer (10 mM Hepes, pH 7.4, 250 mM sucrose, 250 mM KCl, 2.5 mM $MgCl_2$, 1 mM DTT) and recovered by centrifugation through a sucrose cushion for 20 min at 34,000 *g* (0.5 M sucrose in ELB); the membrane pellet was gently loosened and stored in 10-μl aliquots at −70°C. These membranes were estimated as concentrated 10-fold over that found in the crude extract and were therefore used as a 10× stock. Glycogen was purified from the gelatinous pellet derived from the 200,000 *g* centrifugation using a previously described method (Hartl et al., 1994) and was stored at −70°C as a 200 mg/ml stock in ELB.

Annulate Lamellae Formation

For each reaction, cytosol was mixed with the membrane fraction (1:10 or 1:20 dilution of the 10× stock) and purified glycogen (final concentration of 20 mg/ml, 1/10 volume of a 200 mg/ml stock), and allowed to incubate at room temperature for 0–5 h, depending on the experiment. In experiments where either membranes or glycogen were omitted, an equivalent volume of ELB was substituted. A typical assay consisted of 40 μl cytosol, 5 μl membranes, and 5 μl glycogen stock. An ATP-regenerating system was not added; the cytosol fraction has abundant endogenous ATP. At designated time points, 10 μl of the reaction mix was diluted 40-fold in ELB and centrifuged through a 300-μl sucrose cushion (0.5 M sucrose in ELB; 20 min at 34,000 *g*). If the pellets were to be analyzed directly using SDS-PAGE and immunoblotting, the samples were resuspended in loading buffer to a volume 20-fold the original sample volume.

To examine the effect of GTPγS on the ability of nucleoporins to incorporate into the membrane pellet, either GTP or GTPγS was added to a final concentration of 2 mM at $t = 0'$. Both of these reagents were diluted from 50 mM stocks in ELB.

Depletion of WGABPs from Egg Cytosol

The cytosolic fraction was depleted of the WGA-binding proteins (WGA-BPs) by two to three rounds of passage over WGA-sepharose (E.Y. Laboratories, Inc.), as in Finlay et al. (1990), except that the nonspecific binding sites on the WGA-sepharose were blocked by preincubation with 2 volumes of WGA-depleted cytosol. Where indicated, the cytosol was mock-depleted by the inclusion of 125 mM *N*-acetylglucosamine (GlcNAc) and 2 mM trichitotriose (TCT) in high sugar/ELB (HS/ELB) with the WGA-sepharose.

Electrophoresis and Western Blotting

Protein samples in Laemmli gel sample buffer (GSB) were resolved on standard polyacrylamide gels (Laemmli, 1970) and transferred to PVDF in Tris-glycine-SDS buffer. The presence of WGA-binding proteins was detected by prehybridization of the blots with PPT (2% polyvinyl-pyrrolidone, 0.1–0.4% Tween 20 in PBS) and probing with either ^{125}I-labeled WGA or WGA-HRP (1–2 h, RT) in PPT. Con A blots were performed in a similar manner; both lectin-HRP conjugates were obtained from E.Y. Laboratories, Inc. Antibody blots were performed by prehybridization in blocking buffer (3% BSA, 0.2% Tween 20 in PBS) and incubation with the appropriate dilution of antibody in BB for 1 h at room temperature. After extensive washing, the blots were again prehybridized in BB and probed either with ^{125}I-Protein A or with HRP-conjugated goat anti-rabbit IgG. HRP was detected by chemiluminescence (Renaissance kit, New England Nuclear, Boston, MA).

Fractionation of the Annulate Lamellae

In a first attempt to isolate the in vitro synthesized annulate lamellae (AL), the extract containing them was centrifuged through a sucrose gradient (Fig. 3). More specifically, a 300-μl reaction mix was brought to a final concentration of 15% sucrose by the addition of 65% sucrose/ELB (final volume of 0.475 ml). The sample was then loaded onto a step gradient consisting of 0.475 ml each of the following layers: 65, 60, 55, 50, 45, 40, 35, 30, 25, and 20% sucrose in ELB. The gradient was centrifuged at 45,000 rpm in a Ti55 rotor (Beckman Instruments, Inc., Fullerton, CA) for 20 h. The gradient was fractionated from the bottom into 10 × 0.5 ml fractions. GSB was added to two duplicate 15-μl aliquots of each fraction, and the samples were resolved by SDS-PAGE. The gels were transferred to separate PVDF blots and were probed with ^{125}I-Con A to determine the position of gp210, an anti-ribophorin monoclonal antibody to determine the position of ribophorin, or ^{125}I-WGA to determine the position of nup60 and nup200.

Because all of the ConA-binding proteins as well as the ER marker ribophorin comigrated at the same position in the above gradient, it appeared that all of the membrane vesicles had fused into large continuous networks. To disperse these structures into distinct subclasses of membranes, we homogenized the assembly reaction before flotation up through a sucrose gradient. For this, AL assembly assays (100 μl final volume) were incubated at room temperature for 3 h, before dilution with 2 vol of ELB containing 15 μg/ml RNAse A (Sigma, St. Louis, MO) to release ribosomes from the ER. After a 15-min incubation on ice, the samples were homogenized by 8–10 slow passes with a small ground-glass tissue homogenizer (Duall homogenizer, No. K-885450-0020; Kontes, San Leandro, CA), with gentle mixing after each pass. Note that the homogenization step is critical to the separation of AL from ER. Overhomogenization leads to the apparent disruption of pore complexes (Miller, B. R., unpublished observations), while use of a more gentle method such as douncing fails to separate the AL from ER. The samples were then recovered by centrifugation onto a two-step cushion of 2 M sucrose/ELB overlaid with 0.5-M sucrose/ELB, centrifuged at 30,000 *g* for 20 min. The membrane fraction was recovered from the 2 M/0.5 M interface, diluted with 4 vol of 10 mM Hepes, pH 7.4, and pelleted as above. The resulting pellet was carefully but completely resuspended in 125 μl ELB, and adjusted to 65% sucrose with 375 μl of 2.5 M sucrose/ELB. This sample was placed in the bottom of a prechilled SW55 tube and overlaid with one of two sucrose profiles. For the experiments shown in Figs. 4 and 5, the gradient consisted of 0.5 ml each of 60, 55, 50, 45, 42.5, 40, 37.5, 35, and 32.5% sucrose/ELB. The gradients were centrifuged at 300,000 *g* for a maximum of 20 h, and 0.25-ml fractions were collected from the bottom. The gradients in Fig. 8 were modified slightly by substituting a 1 ml block of 35% sucrose for the last two steps. This modification allowed for easier visualization of the difference in density of WGA-depleted and normal AL.

Electron Microscopy

For transmission electron microscopy of ultrathin sections of AL, samples (50 μl) of the extract were diluted 20-fold in ELB and centrifuged through 0.5 M sucrose onto a 2 M sucrose cushion at 34,000 *g* for 20 min. The fluffy layer at the interface was fixed in 2.5% glutaraldehyde (final) in buffer C (0.2 M cacodylate, pH 7.4) for 30 min on ice. After centrifugation for 10

min at 3,000 *g*, the pellet was resuspended in 20 μl of 2% low-melting-point agarose/buffer C and chilled on ice for 3 min. The pellet was then treated with 200 μl 2.5% glutaraldehyde/buffer C for 5 min, washed well with buffer C alone, and then postfixed in 2% osmium tetroxide/buffer C for 3 h. After several washes with ddH_2O, the pellet was treated with 0.5% uranyl acetate for 12–16 h at 4°C. The following day, the pellet was washed several times in water and subsequently dried in a graded series of ethanol washes (50, 70, 90, 95, 99, and 100%). The sample was embedded in Spurrs resin, cut into ultrathin sections, and poststained on grids with 2% uranyl acetate in H_2O for 20 min. Micrographs were taken with an electron microscope (EM 300; Philips Technologies, Cheshire, CT).

For negative staining of AL, samples of extract were diluted in ELB and centrifuged onto a 2 M sucrose cushion, as described previously. The interface was removed, washed, and resuspended in 10 mM Hepes, pH 7.4, 3 mM $MgCl_2$. Ionized, carbon-coated grids were placed face down on a drop of the resuspended material for 1 min, washed in H_2O, and placed on a drop of 2% uranyl acetate for 1 min. The grids were blotted dry and viewed with an electron microscope (EM 300; Philips Technologies).

Densitometry

The blots were scanned using of an 8-bit CCD camera, and the intensities of the bands were quantitated by NIH Image (ver. 1.49) software. To facilitate scaling of the resulting graphs, values are expressed as a percentage of the most intense band.

Results

A Biochemical Assay for the Formation of Annulate Lamellae

To develop a biochemical assay for nuclear pore formation and to study the assembly of known nucleoporins into pore complexes, we focused on their potential incorporation into annulate lamellae (AL). AL reconstitution, as discussed earlier, represents a simplified system for studying nuclear envelope and pore formation in the absence of chromatin. It had been demonstrated that while annulate lamellae are abundant in the cytoplasm of *Xenopus* oocytes, fully mature eggs and extracts harbor no pore-containing membranes (Steinert et al., 1974; Kessel and Subtelny, 1981; Imoh et al., 1983; Larabell and Chandler, 1988; Bement and Capco, 1989; Dabauvalle et al., 1991). During meiotic maturation of the oocyte, AL respond to mitotic signals in a manner similar to the nuclear envelope and, therefore, disassemble. Thus, the nuclear pores are in their disassembled state in a freshly prepared egg extract.

Dabauvalle et al. (1991) showed previously that, in the absence of added chromatin, annulate lamellae can form spontaneously over time in a crude egg extract. The AL were detected visually by either immunofluorescence or immunoelectron microscopy performed on the assembly extract. Their starting extract, though lacking AL, contained large amounts of membrane vesicles, Golgi stacks, coated vesicles, mitochondria, ribosomes, and disassembled nuclear pores (Scheer et al., 1988). To simplify the system, we fractionated the extract by centrifugation and used only those fractions we found were specifically required for AL formation in our assembly reaction. A crude extract was centrifuged at 200,000 *g* for 1.5 h, as previously described (Newmeyer and Wilson, 1991), which separated the extract into a gelatinous pellet consisting predominantly of glycogen and ribosomes, a mitochondrial layer, a membrane vesicle fraction, and a cytosolic fraction (Lohka and Masui, 1984; Newport, 1987; Hartl et al., 1994).

To prepare AL de novo, cytosol was mixed with the membrane fraction and allowed to incubate at room temperature for a period of several hours. We reasoned that if AL were indeed forming in the extract, the soluble disassembled nucleoporins would associate with the membranes over time as they became incorporated into pores. Several years ago the family of *N*-acetylglucosaminylated glycoproteins was shown to be required for pore function (for a review, see Introduction and Rout and Wente, 1994; Featherstone et al., 1988). In *Xenopus* the major members of this family of glycoproteins are the nucleoporins, nup60, nup97, and nup200, which bind to the lectin WGA (referred to here as the WGABPs) nuclear pore complexes. WGA-coated gold particles specifically decorate the NPCs of *Xenopus* nuclei (Scheer et al., 1988; Akey and Goldfarb, 1989; Pante and Aebi, 1993). Antibodies to the most prominent WGA-binding band in *Xenopus*, nup60, localize to the nuclear pore and block transport in vivo (Dabauvalle et al., 1988; Benavente et al., 1989). The glycoproteins nup97 and nup200 are also pore proteins (Powers et al., 1995; Powers, P., C. Macaulay, and D. Forbes, manuscript in preparation). These WGA-binding nucleoporins are initially soluble in the cytosol of egg extracts (Fig. 1, lane *13*). Previously it had been shown that removal of the WGABPs before nuclear reconstitution results in the inability of the resultant nuclei to import protein (Finlay and Forbes, 1990). The proteins nup60, nup97, and nup200 were thus used as examples of typical pore proteins that are initially soluble in an extract.

To initiate a biochemical study of AL assembly, we first asked whether these proteins become associated with the membrane fraction during incubation, as would be expected if they were becoming assembled into pores. The cytosol and membrane fractions of an extract were mixed and incubated at room temperature. At various times, aliquots were removed, diluted, and centrifuged through a sucrose cushion to separate the membranes from the soluble material. Fig. 1 shows an immunoblot of the pelleted membranes, probed with anti-nup60, anti-nup97, and anti-nup200 antibodies. At 0 h, no association of the soluble nucleoporins with the membranes was apparent (Fig. 1, lane *7*). The presence of nucleoporins in the pellet increased with time (Fig. 1, lanes *8–9*). By 90 min, a significant fraction of nup60, nup97, and nup200 had become associated with the membrane pellet. Incorporation of the soluble nucleoporins was in the range of 30–60% of the total, depending on the extract. The ability to pellet the nucleoporins was membrane dependent; it did not occur in the absence of membranes (Fig. 1, lanes *1–6*). We also noted that, although not required, this association seemed enhanced by the addition of glycogen, which was purified from the gelatinous pellet of the 200,000 *g* centrifugation step (Fig. 1; compare lanes *8–9* with lanes *11–12*). Glycogen alone, in the absence of membranes, did not cause any significant pelleting of the nucleoporins (Fig. 1, lanes *4–6*). Because of this seemingly specific effect, glycogen was generally included in the assembly reactions. Interestingly, the optimal concentration of glycogen was found to be 20 mg/ml, the same concentration shown to be optimal for nuclear envelope formation around protein-free lambda phage DNA in a fractionated egg extract (Hartl et al., 1994).

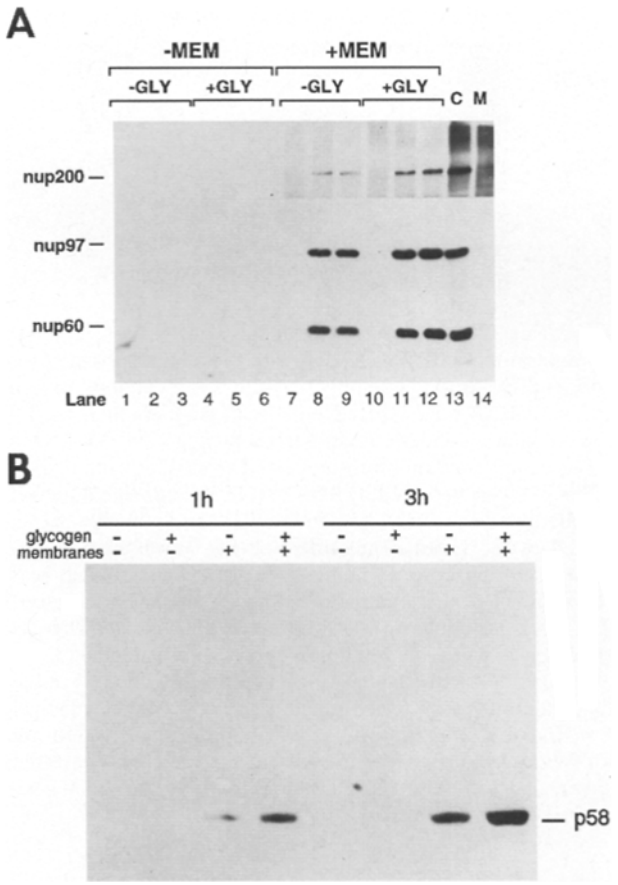

Figure 1. The nucleoporins nup60, nup97, and nup200 associate with the membrane fraction with increased incubation time. (*A*) AL formation assays (50 μl final volume) were incubated in the presence or absence of membranes (1× final concentration) and glycogen (final concentration of 20 mg/ml) at room temperature for several hours. At 0, 1.5, and 3 h, 10-μl aliquots were removed, diluted 40-fold in ELB, and pelleted through a sucrose cushion to separate the membranes from the soluble material. The pellets were resuspended in 200-μl loading buffer, 10 μl was subjected to SDS-PAGE, and the proteins were transferred to nitrocellulose (top portion of the blot) or PVDF (bottom portion of the blot). The top portion of the blot was pretreated with 4 M guanidine hydrochloride for 30 min to make the antigen more reactive. The transferred proteins were probed with affinity-purified anti-nup200 antibodies (*top portion*) or a mixture of affinity-purified anti-nup97 and anti-nup60 antibodies (*bottom portion*). The positions of nup200, nup97, and nup60 are indicated to the left. Lanes *1–13* each contain the equivalent of 0.5 μl of the input reaction mixture. Lanes *1–3* are derived from an assembly mix without added membranes or glycogen (*−MEM, −GLY*) and were incubated for 0, 1.5, and 3 h. Samples in lanes *4–6* contain added glycogen (*+GLY*) and were incubated for 0, 1.5, and 3 h. Lanes *7–9* contain cytosol with added membranes (*+MEM*; 0.05 μl equivalent per lane) and were incubated for 0, 1.5, and 3 h. Lanes *10–12* contain cytosol, with both added membranes and glycogen (*+MEM, +GLY*), and were incubated for 0, 1.5, and 3 h. Lane *13* contains 0.167 μl of the input cytosol, and lane *14* contains 0.05 μl of the input membranes for comparison; the results in lanes *13–14* demonstrate that nup200, nup97, and nup60 were present only in the cytosol prior to the start of the reaction. For calculating the percentage of incorporation of individual nucleoporins, an amount of nucleoporin equal to that in lane *13* would be the equivalent of 30% incorporation. (*B*) AL assembly reactions were incubated at room temperature for up to 3 h. At the designated times, an aliquot was removed, diluted in ELB, and centrifuged through a 0.5 M sucrose cushion. The entire membrane pellet (equivalent to 2 μl membranes) was resuspended in loading buffer containing SDS, boiled for 5 min, and resolved on a 9% polyacrylamide gel. The proteins were transferred to PVDF and probed with the anti–rat nup58 antisera, followed by incubation with an HRP-conjugated goat anti–rabbit IgG secondary antibody. The position of the cross-reacting *Xenopus* 58-kD band is shown.

It appeared from the data above that, upon incubation, the soluble nucleoporins became associated with the membrane fraction. When a similar AL assembly reaction was examined for the presence of nup58, which is found in a 600-kD complex with nup60 in the initial extract (Macaulay et al., 1995; see also Dabauvalle et al., 1990; Finlay et al., 1991; Haino et al., 1993), nup58 was also associated with the membrane pellet (Fig. 1 *B*). This association increased with time and paralleled the incorporation of nup60, nup97, and nup200 into membranes exactly.

Annulate Lamellae Are Present in the Membrane Pellet

To confirm that the association of nucleoporins with the membrane fraction represents true formation of annulate lamellae, we examined ultrathin sections of the pelleted membranes by transmission electron microscopy. Fig. 2 *A* shows an example of a stack of AL found after a 4-h incubation at room temperature. Such stacks were common in the pellet. In agreement with the results of others, we did not detect any pore-containing membranes at $t = 0'$, confirming that intact NPCs were not being introduced by any of the individual components of the reaction mixture (Steinert et al., 1974; Kessel and Subtelny, 1981; Imoh et al., 1983; Larabell and Chandler, 1988; Bement and Capco, 1989; Dabauvalle et al., 1991).

Because the transmission electron microscopy used in this study requires a lengthy sample preparation, we asked whether the presence of AL could be detected by a simple negative-staining procedure. For this, a carbon-coated grid was placed on a drop of the resuspended membrane pellet from a 3-h incubation reaction. We found that, indeed, AL could be identified in this manner. A single lamella is shown in Fig. 2 *B*. These single lamellae were easy to visualize, but perhaps not surprisingly, most of the AL on the grid were present as large stacks, prohibiting good resolution of individual pores. However, the dense hexagonal packing characteristic of AL was clearly visible in the single sheets. In some patches, the eightfold symmetry of individual NPCs was also discernible when observed at higher magnification (Fig. 2 *C*).

Comigration of the WGABPs with the Integral Membrane Nucleoporin, gp210, in a Distinct Membrane Fraction

At this point, a correlation had been established between the presence of the nucleoporins nup58, nup60, nup97, and nup200 in the membrane pellet and detection of AL in this pellet by electron microscopy. There was still a possibility, however, that the pelleting of the nucleoporins with the membranes did not reflect AL assembly but some random process such as nonspecific sticking to membranes or sim-

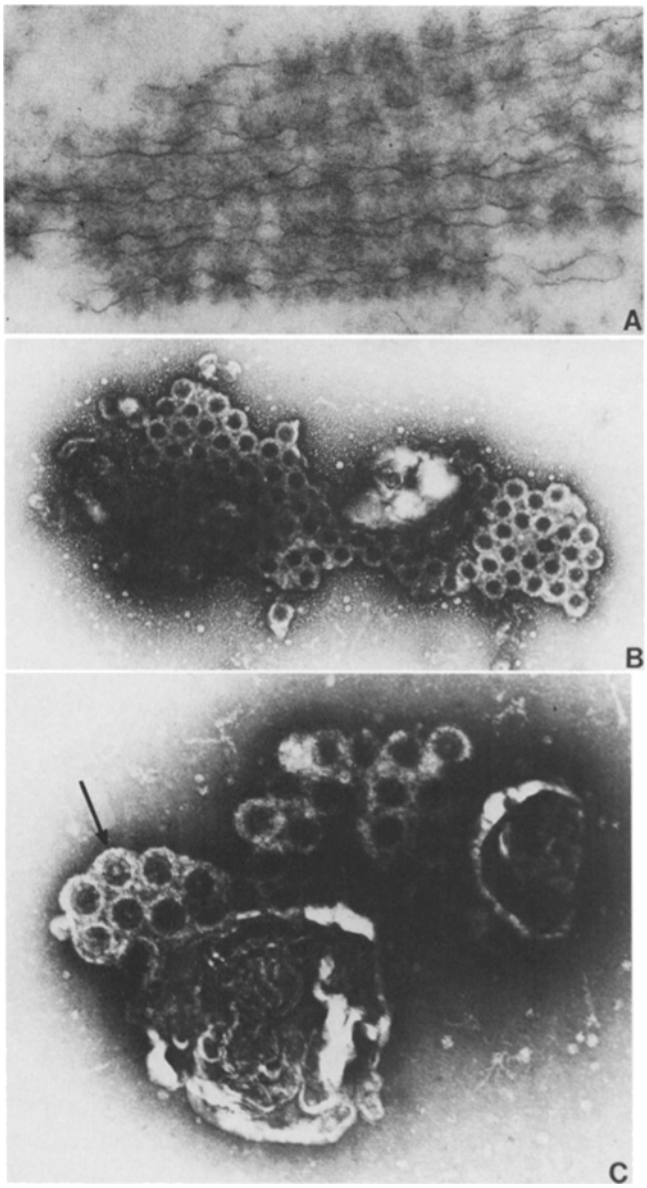

Figure 2. In vitro synthesized AL are present in the membrane pellet. (*A*) Cytosol was incubated with membranes and glycogen for 4 h at room temperature. The assembly reaction was diluted in ELB and centrifuged through a layer of 0.5 M sucrose onto a 2 M sucrose cushion. The fluffy pellet at the interface between the two sucrose layers was removed, mixed with a drop of low-melting agarose, and processed for thin-section transmission electron microscopy as described in Materials and Methods. (*B*) Alternatively, the fluffy pellet was gently homogenized, applied to ionized, carbon-coated grids, washed with H_2O, and negative-stained with uranyl acetate. (*C*) In some views, elements of the eightfold symmetry of individual nuclear pores could be seen.

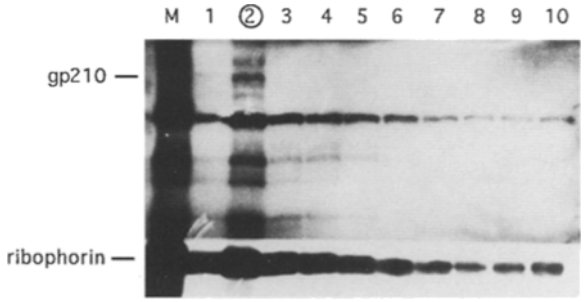

Figure 3. Nup60 and nup200 cosediment with the pore integral membrane protein gp210 as well as with the ER membrane protein ribophorin. An AL assembly reaction was incubated for 4 h, diluted with ELB, and layered onto a sucrose gradient as described in Materials and Methods. After centrifugation at 300,000 *g* for 20 h, 0.5-ml fractions were collected from the bottom. Aliquots (15 μl) were resolved by SDS-PAGE in duplicate. One gel was probed with ^{125}I-WGA to detect nup60 and nup200 (*circled fraction*; data not shown). The other blot (Fig. 3) was cut horizontally at a position corresponding to 70 kD. The top half was probed with ^{125}I-Con A and the bottom with an antibody against the ER marker ribophorin followed by ^{125}I–Protein A. The circled number indicates the fraction that contained the peak of the WGABPs, as determined by Western analysis with WGA. In the WGA blot, nup60, nup97, and nup200 were observed in a distinct peak in fraction 2. The positions of ribophorin and gp210 are shown. With each probe, the majority of the signal was detected in a distinct peak centered on fraction 2. The lane marked *M* contained 2 μl of total membranes.

ple aggregation of the soluble pore proteins into a large, dense pelletable mass. We therefore attempted to isolate the AL away from free vesicles by centrifugation through a sucrose gradient to test whether nup60, nup97, and nup200 enriched together and did so in the fraction that contained AL. When the assembly reaction was centrifuged through the gradient, however, all of the particulate material visible by eye sedimented in one large peak near but not at the bottom of the gradient (at ~45% sucrose). Furthermore, when aliquots of the fractions from the gradient were resolved by SDS-PAGE and probed for various nuclear membrane and ER proteins, all of those proteins peaked in the same fraction (Fig. 3, fraction 2). The probes used were (*1*) WGA, to detect the glycosylated nucleoporins nup60, nup97, and nup200, (*2*) antibody to ribophorin, an integral membrane protein of the ER, and (*3*) Con A, which recognizes complex carbohydrate modifications common to ER integral membrane proteins. Note that gp210, an integral membrane pore protein recognized by Con A (Gerace et al., 1982; Wozniak et al., 1989; Greber et al., 1990), was present in fraction 2 (Fig. 3), as were nup60, nup97, and nup200 (indicated by the circled fraction number; data not shown).

It had been reported previously that in vivo annulate lamellae are often continuous with both the outer nuclear envelope and the ER network (for review see Kessel, 1992). It appeared that a similar phenomenon might be occurring in the extract, since all the vesicles had apparently fused into large membranous structures. It has also been shown that ER vesicles spontaneously fuse into tubules and sheets in this same type of extract reaction mix (Newport and Dunphy, 1992). We wished, therefore, to separate these membrane entities by homogenization, in an attempt to disperse them into discrete subpopulations of membranes. Following this treatment, we would then be able to determine whether the WGA-binding nucleoporins and the integral membrane pore protein gp210 were specifically enriched in a single fraction that was separate from other membrane proteins. To do this, an AL assembly reaction was set up and incubated at room tempera-

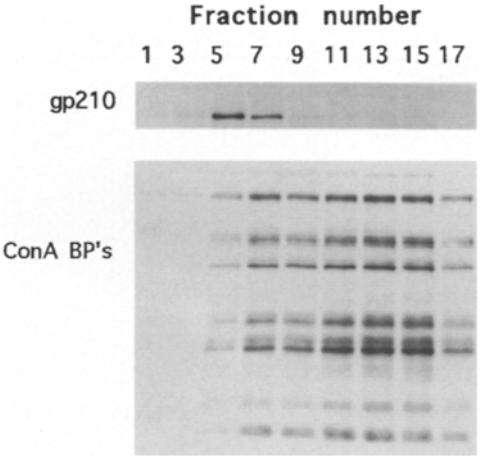

Figure 4. Separation of gp210-containing membranes from the bulk of ER membranes. To attempt to separate AL from ER membranes, an AL assembly reaction was set up and incubated for 3 h at room temperature. The mixture was then homogenized and the membrane fraction recovered, as described in Materials and Methods. The membrane pellet was resuspended in a small volume of ELB, 2 μg HSA was added, and the resuspended membranes were adjusted to 65% sucrose and layered at the bottom of a prechilled SW55 centrifuge tube. The sample (0.5 ml) was overlaid with 0.5 ml blocks of 60, 55, 50, 45, 42.5, 40, 37.5, 35, and 32.5% sucrose (all solutions contained the same buffer composition as ELB), and the gradient was centrifuged at 300,000 *g* for 20 h. Fractions (0.25 ml) were collected from the bottom, and the proteins were precipitated as in Wessel and Flugge (1984). Samples (10% of each fraction) were run on a 7.5% SDS gel and transferred to PVDF. The top portion of the blot corresponding to polypeptides >175 kD was probed with affinity-purified antiserum to a portion of rat gp210 and detected with an HRP-labeled goat anti–rabbit secondary antibody. The remainder of the blot was probed with HRP-ConA. The integral membrane pore protein gp210 was found predominantly in fractions 5–7, while most of the Con A–binding proteins (Con A BPs) were found to enrich near the top of the gradient. Greater than 80% of the loaded amount of any individual nucleoporin was recovered in the peak gradient fractions.

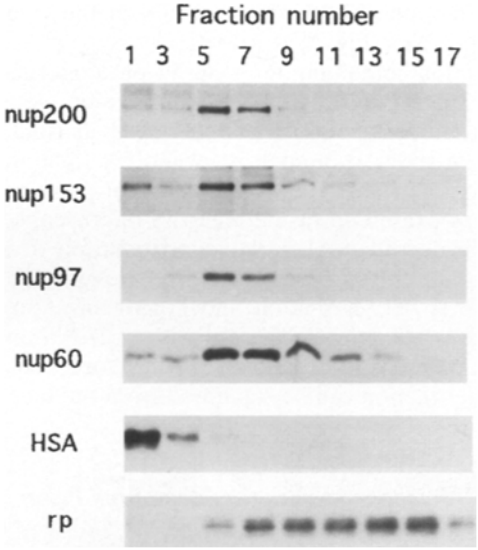

Figure 5. All the nucleoporins tested comigrate with the gp210-containing membrane fraction. Aliquots of the gradient fractions in Fig. 4 were resolved on multiple SDS gels and transferred to either nitrocellulose (for anti-nup200) or PVDF (all other antibodies, i.e., anti–rat nup62, anti–*Xenopus* nup97, mAb 414 to detect nup153, anti-HSA, and anti-ribophorin). The initially soluble pore proteins, nup60, nup97, nup153, and nup200, after incubation to form AL and homogenization were all detected in fractions 5–7 (Fig. 5), coincident with the integral membrane pore protein gp210 (shown in Fig. 4). Distribution of the ER marker ribophorin (rp) was found to follow the pattern of the Con A–binding proteins and peaked in fraction 15. HSA was included in the gradient load zone as a control for nonspecific sticking or entrapment of protein with the membranes. As expected, the HSA was found primarily in fraction 1.

ture for 3 h. The reaction was homogenized as described in Materials and Methods, pelleted, applied to the bottom of a 32–65% concave sucrose gradient, and centrifuged at 300,000 *g* for 20 h to float the membrane-containing structures. As shown in Fig. 4 (*upper portion*), when the reaction mix was homogenized and subjected to flotation, the pore protein gp210 peaked in fractions 5–7 (at a density ~1.20 g/cc). In contrast, the majority of other Con A–binding proteins, as well as ribophorin, were dispersed throughout the gradient, peaking near the top of the gradient in fraction 15 (Fig. 4, *lower portion*).

When the fractions were probed with antisera to the initially soluble glycosylated nucleoporins, it was observed that fractions 5–7 also contained the peak of nup60, nup97, and nup200 (Fig. 5). These results demonstrate that the initial pelleting of the soluble nucleoporins is not due to aggregation or precipitation, because such nonmembranous structures would remain at the bottom of the flotation gradient. Instead, the cosedimentation of the initially soluble nucleoporins with the integral membrane pore protein gp210 in a dense fraction, separate from the bulk of the membrane proteins, supports the conclusion that these nucleoporins are incorporated into pore complexes within the membranes.

The number of vertebrate pore proteins that have been identified, and to which there are antibodies, is limited. One protein, nup153, is a pore protein found in the distal ring of the basket of the pore and on filaments extending from the basket into the nucleoplasm (Cordes et al., 1993; Sukegawa and Blobel, 1993). The Zn^{2+} finger-containing nup153 protein contains GXFXG repeat sequences, as do nup200 and nup60, and is therefore recognized by the anti–GFXFG monoclonal antibody, mAb414. To determine whether nup153 cofractionates with the pore proteins gp210, nup58, nup60, nup97, and nup200, a blot of the same set of membrane vesicle fractions was probed with mAb 414 (Fig. 5). Nup153 can clearly be seen to cofractionate with the other pore proteins. In contrast, the soluble protein human serum albumin (HSA), which was added to the homogenized membranes before flotation as a negative control, did not float up with the membranes but remained at the bottom of the gradient (Fig. 5). Ribophorin, an ER protein, was also present in small amounts in the pore-containing fractions 5–7, as was a small amount of the ER integral membrane proteins visualized with Con A, consistent with the predicted derivation of AL membrane components from the ER (Kessel, 1983). The bulk of ribophorin, however, floated higher in the gradient (Fig.

5), peaking in fractions 13–15 with the bulk of the Con A–binding ER proteins (Fig. 4).

When the fraction containing the peak of nucleoporins was fixed, sectioned, and examined by transmission electron microscopy, it was found to be enriched in small stacks of AL (not shown). In contrast, when the fraction containing the peak of the ConA-binding or ER proteins (fraction 15) was examined in the electron microscope, it was found to be devoid of AL and to consist entirely of a heterogeneous population of vesicles (not shown). We conclude that after AL formation and homogenization, the soluble pore proteins cofractionate with gp210 in an AL-containing membrane fraction. Furthermore, this dense membrane fraction can be separated from the bulk of the ER.

Association of Nucleoporins with the Membrane Pellet Is Blocked by GTPγS

Many cellular processes are known to require small GTP-binding proteins (Dickey and Birnbaumer, 1993). With respect to potential steps in AL formation, GTP might be expected to be involved in one or more steps in the actual assembly of the pore complex, and/or in the fusion of vesicles that would be required for forming a large membranous structure such as the AL (for reviews see Balch, 1989; Hall, 1990). Indeed, Boman et al (1992*a*) have shown that during nuclear envelope assembly in a *Xenopus* egg extract, GTP hydrolysis is required for the fusion of chromatin-bound, nuclear-specific vesicles into a double membrane nuclear envelope.

To test the dependence of AL formation on GTP hydrolysis, GTPγS was added to an assembly reaction at $t = 0'$. GTP was added to a control sample to the same final concentration. AL were allowed to form for 0–3 h as usual, after which the membranes were recovered by centrifugation. Fig. 6 shows an immunoblot of the pelleted membranes, using anti-nup60, anti-nup97, and anti-nup200 antibodies as probes. No incorporation was observed if the reaction was carried out at 0°C (Fig. 6 *A*, lanes *4–6*). Incorporation of the nucleoporins into membranous structures proceeded normally either under control conditions (lanes *1–3*) or in the presence of added GTP (lanes *7–9*). However, the incorporation was essentially blocked by addition of the nonhydrolyzable analogue, GTPγS (lanes *10–12*). When the fractions in Fig. 6 *A* were probed with the mAb414 for the presence of the nucleoporin nup153, this nucleoporin's incorporation into AL was also found to be blocked by GTPγS in the assembly reaction (Fig. 6 *B*, lanes *10–12*). A protein of ≥270 kD, which reacts with the anti-GXFXG mAb 414, also failed to incorporate into the membrane pellet (Fig. 6 *B*). We do not know if this protein is a pore protein but assume it is because it coenriches on sucrose gradients with AL and known pore proteins. These results indicate that AL assembly, measured by pore protein association with the membranes, requires GTP hydrolysis. Indeed, when the samples incubated in the presence of GTPγS were examined by thin-section electron microscopy, no AL were observed. The bulk membranes appeared identical to membranes at $t = 0'$, that is, the vesicles had undergone no membrane fusion (data not shown).

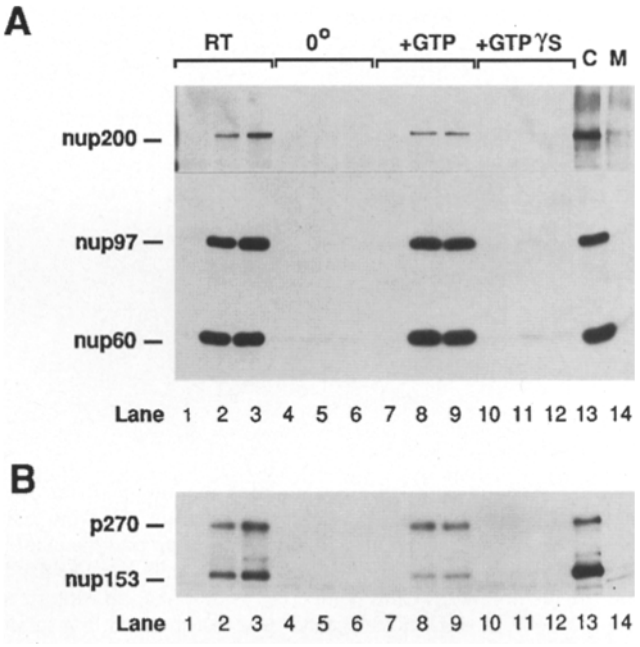

Figure 6. Association of the soluble nucleoporins with AL-containing membranes requires physiological temperature and hydrolyzable GTP. A 100 μl AL mix was assembled on ice and divided into four aliquots. One aliquot remained on ice for the reaction course (lanes *4–6*), while the other three were placed at room temperature after the addition of buffer (lanes *1–3*), GTP (2 mM final, lanes *7–9*), or GTPγS (2 mM, lanes *10–12*). Samples were removed at the indicated times (0, 1.5, and 3 h), diluted 40-fold with ELB, and pelleted as described in Materials and Methods. A sample of the cytosol added to each reaction at $t = 0'$, corresponding to 30% of the input, was loaded in lane *13*. Lane *14* contains an amount of input membranes equivalent to that in lanes *1–12*. Duplicate gels were run, one of which was transferred to nitrocellulose (anti-nup200) and one to PVDF. The blots were probed with either anti-nup200 (6 *A*, *upper portion*), a mixture of anti-nup97 and anti-nup60 antibodies (6 *A*, *lower portion*), and mAb414 (6 *B*). The positions of nup60, nup97, nup200 in Fig. 6 *A*, and p270 and nup153 in Fig. 6 *B* are indicated at the left. As in Fig. 1, an amount of nucleoporin equal to that in lane *13* would be the equivalent of 30% incorporation.

With respect to the amount of incorporation of the different nucleoporins into AL, the efficiency of incorporation of nup153 into AL is somewhat lower than that of the WGABPs (Fig. 6, *A* and *B*; compare lanes *2–3* with lane *13* in both figures; lane *13* contains 30% of the input cytosol). At the present time the significance of this observation is unclear as it is not known whether the cytosolic fraction contains equivalent stoichiometric amounts of each pore protein. However, it is possible that the apparent excess of the WGABPs over nup153 in AL may be related to the way that pore complexes and individual pore proteins are assembled in annulate lamellae. The amount of incorporation of the nup60 and nup97 was relatively high, usually 30–80% (see Fig. 6 *A*). The degree of incorporation of nup200, a presumed component of the cytoplasmic filaments extending from the nuclear pore, was consistently less (Fig. 6 *A*). Possible reasons for the lower amounts of incorporation of nup153 and nup200 are addressed in the Discussion.

Annulate Lamellae Pore Formation Occurs in Extracts Depleted of WGABPs

Finlay and Forbes (1990) showed that when the WGABPs are removed from egg cytosol prior to nuclear reconstitution, an intact nuclear envelope still forms around exogenously added sperm chromatin. This envelope is studded with numerous nuclear pore complexes which are incompetent for import. By transmission electron microscopy, the nuclear pores appeared relatively normal in structure but were less densely stained by uranyl acetate (Finlay and Forbes, 1990). It was concluded that the major structural scaffold of the nuclear pore assembles independent of the WGABPs. This finding corroborated much EM labeling data, which showed a nonscaffold location for the WGABPs in the pore (Finlay et al., 1987; Akey and Goldfarb, 1989; Pante et al., 1994). In other studies, nuclear pores failed to form in a WGA-depleted extract (Dabauvalle et al., 1990) or were structurally defective (Miller and Hanover, 1994). It may be that slight differences in experimental protocol are the cause of these discrepancies, since multiple complex processes must occur for nuclear formation to take place. To analyze the pore protein requirements for AL assembly, we wished to determine whether AL can assemble independent of the WGABPs. Cytosol was depleted of WGABPs by passage over WGA-sepharose. The depleted cytosol was then mixed with membrane vesicles and glycogen, and incubated at room temperature for 4 h. A mock-depleted sample was prepared simultaneously by incubation of the cytosol with the lectin-sepharose in the presence of the competing sugar *N*-acetylglucosamine, followed by incubation with membranes for 4 h. The assembly reactions were diluted and pelleted through a sucrose cushion as described above. The membrane pellets were then prepared for thin sectioning as detailed in Materials and Methods and observed in the electron microscope. Examination of a number of sections revealed that both the mock-depleted and the depleted samples contained stacks of AL, although the AL in both were somewhat smaller and had fewer pores/area than those typically found in untreated cytosol. Fig. 7 *A* shows a cross-section of a representative patch of AL which was formed in the depleted cytosol. An *en face* view is shown in Fig. 7 *B*. This result indicates that the pores of both nuclear envelopes and annulate lamellae can form in the absence of WGABPs.

As stated earlier, previous work indicated that WGABP-depleted pores in nuclei showed a loss of electron density by thin-section electron microscopy (Finlay and Forbes, 1990). When WGABP-depleted AL were formed, homogenized, and then assayed biochemically for their migration in a sucrose density flotation gradient, they were found to have a different buoyant density than control AL. To locate the position of the depleted AL, an immunoblot for nup153 was used (Fig. 8, *A* and *B*, *open circles*). We have observed that this nucleoporin is not significantly removed by WGA-sepharose depletion (Finlay and Forbes, 1990; Fig. 8). As shown in Fig. 8 *B*, the depleted nup153-containing membranes were found at a lighter position in the gradient (fractions 8–11) than in the control reaction, where the majority of the nup153 was found in fractions 6–10 (Fig. 8 *A*). Note that the gradients were altered slightly from those in Figs. 4 and 5 to obtain better separation of depleted AL from control AL (see Materials and Methods). The peak position of the control AL in Fig. 8 corresponds to a density of 1.20 g/cc, whereas the density of the WGA-depleted AL had a density of 1.185g/cc. In both cases, the position of the nup153 pore protein in the gradients corresponded with the position of the integral mem-

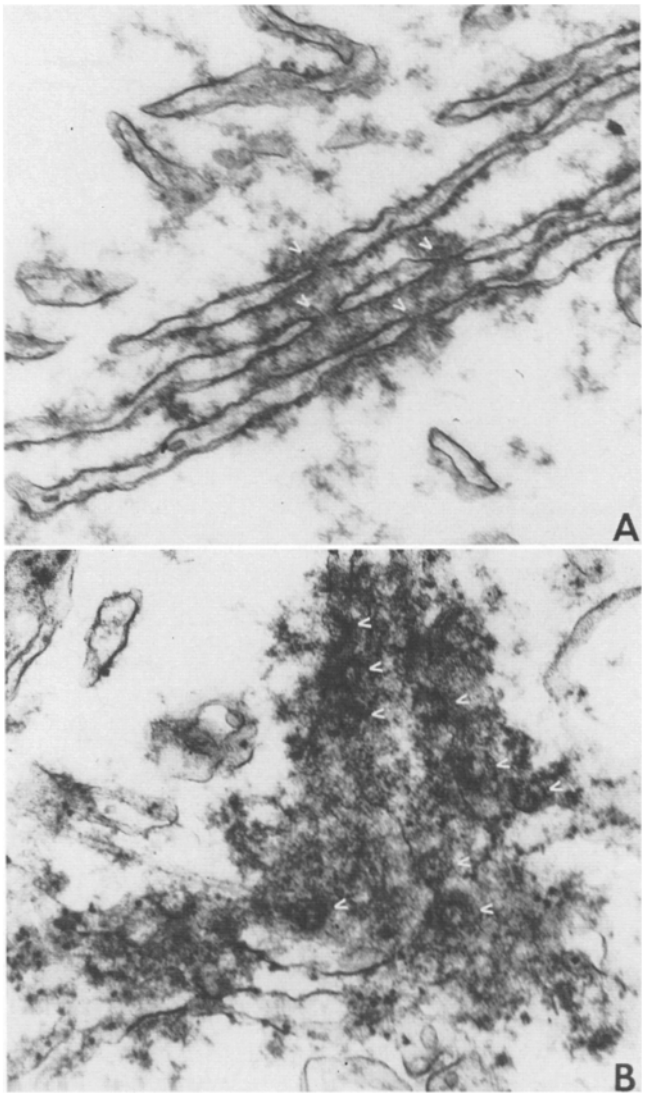

Figure 7. AL form in an extract depleted of WGABPs. Cytosol was depleted of WGABPs by two consecutive 1-h incubations with 0.5 volume WGA-sepharose. A control sample was incubated with WGA-sepharose in the presence of the competing sugars (HS/ELB). The depleted or mock-depleted cytosol was then mixed with glycogen (20 mg/ml final concentration) and membranes (1× final concentration) and allowed to incubate at room temperature for 4 h. The reaction mixes were diluted 20-fold in ELB and centrifuged through a 0.5 M sucrose layer onto a 2 M sucrose cushion. The fluffy pellet at the interface between the 0.5 M and 2 M layers was removed and processed for thin sectioning as described in Materials and Methods. Shown in the upper panel is a cross-section of a small stack of AL formed in the depleted cytosol; shown in the lower panel is an en face view of AL found in the same sample. These stacks are representative of what was found in both the depleted and the mock-depleted samples. The arrowheads mark pore complexes.

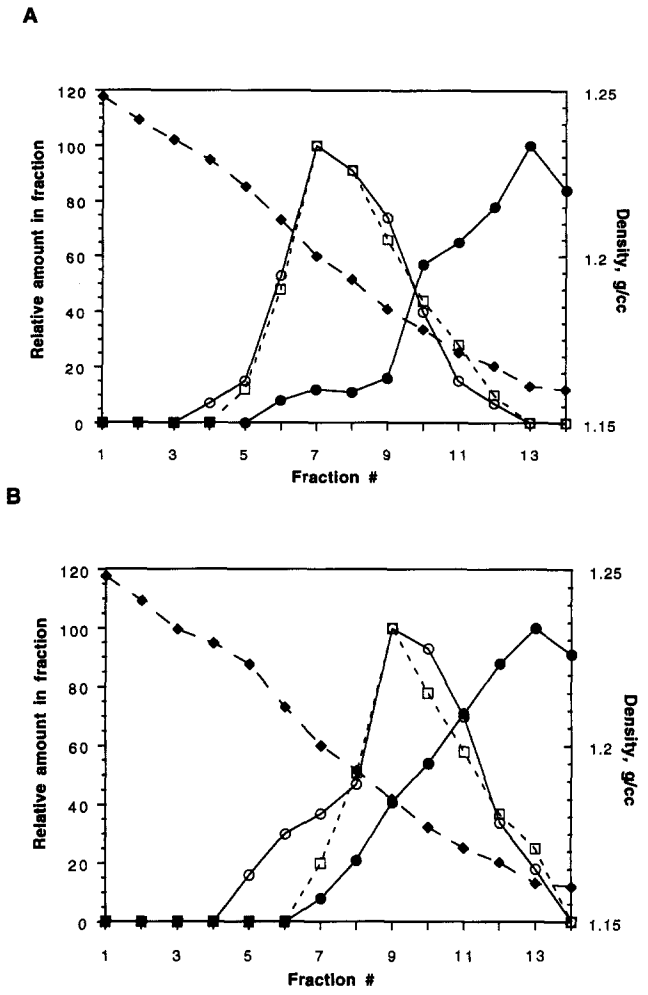

Figure 8. Removal of the pore proteins nup60, nup97, and nup200 results in the formation of AL of lighter buoyant density. AL reactions were set up using (*A*) unfractionated or (*B*) WGABP-depleted cytosol as described in Materials and Methods. At 3 h, the reactions were homogenized and analyzed by flotation through 65–35% sucrose gradients. Samples were processed as in Figs. 4 and 5. The amount of nup153 (*open circles*), gp210 (*open squares*), and ribophorin (*filled circles*) in each fraction was determined by densitometry and is expressed as a percentage of the peak fraction. The density of sucrose (*filled diamonds*) was calculated from the refractive index of each fraction. As in Figs. 4 and 5, the peak of the pore proteins nup153 and gp210 in the control sample occurred at a density of 1.20 g/cc (peaking in fraction 7, *A*). In contrast, the pore proteins nup153 and gp210 in the WGABP-depleted AL peaked in fraction 9 at a density of 1.185 (*B*). There was no significant difference in the sucrose density profile of the gradients. The ER marker protein, ribophorin, showed very similar distributions in both the control and depleted samples, peaking at 1.16 g/cc. The density of pure protein is assumed to be in the range of 1.27–1.3 g/cc, so free proteinaceous structures would remain at the bottom of the gradient (density ~1.24 g/cc).

brane pore protein gp210 (Figs. 8, *A* and *B*, *open squares*). In the control AL reaction, the WGA-binding nucleoporins also peaked in these fractions. The peak of ER membranes, as followed by the marker ribophorin, was much lighter in density and was identical in both gradients (Fig. 8, *A* and *B*). Although the density difference between the WGA-depleted and control AL is small, it was highly reproducible. This indicates that removal of a subset pore proteins can be correlated with a change in the physical properties of the annulate lamellae.

Discussion

In this study, the assembly of soluble nucleoporins into pore complexes was probed using a cell-free extract prepared from *Xenopus* eggs. Specifically, a biochemical assay was developed and used to monitor the incorporation of different, initially soluble nucleoporins into membranes, concomitant with incorporation of an integral membrane pore protein, gp210, into the same membrane population. Upon incubation of an egg extract, all nucleoporins tested became membrane associated in a time- and temperature-dependent manner, as determined by membrane sedimentation. Annulate lamellae formation was observed to occur with the same time course by EM. In the presence of a nonhydrolyzable analog of GTP, visible AL did not form. Importantly, and perhaps due to an inhibitory effect distinct from the observed block in membrane fusion, the membrane association of the soluble nucleoporins also failed to occur. Upon examination of the physical properties of AL in a normal assembly reaction, AL were initially found to sediment in a large membrane population that contained all pore and ER protein markers. This finding is consistent with the one that AL and ER membranes are contiguous in vivo (Kessel, 1983), and with the finding that upon incubation of *Xenopus* egg extracts, the membrane vesicles fuse into large, ER-like reticular structures (Newport and Dunphy, 1992). Homogenization and flotation allowed us to separate a dense membrane fraction enriched in AL from the bulk of the ER and nonlamellar membranes. By immunoblotting, nucleoporins were observed to be incorporated along with gp210 in this dense membrane fraction. When the AL assembly reaction was altered by removing the WGA-binding nucleoporins, we found that AL continued to assemble but had an altered density. Thus, the results described are an initial study of the mechanism of envelope assembly under conditions where all nucleoporins are present and under conditions where a subset is absent.

Molecular Composition of Annulate Lamellae Pores

It is generally believed that the structure and molecular composition of the nuclear pore, at a basic level, is similar to that of pores of annulate lamellae. Because the elaborate imaging and Fourier averaging techniques used to study nuclear pores have not yet been applied to AL and because little biochemical work has been done on AL, this conclusion is based largely on microscopic observations (for review see Kessel, 1992). Such structural work indicates that both types of pores have eightfold radial symmetry. Both consist of two large rings stacked above and below a smaller central ring of spokes (for review, see Hinshaw et al., 1992; Kessel, 1992; Akey and Radermacher, 1993). Movement of substrates through the nuclear pore is thought to be mediated by a transporter located at the center of this spoke ring (Akey and Goldfarb, 1989; Akey and Radermacher, 1993; Dingwall, 1990). Although trans-

port through AL pores cannot be assayed, these pores, like nuclear pores, have been shown to bind gold particles coated with nuclear transport substrate (Feldherr et al., 1984).

From the structural similarity of the pores, the nucleoporins nup60 and nup58, located near the central transporter, and the membrane pore protein gp210 could be predicted to be present in AL, as these proteins are integral to pore structure and function. Previously, AL pores have been shown to be recognized by antibodies to the nucleoporin nup60 (also known as p62; Allen, 1990; Dabauvalle et al., 1991). Indeed, we confirmed biochemically the presence of nup60 in the AL fraction; by immunoblotting, we find that nup58 is also present. In addition, we find that the dense AL-containing fraction contains the integral membrane nucleoporin gp210. All the gp210 present in the egg extract, upon incubation, becomes incorporated in this densely sedimenting membrane population. These results support the conclusion that the nucleoporins are becoming incorporated into AL pores. A further conclusion that can be derived from the presence of gp210 in AL is that AL and nuclear pores must share, at least in part, a common membranous precursor.

Until recently the nuclear pore appeared bilaterally symmetrical. High-resolution electron microscopy has now revealed additional structural elements which indicate that the nuclear pore has a polar nature. On the nucleoplasmic side, eight long filaments of 1,000Å project away from the pore and join at their distal ends to a terminal ring; the entire structure is termed the nuclear basket (Ris, 1990; Goldberg and Allen, 1992; Pante and Aebi, 1993; Pante et al., 1994). Extending from the cytoplasmic ring of the pore is a set of eight short fibers termed the cytoplasmic filaments. Biochemically, the nuclear basket contains the pore protein nup153, while the cytoplasmic filaments contain the proteins CAN/nup214, nup180, and TPR/nup265 (Snow et al., 1987; Cordes et al., 1993; Sukegawa and Blobel, 1993; Wilken et al., 1993; Byrd et al., 1994; Kraemer et al., 1994; Pante et al., 1994). Both CAN and TPR have been associated with oncogenesis in humans when fused to other genes, presenting the intriguing possibility that a new class of oncogenes causes tumorigenesis by altering the function of the nuclear pore.

The asymmetry of the nuclear pore may be generated by internal nuclear constituents. DNA could direct polar assembly of the basket, as at least one component of the basket, nup153, has intrinsic DNA-binding ability (Sukegawa and Blobel, 1993). Alternatively, since the nuclear pore is imbedded in the nuclear lamina, the lamina may play a role in seeding the assembly of the basket so that it occurs exclusively on the nuclear face of the pore. A third possibility is that the newly described nuclear lattice may initiate polarity of the pore (Goldberg and Allen, 1992). A priori there is no reason to assume that the pores of the AL would be polar, that is, that they would contain nuclear baskets and/or cytoplasmic filaments. Indeed, the tight packing of the adjacent membrane stacks in AL might seem to preclude the existence of at least one of these extended structures, there being less than 1,000Å between stacks. The reconstitution assay developed here allowed us to determine whether constituents of the basket and cytoplasmic filaments were present in AL. Upon analysis, we found that both the nucleoporin nup200, which is the *Xenopus* equivalent of CAN/p214 (Powers, M., C. Macaulay, and D. Forbes, manuscript in preparation), and the basket protein nup153 are incorporated into AL. Although we do not know whether egg extracts contain stoichiometric amounts of all the pore proteins, both nup153 and nup200 do seem to be incorporated at lower efficiencies into AL when compared to nup60 (see Figs. 1 and 6). In explanation of this, it is possible that the AL pore is indeed polar but has only remnant (or incipient) structures corresponding to the basket and cytosolic filaments. The tight packing of the stacks would prevent full assembly on all but the outermost stacks. Alternatively, it is possible that, without topological cues such as chromatin or a lamina, the AL pore is inherently an apolar structure. Limited amounts of filament assembly may then occur randomly on either face of the pore, so that each pore contains small amounts of nup153 and nup200 on both sides. Further examination of the specific location of nup153 and nup200 in AL needs to be addressed by immunoelectron microscopy.

Nup153, in addition to being a basket component, is also found on fibers extending from the basket farther into the nucleus (Cordes et al., 1993). The WGA-binding protein, nup97, may have a similar nuclear localization pattern. By immunofluorescence, nup97 is a component of both the nuclear pores and the nuclear interior (Powers et al., 1995). Peptide sequence analysis (Powers et al., 1995) indicates that nup97 has regions of strong similarity to the yeast GLFG nucleoporin, nup116, and other members of the GLFG nucleoporin family (Wente et al., 1992; see Rout and Wente, 1994, for review). When AL were examined biochemically for the presence of nup97, it was found that this protein clearly associates with the dense gp210- and AL-containing membranes. Thus, all the nucleoporins examined, which now include gp210, nup58, nup60, nup97, nup153, and nup200, become incorporated into the same physically distinct, pore-containing fraction in the assembly reaction.

Biochemically Altered Pores of AL

We examined whether AL were able to form in the absence of the WGA-binding nucleoporins nup60, nup97, and nup200. We found that AL do indeed form in such depleted extracts, as judged both biochemically and by electron microscopy. In this instance, nup153 was used as a marker for the assembly of AL. The initially soluble nucleoporin nup153 was found to cofractionate with the integral membrane pore protein gp210, both in normal extracts and in extracts lacking WGA-binding proteins. However, the density of the depleted AL (1.185 g/cc) was lighter than that of control AL (1.20 g/cc). The distribution and peak position of ER membranes in both gradients were identical (peak 1.16 g/cc). These results indicate that depleted AL are still formed as a discrete structure separable from the ER, but that this structure has an altered density.

The density difference between glycoprotein-depleted and normal AL could result from one of two possibilities. The most prominent of the *Xenopus* WGA-binding nucleoporins, nup60, nup97, and nup200, have been shown by gel filtration to be present in the cytosol initially as dis-

tinct, high molecular weight, protein complexes (Dabauvalle et al., 1990; Finlay et al., 1991; Macaulay et al., 1995). Based on the elution profile, nup60 is found with nup58 and nup54 in a 600-kD complex, nup97 in a 450-kD complex, and nup200 in a 1,000-kD complex. A simplistic prediction stemming from the eightfold radial symmetry of the pore might suggest that there are eight copies of each complex in the pore. If this is so, depletion of these proteins would reduce the mass of the pore by 16 MD. Using a value of 120 MD for the mass of an unaltered pore, the reduction would represent an ~13% decrease in protein mass, resulting in a lower overall density for the AL. This estimate is, of course, highly speculative because the exact stoichiometry of the WGABPs in the pores is not known and, as mentioned earlier, because nup200 may be present in lower amounts in AL. Moreover, the lack of these proteins could lead to a greater change in density if they themselves are required for incorporation of other pore or lamella-specific proteins. In an alternate explanation, the biochemical data would be consistent with there being fewer pores/membrane area in the depleted AL. Initial electron microscopy of the mock-depleted and WGA-depleted samples does not support this latter alternative. To determine the actual change in density of the glycoprotein-depleted pores in AL, it would be useful in the future to combine the AL isolated here (Fig. 2 *C*) with the STEM mass determination technique used to calculate the mass of the nuclear pore (Reichelt et al., 1990).

Models for the Assembly of Pore Complexes

How do pore complexes assemble in an envelope? For nuclei, there may be two such mechanisms. There is a great deal of evidence for the de novo assembly of pores in preexisting nuclear envelopes. For example nuclear pore number increases twofold during S phase, in preparation for cell doubling (Maul, 1977*a*). Pore number also increases after lymphocyte activation as well as during oocyte growth (Franke and Scheer, 1970; Maul et al., 1972; Wunderlich et al., 1974). Moreover, in yeast and other organisms that undergo closed mitosis, the increase in pore number before cell division must occur by assembly into an intact envelope, as the nuclear envelope does not break down at mitosis. When modeling a mechanism by which the pore can assemble in intact membranes, it would be logical to propose that a lateral association of individual integral-membrane pore proteins within the bilayer would occur first. This association might then lead to formation of a circular multimer capable of inducing fusion between the inner and outer nuclear membranes, yielding a "donut hole" in the double membrane system. Such holes have been observed in electron micrographs of mitotic cells during disassembly of the pore (Maul, 1977*a*, *b*). Concomitant with the fusion and formation of a membrane hole, the myriad soluble proteins of the pore would then assemble, forming a complete pore complex. An interesting question to ask would be whether the association of any of the soluble nucleoporins is required early in the process, perhaps to induce aggregation of the pore membrane proteins. In our experiments, GTPγS could be acting at one or more of these steps to block incorporation of the initially soluble pore proteins into pore complexes.

A second model for pore formation was proposed by Sheehan et al. (1988). Their model does not explain pore assembly in intact nuclear envelopes or in AL but instead was invoked to explain the assembly of a nuclear envelope around chromatin at the end of an open mitosis. At this point in the cell cycle, it is known that the nuclear envelope forms using chromatin as a substrate. Indeed, a class of nuclear-specific vesicles has been shown to bind to chromatin and fuse to form a nuclear envelope (Lohka and Masui, 1983, 1984; Sheehan et al., 1988; Wilson and Newport, 1988; Newport et al., 1990; Pfaller et al., 1991; Vigers and Lohka, 1991; Boman et al., 1992*a*, *b*; Newport and Dunphy, 1992; Chaudhary and Courvalin, 1993; Sullivan et al., 1993). Sheehan et al. (1988), using a *Xenopus* nuclear assembly system with very limiting amounts of membranes, observed electron-dense, circular structures of pore-like diameter lining the surface of their decondensing chromatin templates. They interpreted these circular structures, which were completely unassociated with membrane, to be immature or "half pores" in the process of formation. Membrane later associated with the half pores, and eventually full pores were formed. It may be that nuclei pore formation occurs by two pathways—a chromatin-dependent pathway used at the end of open mitosis, and a chromatin-independent pathway used more generally in intact nuclei. The formation of pores in AL would be predicted to occur by a mechanism similar to the latter pathway.

Futures Uses for the Assembly System

In summary, we have developed a biochemical assay for annulate lamellae formation and have used it to examine the assembly of specific nucleoporins into pore complexes. In the course of this study we have shown that a dense membrane fraction highly enriched in all nucleoporins tested can be separated from the bulk of other cellular membranes. The assembly system and/or the pore-containing membranes can now be used in a number of ways. First, using immunoblotting, comigration with the pore-containing fraction can be used as a powerful screen for whether a given-candidate pore protein is in fact a nucleoporin. Previously, immunoelectron microscopy was the only method for establishing whether a protein was a component of the pore, a technique dependent on the possession of an antibody functional for electron microscopy. Second, with the recent identification of new nucleoporins, antibodies to their *Xenopus* homologs can be used to deplete these proteins from the extract to determine the role of the proteins in pore assembly. Third, biochemical manipulation of the assembly system should prove useful in trapping intermediates in pore assembly. These can then be used to establish the order of incorporation of specific proteins into pores. Lastly, in vitro–reconstituted AL can be used as starting material for the isolation of vertebrate pores. To date, free pores have been successfully isolated only from the nuclei of budding yeast (Rout and Blobel, 1993), most likely because of differences in the nucleoskeletal components, such as the lamina between yeast and higher eukaryotes. However, the properties unique to yeast nuclear pores, which include smaller size, an absence of WGABPs, and a lack of similar assembly

and disassembly processes due to the closed form of mitosis, may reflect an even greater difference in the substructure of the yeast nuclear pore from that of vertebrates (Allen and Douglas, 1989; Davis and Fink, 1990; Nehrbass et al., 1990; Wente et al., 1992; Rout and Blobel, 1993; for reviews, see Osborne and Silver, 1993; Fabre and Hurt, 1994; Rout and Wente, 1994). Since AL lack a nuclear lamina, contain a high density of pores, and can be separated from contaminating membranes, in vitro–synthesized annulate lamellae hold promise for being the first system from which the pores of higher eukaryotes can be purified.

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