seminars in **CELL & DEVELOPMENTAL BIOLOGY**, Vol 7, 1996: pp 475–486

Reconstitution of nuclear pore assembly and function

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Nuclear pore complexes are the vital communication links that permit the regulated movement of macromolecules between the cytoplasm and nucleus of a cell. As with many other complex cellular events, much of our understanding about pore dynamics and function comes from studying systems that faithfully reproduce these processes in vitro. *Two systems have provided the bulk of our knowledge. The first system reconstitutes nuclear import into permeabilized cultured cells. Import is dependent on exogenously added cytosol, and fractionation of this cytosol has led to the identification and characterization of most of the nuclear transport factors. The second system uses a nuclear reconstitution system derived from Xenopus eggs to study transport and to assemble nuclear pores from their component parts. This system has allowed the characterization of both individual pore components and distinct steps in the pore assembly pathway.*

Key words: membrane / nuclear transport / nucleoporin / pore

THE REGULATED TRANSPORT of protein and RNA between nucleus and cytoplasm is critical for normal cell physiology. Movement between these compartments occurs through nuclear pore complexes: large, multicomponent, proteinaceous channels that span the double membrane of the nuclear envelope. Despite their central role in this vital communication link, little is known about pore complex assembly or function. In higher eukaryotes, pore complexes are disassembled at each mitosis, then reassembled at the end of mitosis. How pores are created, how they disassemble and reassemble with each cell cycle, and how they function to facilitate the movement of material across the nuclear envelope are major questions that need to be addressed.

One of the foremost difficulties in studying the structure, function and dynamics of nuclear pores is their extremely large size and complexity. The mass of a pore is estimated to be 125 MDa, consistent with a composition of ~1000 medium-sized proteins.[1-6] Models of the pore complex show the pore to consist of nuclear and cytoplasmic rings that lie in the plane of the inner and outer nuclear membranes, respectively, bracketing a central ring of spokes (Figure 1). Each ring structure appears to be composed of eight large subunits which confer on the pore an eight-fold rotational symmetry around a line that runs through the center of the pore. This symmetry predicts a reduced complexity; instead of ~1000 different proteins, the pore is likely made up of multiple copies of perhaps 100 different proteins. A detailed description of each identified nuclear pore protein or nucleoporin is beyond the scope of this article (for reviews see refs 2,7-10), but the recent ability to purify large quantities of intact pores from detergent-solubilized yeast nuclear envelopes promises to provide for the rapid cataloging of the protein constituents of the pore, at least in yeast.[11,12] Vertebrate pore proteins, however, differ significantly. Details on both individual yeast and vertebrate pore proteins can be found elsewhere.[7,8,10]

In addition to the core rings, the nuclear pore possesses filaments which extend from the cytoplasmic ring into the cytoplasm. Additional filaments on the nuclear side of the pore are bound together by a small ring-shaped element, creating a basket-like structure. The filaments on the cytoplasmic side of the pore appear to serve in the initial recognition and docking of substrates that are to be transported through the pore complex; the nuclear basket may serve a similar role for RNA and protein export.

One of the most successful approaches to investigating pore function and assembly has been the development of cell-free systems that recapitulate these complex processes *in vitro*. Cell-free systems are amenable to biochemical analysis, permitting both the identification of key components and the characterization of the mechanisms or steps by which the reconstituted process occurs. Two cell-free systems are widely used to examine pore function and assembly, one of them reconstitutes nuclear import into permeabilized cultured cells, while the other uses a

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1084-9521/96/040475+12 $18.00/0

Xenopus nuclear reconstitution system both to examine import and to build functional nuclear pores from their component parts.

The permeabilized cell system for nuclear import

Proteins are targeted to the nucleus by a stretch of basic amino acids termed a nuclear localization sequence or NLS.[13] Nuclear transport has been shown to consist of two steps, binding of NLS-bearing proteins to the exterior of the pore and translocation through the pore.[14,15] That the NLS-dependent movement of a protein into the nucleus requires soluble cytosolic components was first shown using the Xenopus system.[16] Rapid identification of these soluble factors, however, was accomplished using a permeabilized cell nuclear import system. Selective digitonin permeabilization of the plasma membrane of cultured cells results in the release of soluble cytosolic components and leaves an intact nucleus capable of nuclear import.[17] The nuclear accumulation of a fluorescently-tagged nuclear-targeted protein can be used to monitor nuclear import, and this accumulation was found to be dependent on the addition of both cytosol and energy. Fractionation of total cytosol led to the identification of the NLS receptor, a heterodimer consisting of ~60 kDa and ~90 kDa proteins.[18-25] The 60 kDa protein (NLS-receptor, importin 60, karyopherin α, or Srp1p) contains a binding site for the NLS sequence on nuclear-targeted proteins, and thereby acts as a NLS receptor. The 90 kDa protein (importin 90, karyopherin β, or p97) binds this complex to nuclear pores.[20,26,27] On blot overlay assays, the 90 kDa protein can bind directly to a subset of nucleoporins containing multiple copies of short peptide repeats, the FXFG- and the GLFG-type repeats common to certain nucleoporins.[21,27] In sum, in the first step of nuclear transport the 60 kDa subunit binds the NLS-bearing protein to be imported and the 90 kDa subunit targets the NLS-bearing protein to proteins of the nuclear pore.

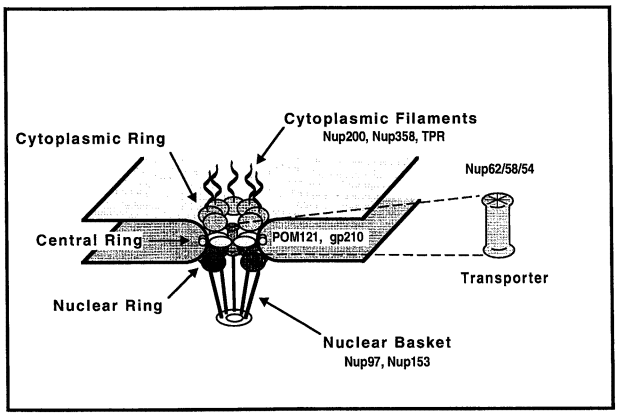

Figure 1. The nuclear pore complex. Nuclear pores span both the inner and outer nuclear membranes at a position where the two membranes are continuous. This allows for the diffusion of integral membrane proteins from their site of synthesis, on the rough endoplasmic reticulum of the outer membrane, to their site of action on the inner membrane. The pore is comprised of three concentric ring structures that lie parallel to the plane of the membrane, as well as filaments that are attached to the cytoplasmic ring and extend away from the pore. A nuclear basket attaches to the nuclear ring. The majority of identified pore proteins are not integral membrane proteins; individual proteins have been localized to the cytoplasmic filaments (Nup200, Nup358, TPR), the nuclear basket (Nup97, Nup153), or the central transporter (Nup62/58/54) which is thought to be directly involved in the transport of material through the central channel. Two integral membrane pore proteins have been identified in vertebrates (POM121, gp210) and they are thought to have a role both in pore assembly and in anchoring the pore to the nuclear membrane.

Purification of the activity required for the second step in import, translocation of the NLS-bearing protein into the nucleus, identified the small GTPase Ran.[28-38] The involvement of a small GTPase explains at least some of the energy requirements for nuclear import. Studies with purified recombinant proteins suggest that Ran-GTP may act by binding to the 90 kDa subunit of the NLS receptor and disrupting its association with the 60 kDa NLS-binding subunit.[39-41] Theoretically, this would release the NLS-bearing protein and the 60 kDa subunit from the pore. In this way, the Ran-GTPase cycle could regulate the reversible binding of an NLS receptor and its cargo to nucleoporins within the pore complex. Other factors involved in regulating Ran-GTPase activity, such as Ran GTPase-activating proteins (GAPs), exchange factors (RCC1), and other regulatory or binding proteins (NTF2/pp15, RanBP1, Nup358/RanBP2), have been identified. These factors are likely to be important in regulating nuclear transport, but their identification has been very recent and the details of how they interact with one another, with NLS-bearing proteins, and with the nucleoporins themselves are just beginning to be established. These topics have been recently reviewed[30-38,42] and will not be considered further here, except to say that simple binding and release interactions do not yet fully explain how proteins are translocated through the pore.

In many ways the nuclear pore behaves as an

elaborate molecular machine that is fixed to the nuclear envelope and translocates macromolecules with the appropriate signals across the envelope. Nucleoporins related to molecular motors have not yet been identified. Although this may be due to the fact that a large number of nucleoporins remain undiscovered, it may also be that the pore does not contain canonical motor proteins. One model predicts that the pore complex as a whole opens and closes like the diaphragm of a camera.[43,44] This opening would presumably occur in response to the NLS receptor and Ran.[38] Normally, only small molecules and proteins (< 40 kDa) can diffuse through the 9 nm channels present in the resting nuclear pore, but larger molecules that contain an NLS, including 25 nm gold particles coated with NLS-bearing proteins, can be specifically imported into the nucleus,[45] indicating that the pore complex is not just a static channel that spans the two nuclear membranes (reviewed in refs 7,10,38). Somehow the pore channel must expand to accommodate these larger molecules. Different pore structures observed microscopically have been proposed to coincide with different opened and closed states of transport.[43,44] However, the way in which the arrangement of nucleoporins changes to accommodate the passage of large molecules or gold particles, as well as the way in which the directionality of transport is achieved, remain the most puzzling problems in nuclear transport.

The Xenopus nuclear reconstitution system

The relationship between nuclear organization and nuclear function is a major question in cell biology. A number of in-vitro systems have been developed to examine individual nuclear processes, such as DNA replication, transcription, and splicing; however, few of these systems examine these processes in the context of an intact nucleus. One in-vitro system in which nuclear processes occur in the context of a fully formed nucleus is the Xenopus nuclear reconstitution system.[46-49] The Xenopus egg contains a large store of nuclear components and is arrested at the second metaphase of meiosis, a point at which all of the stored nuclear components are in a disassembled state. Normally, when a sperm enters the egg during fertilization, a Ca^{2+}-flux is generated which releases the metaphase arrest, thereby activating the egg. This converts the egg to an interphase state and a nuclear envelope assembles around the incoming sperm chromatin.

Xenopus eggs can be activated artificially, or converted to an interphase state, by a number of methods, including electric shock, addition of a Ca^{2+} ionophore, or simply by lysis alone. The resulting activated egg lysate provides access to the stores of disassembled nuclear components that are now competent for nuclear assembly. A crude egg lysate can be further fractionated by high speed centrifugation, separating the membrane or vesicular portion of the lysate from the high speed supernatant (reviewed in refs 50-52). For convenience, this high speed supernatant is referred to as egg cytosol, although this fraction also contains all of the soluble disassembled nuclear components. When condensed chromatin isolated from Xenopus sperm is added to a mixture of egg cytosol and membranes, nuclei spontaneously assemble (Figure 2). In this process, each sperm chromatin swells to expose nuclear vesicle binding sites, acquires a nuclear envelope replete with double nuclear membranes and nuclear pores, and the resulting nucleus imports nuclear-targeted components allowing the nucleus to grow and the DNA to replicate.[50-52] Thus, the system recapitulates, *in vitro*, the post-mitotic assembly of a vertebrate nucleus.

Modularity of the pore complex

In higher eukaryotes, as stated, the nuclear envelope disassembles during mitosis and its components are dispersed throughout the mitotic cell. Lysis of the metaphase-arrested Xenopus egg permits the biochemical characterization of abundant, naturally disassembled, nuclear pore components. However, of the estimated ~100 different nuclear pore proteins, only ~12 vertebrate pore proteins have been identified. A subset of these proteins are modified with O-linked N-acetylglucosamine (GlcNAc) residues and, as a result, bind to the plant lectin wheat germ agglutinin (WGA).[53-56] The ability to purify these nucleoporins by virtue of their binding to WGA-Sepharose has simplified their study. The most prominent WGA-binding proteins in Xenopus eggs are the nucleoporins Nup60, Nup98, and Nup200/CAN.[57-61] These nucleoporins are present in the cytosolic fraction of the egg lysate and can be depleted from this fraction using WGA-Sepharose (see later). The mobility of Nup60, Nup98, and Nup200 on a gel-filtration column indicates that each protein exists in a separate high molecular weight complex.[59,62] The mobilities of the complexes are identical whether they are isolated from interphase or mitotic egg lysates.[62] Thus, the

nuclear pore does not appear to mitotically disassemble into its individual protein constituents, but rather into multiprotein modules. Depending on the size of the modules, this could greatly reduce the complexity of pore assembly in terms of the number of individual components or modules that must coalesce to form a nuclear pore.

The Nup60 WGA-binding protein is in a complex with at least two other nucleoporins, Nup58 and Nup54. This complex has been shown to be essential for nuclear import[63,64] and is located on both sides of the pore.[2,65] It represents one of the modules from

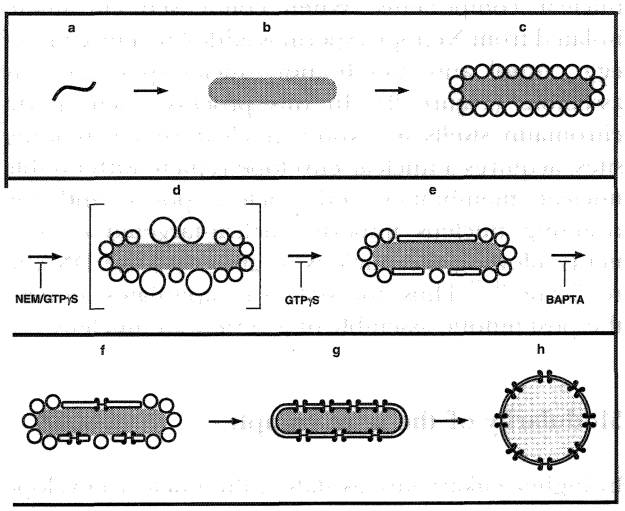

Figure 2. Steps in the assembly of reconstituted nuclei. When highly condensed sperm chromatin (a) is added to a mixture of the cytosolic and membrane fractions of a Xenopus egg lysate, the compact sperm chromatin rapidly swells 25–30 times in volume due to stoichiometric displacement of protamines by the soluble protein nucleoplasmin (b). This decondensation exposes membrane vesicle binding sites allowing the nuclear vesicles to bind to the chromatin surface (c). Adjacent nuclear vesicles fuse to one another (d), but fusion is permanently blocked by the prior treatment of the unfused vesicles with N-ethylmaleimide (NEM). This results in the accumulation of structures depicted in (c). Nuclear vesicle fusion is also inhibited by the presence of GTPγS.[81,83] The fused vesicles flatten onto the chromatin surface (e), and pore-complexes then assemble into the flattened double nuclear membranes (f). The presence of the calcium chelator BAPTA prevents pore assembly so that a completely fused, pore-free, double nuclear membrane encircles the swollen chromatin. A completely enclosed nucleus (g) must form before efficient nuclear import can occur to allow for nuclear growth and DNA replication (h). The fused, rounded vesicles depicted in (d) are a hypothetical intermediate, based on the finding that GTPγS can block pore assembly onto vesicles that have fused in the absence of cytosol, but not those that have fused in the presence of cytosol and BAPTA.

which the pore is assembled.[59,62-64] The Nup60-58-54 module is quite stable; in fact, the homologous rat Nup62 complex can be isolated, intact, from the pores of detergent-solubilized rat liver nuclei.[63,66-68] Characterization of the rat Nup62 complex identified proteins of 62, 58, 54 and 45 kDa. The isolated rat complex appears donut shaped by electron microscopy and has a calculated mass of at least 234 kDa, suggesting a 1:1:1:1 ratio of the component proteins. Importantly, the rat Nup62 complex can functionally substitute for the Xenopus Nup60 complex.[57,63] Therefore, the rat and Xenopus complexes are not only functionally homologous, but the isolated rat complex is also a bone fide module for pore assembly.

The biosynthesis of nucleoporins and their assembly into such modules has not yet been examined. However, the ability of the Xenopus Nup60 or Rat Nup62 complex to act as a module for pore assembly suggests that the proteins which comprise these small multi-protein complexes assemble with one another before they associate with the nuclear pore structure. In one study, the bulk of newly synthesized Nup62 in BRL cells did not associate with the nucleus for 10–12 hrs.[56] Although formation of the Nup62-58-54-45 complex was not examined in this study, the lengthy time for association of Nup62 with the nucleus could be due in part to the time it takes for the prior assembly of the Nup62-58-54-45 complex. This could pose significant problems when trying to analyse individual pore proteins by transient overexpression in tissue culture cells. Most of the Nup62 transiently expressed in cultured cells in one study was found in cytoplasmic aggregates,[69] which may be due to the lack of concurrent overexpression of its partner proteins. Without correct assembly into pore modules, overexpressed pore proteins may not be properly incorporated into the pore complex itself and thus be refractory to study in this manner.

Interestingly, yeast nuclei, unlike higher eukaryotic nuclei, undergo a closed mitosis. The nuclear envelopes do not break down and the pore complexes are not mitotically disassembled. Nevertheless, strong similarities between the smaller yeast nuclear pores and vertebrate pores exist. For example, the yeast nucleoporin Nsp1 has homology to rat Nup62 and, like Nup62, is present in a multiprotein complex containing Nsp1p, Nic96p, Nup57p, and Nup49p.[70,71] A second multiprotein nucleoporin complex isolated from detergent-solubilized yeast nuclei contains Nup85p, Nup84p, Nup120p, Sec13p, Seh1p and Band IV.[72] The metazoan homologues for these complexes

have not yet been established, but the homology between yeast Nsp1 and vertebrate Nup62 suggests that yeast Nup57p and Nup49p may be related to Nup58 and Nup54 of the vertebrate complex. A vertebrate homologue for Nic96p has also been identified, with some proof of an association with the Nup62 complex (Grandi *et al*, in preparation). It remains an open question as to whether these yeast multiprotein complexes assemble prior to pore formation, indicating that the assembly of pore modules is conserved through evolution, or whether each yeast protein assembles into the pore independently, but is extracted as a part of a complex with other nucleoporins.

Nucleoporin function

Once a cellular process has been reconstituted *in vitro*, the role of individual proteins in this process can be examined. Typically, this is accomplished either by using an inhibitor that blocks the activity of a specific protein, or by using a specific antibody to remove a protein from the reconstitution system. For the nuclear pore, an example of this has been the analysis of the subset of the nucleoporins that bind to WGA (see previously). These include Nup200/Can, Nup98, and the Nup62-58-54 complex, which can be depleted from egg cytosol using WGA-Sepharose.[57-59,63,73] Nuclei assembled by adding sperm chromatin and membranes to such depleted cytosol are severely impaired. Although a double nuclear membrane containing pore structures assembles around the sperm chromatin, the pores are not functional. Such nuclei are much smaller than control nuclei. Moreover, they neither import, grow, nor replicate their DNA.

Using nucleoporin-specific antibodies, the contribution of each individual protein to the various nuclear phenotypes detected when all of the WGA-binding proteins are depleted can be examined. For example, Nup98 is the Xenopus homologue of the rat nucleoporin Nup98. This WGA-binding nucleoporin is located on the nuclear side of the pore and in the interior of the nucleus.[58,74] Nuclei lacking Nup98 were assembled using egg cytosol quantitatively depleted of Nup98 with a specific anti-Nup98 antibody.[58] Such Nup98-depleted nuclei were found to be much smaller than nuclei assembled in mock-depleted cytosol. Moreover, DNA replication was completely blocked, but nuclear import was not strongly inhibited. In a similar study, nuclei were assembled using egg cytosol depleted of Nup200/CAN (Powers *et al*, in preparation). This nucleoporin is localized to fibers attached to the cytoplasmic ring (Figure 1) and may have a role in the initial docking of proteins to be imported.[60,61] However, the Nup200-depleted nuclei behaved very similarly to control nuclei. Nuclear import, growth, and DNA replication were not strongly affected. Since nuclei depleted of Nup98 or Nup200 are not strongly inhibited in nuclear import, then the strong block to import found in nuclei depleted of all the WGA-binding proteins must be due to the lack of the Nup60-58-54 complex. The Nup60 protein is known to be important for nuclear import in vivo,[64] and indeed, nuclei assembled in the absence of the Nup60-complex are strongly impaired in nuclear import.[63]

The localization of Nup98 to the inner face of the nuclear pore, as well as the interior of the nucleus, suggests that it may not have a direct role in nuclear import, but could be involved in nuclear export and perhaps other nuclear functions. That nuclei depleted of Nup98 are small, yet not strongly inhibited for nuclear import, suggests that the formation of the nuclear skeleton may be impaired. Alterations in a nuclear scaffold or matrix were not examined directly in the Nup98-depleted nuclei, but a nuclear scaffold is thought to be important in the organization of the interphase nucleus and in DNA replication. For example, when nuclei were assembled using egg cytosol immuno-depleted of the major nuclear skeletal protein lamin LIII, they neither grew nor replicated their DNA.[75,76] The similar phenotypes in the Nup98-depleted and Lamin LIII-depleted nuclei suggests that these two proteins may impinge on common nuclear components. The above examples illustrate one of the most powerful features of the Xenopus nuclear reconstitution system, namely, the way in which nuclear composition can be biochemically manipulated and how the effects of this manipulation on a number of nuclear functions, including assembly, import, growth, and replication, can be measured.

Inhibitors identify steps in the pore assembly pathway

It is obvious that the assembly of a fully functional nucleus is an extremely complex process, involving many separate steps and individual protein–protein interactions. Even the assembly of the nuclear pore, with the coordinate association of multiple copies of perhaps a hundred different components, is itself very

complex. A powerful feature of the Xenopus nuclear reconstitution system is that individual steps which make up the nuclear assembly pathway can be examined separately.[47-49,77-80] In an early study which varied the amount of membrane vesicles in a nuclear assembly reaction, pore assembly was found to require the presence of the membrane vesicle fraction and it was suggested that partially formed pore complexes may have a role in targeting nuclear vesicles to the chromatin surface.[49] However, the binding of nuclear vesicles to the surface of swollen chromatin can occur in the absence of egg cytosol, indicating that pore complexes are not required for nuclear vesicle targeting.[47,78,79,81,82]

A priori, the assembly of nuclear pores and the double nuclear membrane could theoretically occur in one of two ways: (1) assembly might occur *concurrently*, where the formation of pore complexes and the double nuclear membrane occur at the same time, but independently of one another, or (2) assembly may occur in an *ordered* fashion, where the formation of a double nuclear membrane must take place prior to pore assembly. The *ordered* and *concurrent* models for assembly of the double nuclear membrane and nuclear pores have now been addressed in the Xenopus nuclear reconstitution system by using inhibitors that block membrane assembly. In the course of this study, inhibitors which block pore assembly were also identified.[80] The premise on which the study was based was that if pores could still assemble when double nuclear membrane formation is blocked, then these two nuclear envelope structures must assemble independently of one another, i.e. in a concurrent fashion. However, if pores do not assemble when formation of the nuclear membrane is blocked (but a nuclear membrane can assemble when pore formation is blocked), then they must assemble in a sequential, or ordered, fashion.

Three inhibitors reported to interfere with nuclear membrane assembly, NEM (N-ethylmaleimide), GTPγS, and the calcium chelator BAPTA,[79,81,83,84] were specifically tested for their effects on nuclear pore formation.[80] The occurrence of nuclear pores on nuclear intermediates assembled in the presence of the inhibitors was assayed in two ways: (1) by immunofluorescence using specific anti-pore antibodies, and (2) by direct visualization of the pores through electron microscopy. Previously it has been demonstrated that when the membrane fraction of a Xenopus egg extract is treated with NEM before addition to a nuclear assembly reaction, nuclear vesicle fusion is blocked and unfused nuclear vesicles coat the surface of the swollen sperm chromatin.[79,80] When these structures were examined for the presence of pores with the methods described above, pore complexes were absent.[80] This indicated that either pore assembly requires prior formation of a fused nuclear membrane or, alternatively, that NEM treatment of the membrane fraction blocks both nuclear vesicle fusion *and* pore formation, independently of one another. These two possibilities were distinguished by asking whether pore assembly would be blocked by NEM treatment of an intermediate containing patches of already fused nuclear membranes. Such an intermediate substrate was created by mixing together sperm chromatin, membrane vesicles, and the egg protein nucleoplasmin. In the presence of nucleoplasmin, the highly condensed sperm chromatin swells 25–30 fold to expose nuclear vesicle binding sites.[79,85] In the absence of egg cytosol, nuclear vesicle fusion occurs, but is inefficient; only patches of fused double nuclear membrane form on the surface of the swollen sperm chromatin (refs 47,80,81; see Figure 2e). The fused patches do not contain pores due to the absence of egg cytosol which contains all the soluble nuclear pore components. Complete nuclear envelope assembly can be induced by the addition of egg cytosol to the intermediates; such nuclei contain normal nuclear pores. Importantly, when intermediates containing patches of fused nuclear membrane were treated with NEM before the addition of cytosol, subsequent pore assembly was not blocked, i.e. the nuclear intermediates resembled that depicted in Figure 2f by electron microscopy.[80] Two conclusions can be drawn from this: (1) a double nuclear membrane must form before pore assembly can take place, and (2) once the double nuclear membrane has formed, there are no further NEM-sensitive steps in pore assembly. Thus, the nuclear membrane and the nuclear pores must assemble in an ordered fashion.

The addition of the nonhydrolysable GTP analog, GTPγS, to a standard nuclear assembly reaction has been shown to block nuclear vesicle fusion.[79,81,83] In an extension of the study above, GTPγS was found to inhibit pore assembly at two separate steps (Figure 2, legend). First, the known GTPγS block to nuclear vesicle fusion[79,81,83] prevented the formation of a fused double nuclear membrane and thus the assembly of nuclear pores, reiterating the result with NEM that a double nuclear membrane is required to form prior to pore assembly (ref 80). In addition, pore assembly was also blocked when nuclear intermediates bearing patches of fused nuclear membranes were

added to egg cytosol containing GTPγS,[80] indicating that GTPγS can block pore assembly at a second step that occurs after nuclear vesicle fusion.

When the calcium chelator BAPTA was added to a normal nuclear assembly reaction, it slowed the rate at which nuclear vesicles fuse.[84] Interestingly, once vesicle fusion occurred, it was clear that nuclear pore assembly itself was completely blocked.[80] The resulting nuclear structures consisted of chromatin surrounded by completely fused, but pore-free, double nuclear membranes. Using a novel anchored nuclear assembly assay,[80] it was possible to show that the effect of BAPTA on nuclear pore assembly was reversible. In the anchored nuclear assay, sperm chromatin are swollen by the addition of nucleoplasmin, then attached to a poly-lysine coated glass slide. The chromatin anchored to the glass slide act as substrates for nuclear envelope assembly when incubated under a drop containing a mixture of egg cytosol and membranes. The advantage of such a system is that a series of multiple nuclear assembly conditions can be examined. For example, if anchored nuclei are first assembled using a mixture of egg cytosol, membranes and BAPTA, pore-free double nuclear membranes assemble on the anchored chromatin. The residual cytosol and membranes can be removed, the anchored BAPTA-nuclei washed, then complete egg cytosol lacking BAPTA added to the anchored pore-free structures. When the assay was performed in this manner, the nuclear envelopes acquired nuclear pores, indicating that BAPTA inhibition of pore assembly is reversible[80]. Moreover, if the pore-free BAPTA-nuclei were treated with NEM before the addition of the fresh BAPTA-free cytosol, pore assembly also took place, confirming that NEM does not block pore assembly once a fused double nuclear membrane has formed.

To order the BAPTA-sensitive and GTPγS-sensitive steps in pore assembly, anchored BAPTA-nuclei containing pore-free double nuclear membranes were formed. When the BAPTA-containing extract was washed away from the anchored nuclei and replaced with egg cytosol containing GTPγS, pore assembly occurred.[80] This indicated that GTPγS could not block pore assembly onto fused double nuclear membranes that assemble in the presence of egg extract containing BAPTA. Therefore, in the ordered nuclear envelope assembly pathway, the BAPTA-sensitive step in pore assembly must occur downstream from the GTPγS-sensitive steps.

The use of inhibitors has allowed three distinct steps in nuclear envelope assembly to be identified and now ordered (Figure 2). The first step is fusion between the chromatin-bound nuclear vesicles. This step is sensitive to either NEM treatment of the membranes or the presence of GTPγS.[79-81,83,84] After nuclear vesicle fusion, a second GTPγS-sensitive step has been detected.[80] It remains to be determined whether this second GTPγS-sensitive step involves inhibiting further assembly of the double nuclear membrane or assembly of the nuclear pore complexes themselves. In either case, the second GTPγS-sensitive step occurs prior to the third identified step in nuclear envelope formation, the BAPTA-inhibition[80] of pore assembly into a completely fused double nuclear membrane.

The above results indicate that the in-vitro assembly of the nuclear envelope occurs in an ordered hierarchical fashion. Blocking one step in the multistep envelope assembly pathway does not block the steps that occur upstream in the pathway, but does inhibit downstream steps. Extrapolating from the Xenopus model system, the assembly of pores in the reforming nuclear envelopes of a somatic cell during telophase would occur only after formation of a double nuclear membrane. This mechanism of assembly, i.e. pore assembly into existing double nuclear membranes, likely occurs then by the same mechanism that must occur in somatic cells during interphase, where the number of pores in the intact nuclear membrane doubles at S-phase in preparation for cell division.[86] Lastly, yeast undergo a closed mitosis meaning that pores in that system must, by definition, assemble into an intact double nuclear membrane. We conclude that this method of pore assembly is likely conserved among eukaryotes and that the Xenopus nuclear reconstitution system provides a way to biochemically examine this complex process.

Potential targets for NEM inhibition

The NEM sensitivity of nuclear vesicle fusion is reminiscent of that seen with the NEM-sensitive ATPase, NSF (NEM-sensitive factor). Members of the NSF family of proteins are required for vesicle fusion events at many steps in the endocytic and exocytic pathways.[87-90] It is not known if NSF is required for nuclear vesicle fusion. Existing experimental data has not ruled it out, but if NSF is required it is not likely to be the only NEM-sensitive protein involved in nuclear vesicle fusion. Specifically, both NSF and the NSF-related protein p97 (Cdc48p) are present in the cytosolic fraction of the egg [ref 91 and C. Macaulay,

unpublished observations], yet NEM treatment of the vesicular fraction of the egg permanently blocks nuclear vesicle fusion. Therefore, the NEM-sensitive membrane fusion activity is either a form of NSF or p97 that is unexchangeable with its cytosolic counterpart, or the fusion activity is not NSF, but a novel component tightly associated with nuclear vesicles. It is possible that the NEM-sensitive component is an integral membrane protein that is directly involved in nuclear vesicle fusion.

Potential targets for GTPγS inhibition

As described above, the presence of GTPγS in a nuclear assembly reaction can block nuclear envelope formation at two steps: nuclear vesicle fusion and nuclear pore assembly.[80] A small soluble GTPase termed ADP-ribosylation factor or ARF is thought to mediate the block by GTPγS on nuclear vesicle fusion.[81,83] ARF refers to a family of related GTPases which are involved in vesicle formation at multiple steps during protein secretion (reviewed in refs 89,90,92). In other systems, ARF-GTP binds to membranes and mediates the binding of coat proteins, which induces vesicle formation. Such coated vesicles cannot fuse. The subsequent hydrolysis of GTP by ARF releases both ARF and the bound coat proteins, allowing the vesicles to fuse to their target membranes. In the Xenopus nuclear reconstitution system, ARF-GTPγS is thought to induce the irreversible binding of coat proteins to membrane vesicles, including nuclear vesicles, and inhibit any subsequent fusion of those vesicles, thus inhibiting the formation of a nuclear membrane. It has not yet been determined whether ARF plays a role in the *normal* formation of the double nuclear membrane.

How GTPγS blocks the second step in nuclear envelope formation is not known. It may act to block further formation of the double nuclear membrane, or it may block pore assembly itself. One hypothetical intermediate step in the formation of the nuclear membrane is the flattening of the fused nuclear vesicles (Figure 2d–e). Flattening must occur after nuclear vesicle fusion, since if fusion is blocked by NEM treatment of the membrane vesicles, only round vesicles are seen bound to the chromatin surface.[80] Therefore, one possibility is that the ARF-GTPγS-induced binding of coat proteins prevents the fused nuclear vesicles from flattening. Many alternate hypothesis, however, also exist, including a direct interference with pore assembly. This latter would have to occur at an early step in pore assembly, prior to the BAPTA-sensitive step.

Potential targets for BAPTA inhibition

The mechanism by which BAPTA blocks pore assembly remains a crucial question, but two possibilities are suggested. One is that BAPTA buffers a calcium gradient essential for pore assembly, the second is that BAPTA binds as an antagonist to a component involved in pore assembly.[80] The free calcium levels in egg lysates have been measured to be 450 nM, and the addition of either BAPTA or EGTA reduces the free calcium concentration to ~375 nM.[84] Unlike BAPTA, EGTA does not inhibit nuclear pore assembly;[80] therefore, pore assembly is not likely blocked by the simple chelation of free calcium in the egg lysate. The rate of calcium binding for BAPTA is 2–3 orders of magnitude faster than for EGTA.[93] If a very transient, localized, calcium gradient is required at the site of pore assembly, it may be suppressed by the fast-binding BAPTA, but not by slower-binding EGTA. Such a model has been suggested for the slowing of vesicle fusion caused by BAPTA.[84] On the other hand, 4–5 mM BAPTA is required to inhibit pore assembly; 1–2 mM is not sufficient.[80,84] This requirement for high levels of BAPTA is consistent with the idea that BAPTA reversibly binds to a ligand with low affinity, and this binding blocks pore assembly. The identity of such a ligand or ligands has yet to be determined. However, in addition to the block to nuclear pore assembly, BAPTA can affect the binding of a chromasomal protein to the chromatin, even in the absence of added egg membranes (C. Macaulay, unpublished observations). Whether BAPTA's several effects on reconstituted nuclei are due to interactions with a single ligand, or with multiple ligands, remains an open question.

Recently, one ligand that BAPTA has been reported to be able to bind to is the IP3 receptor.[94] These receptors is found on a number of cellular membranes, including nuclear membranes, from which it can release lumenal calcium stores in response to IP3 simulation.[95-97] Interestingly, antibodies to IP3 receptors which block IP3 stimulated calcium release have been reported to block nuclear growth when added to the Xenopus nuclear reconstitution system.[98] Nuclear growth requires multiple steps in the nuclear assembly pathway, including pore assembly. It would be very interesting to determine if any of the steps affected by these antibodies are the same as the steps affected by

BAPTA. An equally interesting alternative possibility is that BAPTA instead binds to a pore protein, either integral membrane or soluble, required at an early step in pore formation, and thereby blocks assembly.

Nuclear pore assembly

At sites in the nuclear envelope where fully assembled pore complexes span the inner and outer nuclear membranes, the two membranes are continuous or fused with one another (Figure 1). This allows for the diffusion of integral membrane proteins between the outer and inner nuclear membranes. Given that we now know that pores assemble after formation of the double nuclear membrane, the inner and outer nuclear membranes must come together and fuse during the pore assembly process. This fusion step does not occur when nuclei are assembled in the presence of BAPTA. Because BAPTA is not membrane permeant, it is not likely to be acting in the lumen of the double nuclear membrane where fusion between the two membrane bilayers must begin. Instead, it is reasonable to consider that BAPTA is acting on the nuclear and/or cytoplasmic side of the double nuclear membrane to inhibit signals which are required to trigger this membrane fusion step. Such a requirement for signaling across the nuclear membrane suggests an important role for integral membrane proteins in the pore assembly process. A series of pore assembly steps have been proposed in which the pore membrane or POM proteins (integral membrane proteins located at the nuclear pore) act as receptors for the binding of soluble pore proteins, and that this binding leads to a signal for fusion between the inner and outer nuclear membranes.[80,99,100] The BAPTA-sensitive step in the generation of the fusogenic signal remains to be determined.

In vertebrates, only two POM proteins have been identified to date, POM121 and gp210.[101-104] Of these, POM121 has a large cytoplasmic domain and can bind to WGA.[101] In one study, nuclei were assembled around a lambda DNA substrate using a mixture of Xenopus egg cytosol and membranes that had been depleted with WGA-Sepharose.[59] When examined by electron microscopy, these nuclei were found to be surrounded by a pore-free double nuclear membrane. Others have found that the cytosolic WGA-binding proteins are not essential for the assembly of a basic pore structure.[57] In this latter case, nuclei were reconstituted around a sperm chromatin substrate using *untreated* membranes, but Xenopus egg cytosol depleted of the WGA-binding proteins. It is tempting to consider that in the first case, the depletion of nuclear vesicles containing POM121 prevented pore assembly; however, the different DNA substrates used to reconstitute nuclei in these two studies could have contributed to the different findings. A third study found that depletion of the cytosolic WGA-binding proteins resulted in nuclei that contained fewer pores and were less active at nuclear import.[73] However, this study also found that these depleted nuclei replicated their DNA as well as control-depleted nuclei. Given that DNA replication requires functional pores capable of nuclear transport, as well as the WGA-binding Nup98 protein,[58] it is likely that the egg extracts in this study were not quantitatively depleted. With the development of specific antibody reagents to the cytoplasmic domains of the POM proteins, it will be possible to investigate the role of membrane vesicles containing POM proteins and the POM proteins themselves in nuclear pore assembly.

Future directions

The use of in-vitro systems that reconstitute nuclear import and nuclear pore assembly has proven to be extremely valuable for the characterization of these complex cellular processes. It has not only allowed for the identification of key components involved in each process, but also the initial characterization of the mechanisms by which each process occurs. Continuing with such an approach to examine nuclear import should not only provide further details of how the various soluble nuclear-import factors interact with NLS-bearing proteins and nuclear pore components, but should also begin to address how the directionality for nuclear transport is established. One exciting prospect will be to use an analogous approach to establish how nuclear export occurs. Interesting questions include how many export pathways exist, what the export signals for each pathway are, what receptors interact with each export signal, and how these receptor-export signal complexes interact with nuclear pore proteins during translocation into the cytoplasm?

In terms of pore assembly, the Xenopus nuclear reconstitution system is powerful and can facilitate the identification of novel inhibitors in the assembly process. Identification of the targets of the various

inhibitors should help illuminate the major molecular interactions required for pore assembly.

Nuclear pore assembly is also a regulated process and the number of pores in the nuclear envelope can change with the needs of the cell. When resting peripheral lymphocytes are stimulated with PHA, the number of nuclear pores doubles to accommodate the increase in nuclear activity of the stimulated cell.[86] Pore number might be controlled simply by regulating the synthesis of pore components, but experiments with the Xenopus nuclear reconstitution system suggest it is more complex. The electron microscopic examination of nuclei reconstituted *in vitro* indicates that the number of pores in the nuclear envelopes depends on the assembly conditions.[75,105] The density of pores in the envelopes of nuclei assembled using egg cytosol immuno-depleted of lamin LIII was found to be 2–3 fold greater than in nuclei assembled using control cytosol.[75] Although an association between pore complexes and the nuclear lamina of vertebrate cells has been known for many years, how the nuclear lamina could regulate pore number is not known. In addition, nuclear vesicles containing POM proteins are predicted to be important in the nuclear pore assembly process. Regulating the incorporation of such specific classes of nuclear vesicles could be important in determining pore number.[105-107] With modern techniques such as image analysis, it should soon be possible to quantitate the number of pores in a nucleus by immunofluorescence. This will allow for the relatively rapid evaluation of conditions that modulate pore number and, perhaps, the determination of the mechanism by which pore number is regulated.

Acknowledgements

The authors apologize to all those whose work was not discussed or cited directly due to limitations of space. Work in the author's lab was supported by a Centennial Fellowship from the Medical Research Council of Canada to C. Macaulay, and a grant (GM33279) from the National Institutes of Health to D. Forbes.

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