Assembly of the Nuclear Pore: Biochemically Distinct Steps Revealed with NEM, GTPγS, and BAPTA

Colin Macaulay and Douglass J. Forbes

Department of Biology 0347, University of California San Diego, La Jolla, California 92093-0347

Abstract. A key event in nuclear formation is the assembly of functional nuclear pores. We have used a nuclear reconstitution system derived from Xenopus eggs to examine the process of nuclear pore assembly in vitro. With this system, we have identified three reagents which interfere with nuclear pore assembly, NEM, GTPγS, and the Ca^{++} chelator, BAPTA. These reagents have allowed us to determine that the assembly of a nuclear pore requires the prior assembly of a double nuclear membrane. Inhibition of nuclear vesicle fusion by pretreatment of the membrane vesicle fraction with NEM blocks pore complex assembly. In contrast, NEM treatment of already fused double nuclear membranes does not block pore assembly. This indicates that NEM inhibits a single step in pore assembly—the initial fusion of vesicles required to form a double nuclear membrane. The presence of GTPγS blocks pore assembly at two distinct steps, first by preventing fusion between nuclear vesicles, and second by blocking a step in pore assembly that occurs on already fused double nuclear membranes. Interestingly, when the Ca^{2+} chelator BAPTA is added to a nuclear assembly reaction, it only transiently blocks nuclear vesicle fusion, but completely blocks nuclear pore assembly. This results in the formation of a nucleus surrounded by a double nuclear membrane, but devoid of nuclear pores. To order the positions at which GTPγS and BAPTA interfere with pore assembly, a novel anchored nuclear assembly assay was developed. This assay revealed that the BAPTA-sensitive step in pore assembly occurs after the second GTPγS-sensitive step. Thus, through use of an in vitro nuclear reconstitution system, it has been possible to biochemically define and order multiple steps in nuclear pore assembly.

NUCLEAR pore complexes are critical to many aspects of nuclear function, yet the molecular structure of the pore and the mechanism of its assembly are poorly understood. The physical structure of the pore, estimated to be 125 million Da in mass, involves a permanent and specific membrane hole connecting the two nuclear membranes, with ~1,000 protein molecules (~60–100 different proteins) assembled within this hole to create the pore complex (Reichelt et al., 1990; Pante and Aebi, 1994; for reviews see Forbes, 1992; Gerace, 1992; Osborne and Silver, 1993; Fabre and Hurt, 1994; Rout and Wente, 1994). The great size of the pore clearly contributes to the complex nature of the assembly process, but an additional complexity is the need to establish structural and functional polarity to the pore, such that nuclear proteins are imported and mRNAs are exported.

The structure of the nuclear pore becomes disassembled at mitosis. Immunofluorescence studies on cultured cells with anti-pore antibodies indicate that both the peripheral and integral membrane proteins of the pore distribute throughout the cell during mitosis (Gerace et al., 1982; Davis and Blobel, 1986; Park et al., 1987; Chaudhary and Courvalin, 1993; Cordes et al., 1993; Radu et al., 1993; Byrd et al., 1994). Reassembly of the nuclear envelope and nuclear pore is presumed to occur in a hierarchical fashion, with the assembly of the first components creating the target for binding and subsequent assembly of the later components, until a complete nuclear envelope is formed. For the nuclear pore, with only a few exceptions (Chaudhary and Courvalin, 1993; Byrd et al., 1994), the order and manner of this assembly is largely unknown.

Cell-free nuclear reconstitution systems have proven quite useful for identifying distinct biochemical steps in both the mitotic breakdown and reassembly of nuclei. When chromatin from demembranated Xenopus sperm is mixed with the soluble and vesicular components of egg lysates, nuclei spontaneously assemble (Lohka and Masui, 1983). The resulting nuclei import nuclear-targeted proteins, replicate their DNA, and break down in response to mitotic signals (Lohka and Masui, 1984; Lohka and Maller, 1985; Blow and Laskey, 1986; Finlay et al., 1987; Newport, 1987; see Newmeyer and Wilson, 1991, and references therein). When condensed sperm chromatin is added to an assembly reaction, the first step in nuclear assembly is a swelling or decondensation of the chromatin. This decon-

Please address all correspondence to Dr. D. J. Forbes, Department of Biology 0347, University of California San Diego, La Jolla, CA 92093-0347. Tel.: (619) 534-3398. Fax: (619) 534-0555.

densation is mediated by nucleoplasmin, an acidic, heat-stable protein present in egg cytosol (Laskey et al., 1978; Philpott et al., 1991). Nucleoplasmin contains tracts of poly-glutamic acid residues which are thought to displace protamines from the sperm chromatin. Indeed, poly-glutamic acid itself causes the decondensation of sperm chromatin when used in vitro (Dingwall et al., 1987; Pfaller et al., 1991; Philpott et al., 1991). In the presence of nucleoplasmin, sperm chromatin swells 25–30-fold in size and exposes multiple sites for nuclear membrane vesicle binding (Pfaller et al., 1991; Newport and Dunphy, 1992; Philpott et al., 1991).

Nuclear membrane vesicles are targeted to the chromatin through a trypsin-sensitive receptor (Wilson and Newport, 1988). Evidence exists that a distinct class of nuclear-specific vesicles is involved, vesicles presumably formed by the mitotic breakdown of the previous nuclear membranes (Wilson and Newport, 1988; Vigers and Lohka, 1991; Lourim and Krohne, 1993). (Nuclear lamins will not be discussed here as they have been recently reviewed; Hutchinson et al., 1994; Lourim and Krohne, 1994.) The binding of nuclear vesicles to chromatin is regulated in a cell cycle–dependent manner through the mitotic phosphorylation of vesicular components (Pfaller et al., 1991; Vigers and Lohka, 1992; Foisner and Gerace, 1993; Gerace and Foisner, 1994). Phosphorylation also appears to play a role in pore complex dynamics. We have recently shown that two nuclear pore proteins, Xenopus nup200 (214/CAN) and nup97, are phosphorylated at mitosis and, moreover, are specific targets for the mitotic kinase Cdc2/cyclin B in vitro (Macaulay et al., 1995). It is likely that phosphorylation promotes the disassembly of the nuclear pore, as it does the disassembly of other nuclear envelope components.

During nuclear assembly, once nuclear-targeted vesicles have bound to the chromatin surface, adjacent vesicles then fuse to form a double nuclear membrane. A number of studies have identified reagents that interfere with this fusion. Specifically, pretreatment of the membrane vesicles with the alkylating agent N-ethylmaleimide (NEM)[1] produces vesicles which can bind to the decondensed chromatin, but fail to fuse (Newmeyer and Forbes, 1990; Newport and Dunphy, 1992; Vigers and Lohka, 1992). Similarly, the presence of GTPγS in an assembly reaction blocks fusion between chromatin-bound vesicles (Pfaller et al., 1991; Boman et al., 1992*a*,*b*; Newport and Dunphy, 1992; Sullivan et al., 1993). The activity responsible for GTPγS inhibition of vesicle fusion behaves very much like the ADP-ribosylation factor, ARF (Boman et al., 1992*a*; Sullivan et al., 1993). In other cellular fusion events, GTPγS-ARF is thought to cause an irreversible binding of coatomer proteins to membrane vesicles, thereby blocking subsequent fusion (Moss and Vaughan, 1995; Boman and Kahn, 1995). Although ARF appears responsible for the inhibitory activity of GTPγS on nuclear vesicle fusion, ARF may not normally play a role in nuclear membrane dynamics, as its depletion causes no defect in nuclear assembly (Boman et al., 1992*b*). Recently, the Ca^{2+} chelator, BAPTA, has also been reported to inhibit fusion between chromatin-bound nuclear membrane vesicles (Sullivan et al., 1993). It was postulated that BAPTA might act by buffering local Ca^{2+} gradients required for the vesicle fusion events of nuclear assembly (Sullivan et al., 1993).

In steps subsequent to vesicle fusion, a nuclear envelope forms complete with lamina and nuclear pores. Nuclear-targeted proteins are imported, the nucleus grows in size, chromatin further decondenses, and the genomic DNA is replicated. Given that nuclear transport is required for all of these events, nuclear pores must assemble either during or shortly after the formation of the double nuclear membrane. Very little is known of the way in which the pore complex assembles. A major question in nuclear pore formation is: (*a*) whether formation of a double nuclear membrane occurs first, followed later by an intermembrane fusion between the inner and outer nuclear membranes to form the hole in which the pore complex then assembles, or (*b*) whether a partial pore forms on the surface of the chromatin recruiting membrane vesicles around it which then fuse to create a nuclear pore and a double membrane. One electron microscopic study of nuclear assembly, done under conditions of very limiting membranes, suggested that nuclear membrane vesicles may first bind to round pore-like precursors on the chromatin surface of Xenopus sperm (Sheehan et al., 1988), as in the second model. We have used the Xenopus nuclear reconstitution system to more closely examine the mechanism of pore complex assembly in newly forming nuclei, focusing specifically on the role of the nuclear membrane in this assembly.

Three different reagents reported to block membrane fusion, NEM, GTPγS, and BAPTA, were tested for an effect on the nuclear incorporation of pore proteins and the appearance of visible nuclear pores. By using immunofluorescence and electron microscopy to examine various nuclear assembly intermediates, we found that fusion between adjacent membrane vesicles is a required first step in nuclear pore assembly. In addition, two of the inhibitors, GTPγS and BAPTA, were found to block additional biochemically distinguishable downstream steps in pore assembly. Using a novel anchored nuclear assembly assay developed for the purpose, it was possible to order the GTPγS- and BAPTA-sensitive steps, further defining the assembly pathway of the nuclear pore.

Materials and Methods

Components of the Xenopus In Vitro Nuclear Reconstitution System

Both the cytosolic and membrane vesicle fractions of a Xenopus egg lysate were prepared as previously described (Powers et al., 1995). Membrane-free egg cytosol was frozen in 50-μl aliquots in liquid nitrogen. The washed membrane vesicle fraction was frozen as a 20× concentrated stock in 5-μl aliquots in liquid nitrogen. After freezing, both fractions were stored at −70°C. Demembranated condensed sperm chromatin was isolated from Xenopus testes, as described by Smythe and Newport (1991). The chromatin was distributed into 5-μl aliquots at a concentration of 50,000 sperm/μl, frozen in liquid nitrogen, and stored at −70°C. To swell the condensed sperm chromatin and expose the nuclear membrane vesicle binding sites, a crude preparation of nucleoplasmin was used (Newport and Dunphy, 1992). Nucleoplasmin is a nuclear protein released into the Xenopus egg cytosol at mitosis; it is responsible for the initial swelling of the highly condensed sperm chromatin (Philpott et al., 1991). Since nucleoplasmin is the major protein remaining soluble when total egg cytosol is

1. *Abbreviations used in this paper*: ARF, ADP-ribosylation factor; BAPTA, 1,2-bis-(o-Aminophenoxy)-ethane-N,N,N′,N′-tetra acetic acid; EGS, ethylene glycol*bis* (succinimidyl-succinate); NEM, N-ethylmaleimide.

denatured by heating, crude nucleoplasmin was prepared by heating cytosol to 100°C for 5 min. The denatured proteins were removed by centrifugation at 200,000 *g* for 30 min (Laskey et al., 1978; Newport and Dunphy, 1992) and the crude nucleoplasmin was stored at −70°C until use.

Assembly of Nuclei and Nuclear Intermediates In Vitro

Unless otherwise stated, all assembly components were kept on ice before use. Normal nuclei (Fig. 2 *A*) were assembled in vitro by combining sperm chromatin with the cytosol and membrane vesicle fractions of an egg lysate. For this, 5 μl of membrane vesicles was diluted into 25 μl of crude nucleoplasmin, supplemented with 0.1 mM GTP, and an ATP-regenerating system (1 mM ATP, 20 mM phosphocreatine, and 50 μg/ml creatine kinase final concentration). A 10-μl aliquot of the membrane-nucleoplasmin mixture was added to 40 μl of egg cytosol that had been supplemented with an ATP-regenerating system. Sperm chromatin was added to a final concentration of 1,000/μl and nuclei were allowed to assemble by incubation at room temperature. After assembly for 1 h, the mixture was placed on ice and the nuclei were processed for immunofluorescence or electron microscopy (see below).

A nuclear intermediate that consists of swollen sperm chromatin surrounded by membrane vesicles which have fused to a limited extent (Intermediate II, Fig. 2 *C*) was assembled by omitting complete egg cytosol from the nuclear reconstitution reaction. For this, 5 μl of membrane vesicles were diluted into 25 μl of crude nucleoplasmin supplemented with an 0.1 mM GTP and an ATP-regenerating system. Sperm chromatin was added to a final concentration of 5,000/μl and the mixture incubated at room temperature for 1 h. During this incubation, nucleoplasmin causes the condensed sperm chromatin to swell and the nuclear membrane vesicles then bind to the swollen chromatin. In the presence of ATP and GTP, a certain amount of fusion occurs between the bound vesicles (Newport and Dunphy, 1992; Coverley et al., 1993). Further steps in nuclear assembly (complete membrane fusion, pore complex assembly, and nuclear import) were accomplished by diluting 10 μl of the chromatin-bound fused vesicles (Intermediate II, Fig. 2 *C*) into 40 μl of complete cytosol containing an ATP-regenerating system. This assembly reaction was incubated at room temperature for 1 h, placed on ice, and processed for immunofluorescence or electron microscopy.

NEM Treatment

To examine the effect that treatment of the membrane vesicle fraction with the alkylating agent N-ethyl maleimide (NEM) had on nuclear pore assembly, 5 μl of membrane vesicles were diluted into 25 μl of nucleoplasmin, and this was then divided into two 15-μl aliquots. A 1-μl sample of freshly made 75 mM NEM was added to one of the aliquots, incubated at room temperature for 5 min, and the excess NEM was quenched by the addition of 1 μl of 125 mM DTT and a further incubation at room temperature for 2 min.

As a control, the NEM and DTT were mixed together for 2 min before their addition to the second 15-μl membrane-nucleoplasmin aliquot. The ability of the NEM-treated (or control) membranes to assemble nuclei was tested by the addition of 10 μl of the membrane mixture to 40 μl of egg cytosol supplemented with an ATP-regenerating system. Sperm chromatin was added to a final concentration of 1,000/μl and Intermediate I (Fig. 2 *B*) was allowed to assemble at room temperature. After 1 h, the intermediates were placed on ice and examined for the presence of pore proteins or nuclear pores by immunofluorescence or electron microscopy, respectively.

In the initial experiments done to titrate the amount of NEM needed to inhibit nuclear formation, treatment with 1 mM NEM was sufficient to inhibit subsequent nuclear formation when the membrane vesicle fraction was in buffer. However, when the membrane vesicle fraction was in a reducing environment such as egg cytosol or the nucleoplasmin preparation, 1 mM NEM did not inhibit nuclear formation. In this case, 5 mM NEM treatment of the membranes was required to permanently block nuclear formation.

We examined the ability of NEM to inhibit further membrane fusion and/or pore protein assembly on a nuclear intermediate consisting of chromatin-bound vesicles that had fused to a limited extent (Intermediate II, Fig. 2 *C*). To accomplish this, the nuclear intermediate was first assembled by mixing membrane vesicles (5 μl), crude nucleoplasmin (25 μl), sperm chromatin (5,000/μl), 0.1 mM GTP, and an ATP-regenerating system. This mixture was divided into two 15-μl aliquots and incubated for 1 h at room temperature. One aliquot was then treated with 1 μl of 75 mM NEM for 5 min at room temperature and the unreacted NEM was quenched by adding 1 μl of 125 mM DTT. The control aliquot was treated with 2 μl of the NEM and DTT samples that had been premixed. A 10-μl aliquot of the NEM-treated (or control) nuclear intermediates were diluted into 40 μl of egg cytosol supplemented with an ATP-regenerating system and incubated for 1 h at room temperature. The nuclei were then placed on ice and processed for immunofluorescence or electron microscopy.

BAPTA Treatment

To test the effect on pore assembly of adding the Ca^{2+} chelator BAPTA to a nuclear assembly reaction, BAPTA (Calbiochem, La Jolla, CA) was prepared as a 0.5-M stock solution in 10 mM Hepes, pH 7.4, and stored at −20°C. Nuclear assembly reactions were prepared by diluting 5 μl of membrane vesicles into 25 μl of crude nucleoplasmin and dividing this into two 15-μl aliquots. To one aliquot, BAPTA was added to a final concentration of 5 mM. To the second (control) aliquot, EGTA was added to a final concentration of 5 mM. A 10-μl sample of the diluted membranes was mixed with 40 μl of egg cytosol containing an ATP-regenerating system and either 5 mM BAPTA or EGTA. Sperm chromatin was added to a final concentration of 1,000/μl and incubated for 1 h at room temperature. The resulting assembled structures were then placed on ice and processed for immunofluorescence or electron microscopy.

We also examined the effect of BAPTA on membrane fusion and pore protein assembly when added to an intermediate in nuclear formation that consists of chromatin-bound vesicles that have fused to a limited extent (Intermediate II, Fig. 2 *C*). The nuclear intermediates were first assembled by mixing membrane vesicles (5 μl), crude nucleoplasmin (25 μl), sperm chromatin (5,000/μl), 0.1 mM GTP, and an ATP-regenerating system. This mixture was divided into two 15-μl aliquots and incubated for 1 h at room temperature. To one aliquot BAPTA was added to a final concentration of 5 mM, and to the second control aliquot EGTA was added to a final concentration of 5 mM. A 10-μl sample of each set of nuclear intermediates was diluted into 40 μl of egg cytosol containing an ATP-regenerating system and either 5 mM BAPTA or EGTA. After incubation for 1 h at room temperature, the resulting structures were placed on ice and processed for immunofluorescence or electron microscopy.

GTPγS Treatment

The effects of GTPγS addition on both membrane fusion and pore protein assembly were examined. GTPγS (Boehringer Mannheim, Indianapolis, IN) was prepared as a 40-mM solution in 100 mM Hepes, pH 7.1, 1 mM DTT, and stored in small aliquots at −70°C. Nuclear assembly reactions were prepared by diluting 5 μl of membrane vesicles into 25 μl of crude nucleoplasmin and dividing this into two 15-μl aliquots. To one aliquot, GTPγS was added to a final concentration of 2 mM. To the second aliquot, 2 mM GTP was added as a control. A 10-μl sample of each set of diluted membranes was mixed with 40 μl of egg cytosol containing an ATP-regenerating system and either 2 mM GTPγS or GTP. Sperm chromatin was added to a final concentration of 1,000/μl and the reactions were incubated for 1 h at room temperature. The assembled structures were then placed on ice and processed for immunofluorescence or electron microscopy.

The effect of GTPγS on membrane fusion and pore protein assembly when added to Intermediate II (Fig. 2 *C*) was also tested. Nuclear intermediate II, assembled in a 30-μl reaction containing membrane vesicles, crude nucleoplasmin, sperm chromatin, 0.1 mM GTP, and an ATP-regenerating system, as described above, was divided into two 15-μl aliquots and incubated for 1 h at room temperature. To one aliquot, 2 mM GTPγS was added, and to a second control aliquot 2 mM GTP was added. A 10-μl sample of each set of nuclear intermediates was diluted into 40 μl of egg cytosol containing an ATP-regenerating system and either 2 mM GTPγS or GTP. After incubation for 1 h at room temperature, the resulting structures were placed on ice and processed for immunofluorescence or electron microscopy.

An Anchored Nuclear Assembly Assay

An assay which consists of adding the cytosol and membrane vesicle fractions from a Xenopus egg lysate to swollen sperm chromatin that have been attached to a poly-lysine–coated glass slide was developed to allow the stepwise assembly of a nucleus. Nuclear assembly takes place on the surface of the glass slide. With the nuclei attached to a rigid surface, it is relatively easy to change the buffer or cytosol components without damaging the nuclei. For this assay, sperm chromatin was diluted into 25 μl of crude nucleoplasmin (10,000/μl) and allowed to swell for 5 min at room temperature. The swollen sperm chromatin was then diluted in ELB (10 mM

Hepes, pH 7.4, 50 mM KCl, 2 mM $MgCl_2$) to a final concentration of 800/μl. A 25-μl aliquot was placed in each well of a 10-well slide (ICN, Costa Mesa, CA; No. 6041805) that had been pretreated with 0.2 mg/ml poly-L-lysine for 15 min at room temperature and rinsed with H_2O. The slide was incubated in a humidified chamber for 1.5 h at room temperature to allow the swollen sperm chromatin to settle onto the slide surface and bind to the poly-lysine. Unbound chromatin was removed and the excess poly-lysine was blocked for 15 min with 25 μl of 5% BSA in ELB. Each well was gently washed five times with 25 μl of ELBK (10 mM Hepes, pH 7.4, 125 mM KCl, 2 mM $MgCl_2$). Nuclear assembly around the poly-lysine tethered chromatin was initiated by adding a 25-μl aliquot of a mixture of egg cytosol and membranes (see below).

Ordering the Positions at Which BAPTA and GTPγS Block Nuclear Assembly

The anchored nuclear assembly assay was used to determine whether the fused double nuclear membrane that forms around chromatin in the presence of BAPTA (Intermediate III, Fig. 2 *D*) is a substrate for nuclear pore assembly and, if so, if this process is inhibited by GTPγS. Intermediate III was first assembled by adding 25 μl of a mixture containing egg cytosol (100 μl), membranes vesicles (5 μl), and 5 mM BAPTA to the poly-lysine bound chromatin. After a 1-h incubation at room temperature, the cytosol-membrane-BAPTA mixture was removed and each well was washed five times with 25 μl of ELBK. A 25-μl aliquot of membrane-free egg cytosol containing an ATP-regenerating system and either no inhibitor, 5 mM BAPTA, or 2 mM GTPγS was then added to the bound BAPTA nuclei (Intermediate III). Alternatively, the washed BAPTA-nuclei were treated with 5 mM NEM, quenched with 7.5 mM DTT, and 25 μl of membrane-free egg cytosol without any inhibitor was added. All four sets of nuclei were incubated for 1 h at room temperature, washed five times with ELBK (25 μl), fixed with 4% formaldehyde for 20 min at room temperature, and processed for immunofluorescence with mAb 414.

Immunofluorescence Microscopy

Nuclei or nuclear intermediates assembled in vitro were assayed for the acquisition of pore proteins by immunofluorescence with the anti-pore monoclonal antibody 414 (BAb Co, Richmond, CA; Davis and Blobel, 1986; Dabauvalle et al., 1990). For nuclei or intermediates assembled in solution, the nuclear reconstitution reaction (25–50 μl) was chilled on ice for 15 min to depolymerize microtubules for better viewing. (This step was not necessary for the observed mAb 414 binding, or lack thereof in negative cases, but aided visualization of the mAb 414 signal.) After this, the nuclei/intermediates were fixed by dilution into 1 ml of ELB containing freshly diluted 2 mM ethylene glycol*bis*(succinimidyl-succinate) (EGS) and incubation for 40 min at room temperature. The cross-linker EGS (Pierce #21565) was prepared as a 0.2-M stock solution in dry DMSO. After fixation, the 1-ml sample was layered onto a cushion of ELB containing 25% sucrose. The nuclei/intermediates were pelleted through the sucrose onto a poly-lysine–coated coverslip. The coverslip was rinsed in PBS, postfixed with 4% paraformaldehyde in PBS for 10 min at room temperature, and then blocked with PBS containing 10% FCS, 0.1 M glycine, and 0.1% Triton X-100 for 15 min at room temperature. The nuclei/intermediates adhering to the coverslip were incubated for 1 h at room temperature with the anti-pore mAb 414 diluted 1:2,000 in PBS containing 10% FCS. Coverslips were rinsed with PBS, then incubated for 1 h at room temperature with a secondary RITC-conjugated goat anti–mouse IgG (Boehringer Mannheim, diluted 1:100 in PBS containing 10% FCS). After rinsing with PBS, coverslips were mounted in 90% glycerol, 10% PBS, and 1 mg/ml *p*-phenylenediamine (to prevent quenching), and sealed with nail polish.

For nuclei or intermediates that were assembled around chromatin prebound to poly-lysine–coated glass slides, the nuclei/intermediates were washed five times with 25 μl of ELBK, and then fixed in ELB containing 4% paraformaldehyde for 20 min at room temperature. After fixation, the slide was blocked with PBS containing 10% FCS, 0.1 M glycine, and 0.1% Triton X-100 for 15 min at room temperature. The nuclei/intermediates were incubated with mAb 414 in the same manner as for nuclei reconstituted in solution and pelleted onto coverslips. For both assays, immunofluorescence of the resulting structures was viewed using a Zeiss photomicroscope III with an attached CCD camera (Cohu, San Diego, CA) and an in-line image integrator (Micron Instruments, San Diego, CA). The images were printed on heat-sensitive paper using a Sony videographic printer. For each experiment, the images were obtained using the same magnification and integration values.

Electron Microscopy

To determine whether nuclei or nuclear intermediates contained fused membrane vesicles and/or nuclear pore complexes, the various structures were prepared for electron microscopy. A 25–50-μl assembly reaction containing reconstituted nuclei or nuclear intermediates was placed on ice for 15 min to depolymerize the microtubules. The nuclei were fixed by the addition of 0.4 ml of ice cold fixation buffer (25 mM Hepes, 25 mM Pipes, pH 7.2, 1 mM EGTA, 50 mM KCl, 2 mM Mg acetate, and 5% sucrose) containing 1% E.M. grade glutaraldehyde (Sigma Chem. Co., St. Louis, MO), and incubation on ice for 1 h. The fixed nuclei were pelleted for 5 s using a horizontal microfuge (model E; Beckman Instrs., Fullerton, CA). The supernatant was carefully removed and 0.2 ml of ice cold fixation buffer containing 10 mg/ml tannic acid and 2.5% E.M. grade glutaraldehyde was added to the nuclear pellet. This was incubated on ice for 2 h, and then microfuged for 5 s to pellet any resuspended nuclei. The nuclear pellet was then washed with ice cold 0.1 M Na cacodylate, pH 7.2 (4 × 0.4 ml), and postfixed with 1% OsO_4 on ice for 1 h. The nuclear pellet was washed with ice cold water (4 × 0.4 ml) and stained with 0.5% uranyl acetate overnight at 4°C. Each sample was dehydrated through a graded ethanol series, embedded in Spurr's resin, sectioned, stained with uranyl acetate and lead citrate, and then viewed on a 2300 transmission electron microscope (JEOL, Peabody, MA).

Results

NEM and GTPγS Block Nuclear Pore Assembly

As a first step in examining the process of nuclear pore assembly, we have used a Xenopus nuclear reconstitution system to identify the stages in nuclear formation at which pore complexes can assemble. Specifically, we have examined the role of the double nuclear membrane in pore assembly by creating nuclear formation intermediates that contain different amounts of fused double nuclear membrane. Each of these intermediates was then examined for the presence of pore proteins, using immunofluorescence with an anti-pore mAb 414, and for the presence of pore structures, using electron microscopy. Raised to the nuclear pore-lamina complex of rat liver nuclei, mAb 414 binds to the pore complexes of rat, Xenopus, and yeast nuclei, and is thought to recognize the consensus peptide sequence XFXFG (Davis and Blobel, 1986; Akey and Goldfarb, 1989; Aris and Blobel, 1989; Rout and Wente, 1994). In Xenopus, mAb 414 recognizes four nuclear pore proteins, nup60, nup153, nup200, and nup270 (Finlay and Forbes, 1990; Meier et al., 1995). These proteins are initially soluble in egg cytosol, but assemble and become pelletable as pore assembly proceeds (Finlay and Forbes, 1990; Meier et al., 1995). To confirm that mAb 414 antibody would detect the assembly of pore proteins on normal nuclei by immunofluorescence, as expected, nuclei were formed in a standard assembly reaction by mixing egg cytosol, membrane vesicles, and demembranated sperm chromatin. After a 1-h incubation at room temperature, the reconstituted nuclei were fixed, centrifuged through a sucrose cushion onto a coverslip, and tested for their ability to bind the anti-pore monoclonal antibody 414. By immunofluorescence, the nuclei were seen to have acquired multiple mAb 414 binding sites. The nucleus shown in Fig. 1 *A* is an example of a surface stain with mAb 414. Individual nuclei had either a surface staining pattern, as shown, or a nuclear rim staining pattern, which depended on the plane of focus of the microscope and the deformation of the nuclei incurred by striking the coverslip.

The inhibition of vesicle fusion is known to block forma-

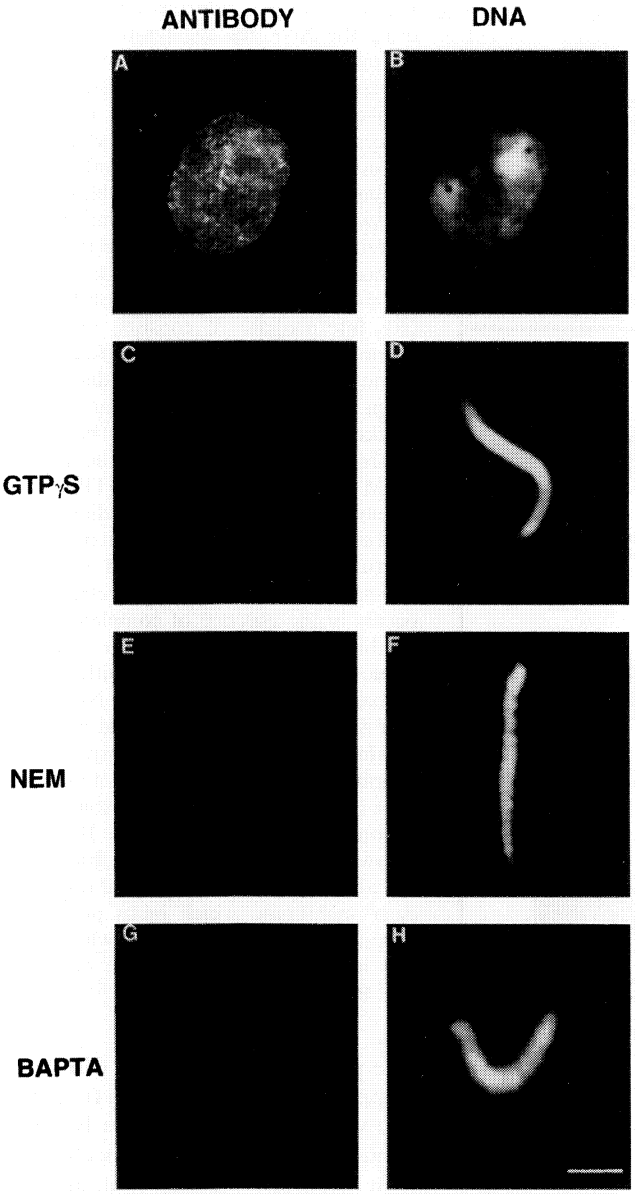


Figure 1. Pore proteins fail to assemble onto nuclei in the presence of inhibitors of nuclear envelope formation. Nuclei were reconstituted by mixing sperm chromatin with the cytosolic and membrane fractions of an egg lysate. The assembly reactions included either no inhibitors (*A* and *B*), 2 mM GTPγS (*C* and *D*), 5 mM BAPTA (*G* and *H*), or the membrane vesicle fraction was pretreated with 5 mM NEM for 5 min at 23°C (*E* and *F*). After forming for 1 h, each nuclear assembly reaction was incubated on ice for 15 min to depolymerize the microtubules. The nuclei were fixed for 40 min at room temperature by a 20-fold dilution into egg lysis buffer containing 2 mM EGS. The nuclei were then centrifuged onto a poly-lysine–coated glass coverslip, and assayed for mAb 414 binding by immunofluorescence. Panels on the left show the localization of mAb 414. Each image was exposed to the same extent and at the same magnification. Panels on the right show the corresponding DNA staining pattern visualized using HOECHST 33258. The scale bar in panel *H* represents 10 μm.

tion of a double nuclear membrane and subsequent growth of the nucleus. Specifically, the action of three reagents has been reported to inhibit nuclear vesicle fusion in vitro: (*a*) treatment of the membrane fraction with the alkylating agent NEM, (*b*) presence of GTPγS in the assembly reaction, and (*c*) presence of the Ca^{2+} chelator BAPTA in the assembly reaction (Newmeyer and Forbes, 1990; Boman et al., 1992*a*,*b*; Newport and Dunphy, 1992; Vigers and Lohka, 1992; Sullivan et al., 1993). To determine the role of the double nuclear membrane in nuclear pore formation, the effects of each of these reagents on nuclear pore assembly were tested.

To analyze the effect of NEM, the membrane fraction of an egg extract was pretreated with 5 mM NEM for 5 min at room temperature. Unreacted NEM was then quenched with 7.5 mM DTT. Cytosol, sperm chromatin, and the NEM-pretreated membranes were mixed and incubated for 1 h at room temperature. We found, as expected, that nuclear assembly did not occur. Instead, membrane vesicles bound to the swollen sperm chromatin and the vesicle-bound chromatin remained small (Fig. 1 *F*; Newport and Dunphy, 1992). This structure is subsequently referred to as nuclear Intermediate I (Fig. 2 *B*) and consists of swollen sperm chromatin bound by unfused membrane vesicles. The structures assembled using NEM-treated membranes (Intermediate I) remained in a worm-like shape for up to 4 h, indicating that the block to nuclear growth was effectively permanent. Importantly, when the structures assembled with NEM-treated membranes were then examined by immunofluorescence, they failed to bind the anti-pore antibody mAb 414 (Fig. 1 *E*). In a control experiment, where the NEM was quenched with DTT before its addition to the membrane fraction, normal nuclei formed and readily bound mAb 414 (not shown; identical to Fig. 1 *A*). Thus, in the absence of membrane fusion, the mAb 414-binding pore proteins do not assemble onto nuclear intermediate I.

It has been well documented that GTPγS irreversibly inhibits the fusion of nuclear membrane vesicles without inhibiting their chromatin binding (Boman et al., 1992*a*,*b*; Newport and Dunphy, 1992). To examine the effect of GTPγS inhibition of vesicle fusion on pore assembly, sperm chromatin, membrane vesicles, and cytosol were mixed and incubated in the presence of 2 mM GTPγS for 1 h at room temperature. As was the case for NEM, membrane vesicles in the presence of GTPγS bound to swollen sperm chromatin but the intermediates did not grow in size (Fig. 1 *D*). Moreover, when tested for immunofluorescence with mAb 414, these Intermediate I structures were found to lack pore protein antigens (Fig. 1 *C*). When 2 mM GTP was added to the assembly reaction as a control in place of GTPγS, normal nuclear assembly, growth, and mAb 414 binding were observed (not shown; identical to Fig. 1 *A*). We conclude that both NEM pretreatment of the membranes and GTPγS prevent nuclear pore assembly.

BAPTA Allows Nuclear Membrane Vesicle Fusion, but Blocks Pore Assembly

In addition to NEM and GTPγS, the Ca^{2+} chelator BAPTA has been reported to inhibit the fusion, but not the chromatin binding, of nuclear membrane vesicles (Sullivan et al., 1993). Interestingly, the Ca^{2+} chelator EGTA has no inhibitory effect on nuclear assembly (Sullivan et al., 1993). To examine the effect of BAPTA on pore assembly, sperm chromatin, membrane vesicles, and cytosol were

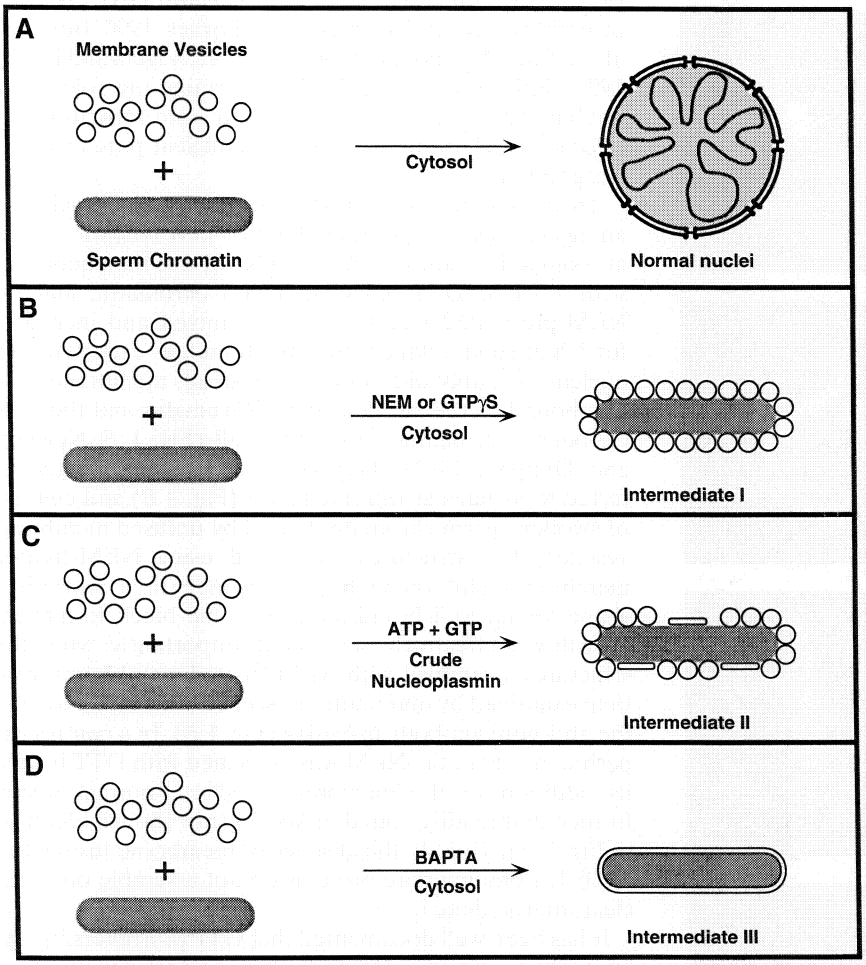


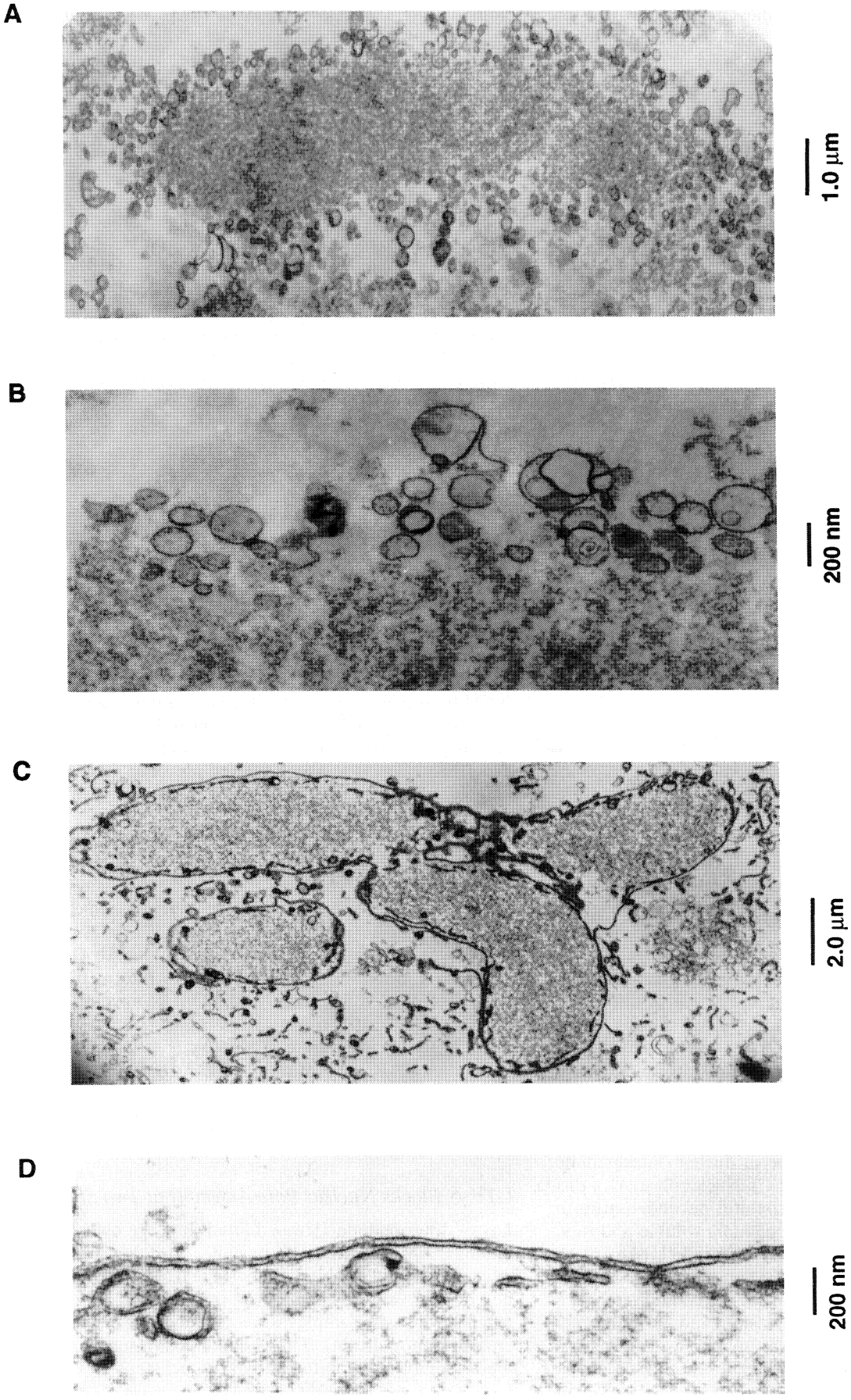
Figure 2. Nuclear intermediates assembled using the Xenopus egg lysate system. (*A*) Normal nuclei form when sperm chromatin is mixed with the membrane vesicle and cytosolic fractions of an egg lysate. During this process, a normal nuclear envelope that consists of a double nuclear membrane containing pore complexes assembles around the swollen sperm chromatin. The import of proteins into the nucleus allows for its subsequent growth, further decondensation of the chromatin, and replication of the DNA. (*B*) Intermediate I consists of swollen sperm chromatin bound by unfused membrane vesicles. This intermediate forms when fusion between the nuclear vesicles is blocked. Vesicle fusion can be blocked by pretreating the membrane fraction with NEM, or by the presence of GTPγS in the assembly reaction. (*C*) Intermediate II consists of swollen sperm chromatin bound by a mixture of fused and unfused membrane vesicles. A limited amount of vesicle fusion occurs in the absence of complete cytosol. In this case, sperm chromatin and membrane vesicles were mixed with a crude preparation of nucleoplasmin. Nucleoplasmin is the cytosolic protein that causes the sperm chromatin to swell exposing the membrane vesicle binding sites. In the presence of added ATP and GTP, some vesicle fusion occurs. (*D*) Intermediate III consists of swollen sperm chromatin that is surrounded by an extensively fused double nuclear membrane which lacks pores. This pore-free intermediate forms when a normal nuclear assembly reaction contains 5 mM BAPTA (see text).

mixed and incubated in the presence of 5 mM BAPTA for 1 h at room temperature. We expected that this treatment would result in the formation of Intermediate I structures (Fig. 2 *B*), as had NEM and GTPγS and, indeed, we found that the structures formed looked similar in the light microscope. The structures did not grow. When examined by immunofluorescence microscopy, the BAPTA-treated intermediates failed to bind mAb 414 (Fig. 1, *G* and *H*). The use of 5 mM EGTA, instead of BAPTA, did not inhibit nuclear assembly, growth, or mAb 414 binding (not shown). Thus, the three reagents NEM, GTPγS, and BAPTA all block the assembly of the mAb 414-binding pore proteins onto the nuclear envelope.

In previous studies, the fluorescent membrane dye DHCC has been used to distinguish between fused and unfused chromatin-bound membrane vesicles (Wilson and Newport, 1988; Boman et al., 1992*a*; Newport and Dunphy, 1992). Using this dye, Intermediate I structures, such as those formed in the presence of GTPγS or with NEM-treated membranes, appear to be bounded by a fuzzy layer of unfused membrane vesicles (not shown; see Boman et al., 1992*a*; Newport and Dunphy, 1992). Unexpectedly, we found that at 60 min in assembly reactions containing 5 or 10 mM BAPTA, the chromatin was surrounded by a smooth rim of DHCC membrane dye, characteristic of a fused double nuclear membrane (not shown). This was in contrast to the previously reported effect of BAPTA on nuclear membrane formation at 30 min (Sullivan et al., 1993), although those investigators did see fusion at 1.5–2 h. When we examined the intermediates in our extracts at 15–30 min with DHCC, we found that BAPTA had slowed vesicle fusion, but had much less of an effect on vesicle fusion than GTPγS (not shown). Complete fusion took place by 60–90 min with BAPTA.

To more precisely examine the state of the membrane vesicles on nuclear intermediates assembled with the dif-

Figure 3. Nuclei reconstituted using membranes pretreated with 5 mM NEM have bound unfused vesicles, whereas nuclei reconstituted in the presence of 5 mM BAPTA have fused double nuclear membranes. Nuclei were reconstituted in a standard assembly reaction, either using membranes that were pretreated with 5 mM NEM for 5 min at 23°C (*A* and *B*), or in the presence of 5 mM BAPTA (*C* and *D*). After formation for 60 min, the nuclei were fixed with glutaraldehyde, embedded in Spurr's resin, and processed for electron microscopy as described in Materials and Methods. In the nucleus in *C*, at many places two double nuclear membranes are forming, indicating highly active vesicle fusion.


A
1.0 µm
B
200 nm
C
2.0 µm
D
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ferent inhibitors, we prepared each sample for electron microscopy. The structures assembled in a nuclear reconstitution reaction using membranes pretreated with 5 mM NEM were seen to consist of swollen sperm chromatin surrounded by a relatively uniform layer of unfused membrane vesicles 100–200 nm in diameter (Fig. 3 *A*), as expected for Intermediate I. At a higher magnification, the membrane vesicles were seen to be in direct contact with chromatin (Fig. 3 *B*). As has been documented by others, we also found that the structures assembled in a nuclear reconstitution reaction containing GTPγS were of the Intermediate I type, and consisted of swollen sperm chromatin surrounded by unfused vesicles (not shown; identical to those of Fig. 3, *A* and *B*; see Boman et al., 1992*a*; Newport and Dunphy, 1992).

In contrast, we found that at 60 min the chromatin structures assembled in a nuclear reconstitution reaction containing 5 mM BAPTA were surrounded by extensively fused double nuclear membranes (Fig. 3, *C* and *D*). Most interestingly, nuclear pores were not present in any of the fused double nuclear membranes assembled in the presence of BAPTA. This correlates with the lack of mAb 414 binding to these nuclei. Pores were readily observable in the membranes of nuclei assembled in the presence of EGTA (see Fig. 5 *D*). Thus, BAPTA delays but does not inhibit fusion between chromatin-bound membrane vesicles. BAPTA does, however, completely block the assembly of nuclear pore proteins and nuclear pore structures onto the double nuclear membrane. The structure formed in the presence of BAPTA we termed Intermediate III (Fig. 2 *D*) in further discussions.

Pore Assembly Requires Fusion between Chromatin-bound Membrane Vesicles

During the in vitro formation of the nuclear envelope, inhibition of membrane vesicle fusion by either NEM-pretreatment or the presence of GTPγS clearly blocked pore assembly, as determined by mAb 414 binding and electron microscopy. These results could indicate that pore assembly occurs after a double nuclear membrane is generated by fusion of the chromatin-bound vesicles. In this model, interference with vesicle fusion would consequently prevent pore protein assembly. On the other hand, it was possible that either NEM pretreatment of the membranes or the presence of GTPγS inhibits pore assembly directly, in addition to an effect on membrane fusion. To distinguish an effect of NEM or GTPγS on pore assembly from their effects on nuclear vesicle fusion, we took advantage of the finding that when sperm chromatin and membrane vesicles are mixed with a crude preparation of nucleoplasmin (instead of cytosol), a limited amount of vesicle fusion occurs, giving rise to another type of nuclear intermediate, denoted Intermediate II (Fig. 2 *C*). Nucleoplasmin, a protein which mediates sperm chromatin decondensation in the egg, is the major protein remaining soluble and functional when egg cytosol is heat denatured (Laskey et al., 1978; Philpott et al., 1991; Newport and Dunphy, 1992; see Materials and Methods). When highly condensed sperm chromatin is mixed with nucleoplasmin and the membrane fraction of an egg lysate, the chromatin swells to expose many vesicle-binding sites; in the presence of ATP and GTP, limited fusion occurs between adjacent chromatin-bound vesicles to form Intermediate II (Fig. 2 *C*; Newport and Dunphy, 1992; Coverley et al., 1993). We prepared Intermediate II in this manner to test whether the various reagents used above would block pore formation in an already assembled double nuclear membrane. When Intermediate II was diluted into standard egg cytosol without added reagents and incubated for 1 h, a normal nuclear envelope formed, as expected (Newport and Dunphy, 1992). Such structures were always more elongated than nuclei assembled in complete cytosol (i.e., without the 60-min preincubation in nucleoplasmin plus membranes). When the resulting structures were tested for pore protein assembly by immunofluorescence with mAb 414, we found that the partially fused intermediates, once incubated in cytosol, bound mAb 414 (Fig. 4, *A* and *B*). Neither nucleoplasmin, sperm chromatin, nor membrane vesicles contain mAb 414-reactive proteins, so before dilution into complete egg cytosol the partially fused intermediates did not stain with mAb 414 (not shown). We conclude that when Intermediate II is incubated in cytosol, pore protein assembly takes place.

If Intermediate II was treated with 5 mM NEM for 5 min before dilution into complete egg cytosol, further fusion between membrane vesicles should be blocked. We found this to be true. Interestingly, however, after incubation in cytosol the resulting structures bound mAb 414 (Fig. 4, *E* and *F*). Indeed, when these samples were prepared for electron microscopy the patches of fused membranes present at the periphery of the sperm chromatin contained nuclear pores, correlating with the acquisition of mAb 414 binding (Fig. 5, *A* and *B*). We conclude that NEM treatment after vesicle fusion does not block nuclear pore assembly. In addition, we conclude that fusion of chromatin-bound vesicles to create patches of double nuclear membrane is required for and precedes nuclear pore complex assembly, since pores could not form on Intermediate I.

When reactions containing Intermediate II were diluted into cytosol containing 5 mM BAPTA, we found that Intermediate II was converted into a nucleus having an extensively fused double nuclear membrane, which contained no nuclear pores (Fig. 5, *C* and *D*). Correlating with this, the nuclear intermediates did not bind mAb 414 (Fig. 4 *G*). This structure appears identical to Intermediate III (Fig. 2 *D*). For comparison, a segment of envelope from nuclei assembled in the presence of EGTA shows that pores, when present, are easily visible (Fig. 5 *E*). We conclude that BAPTA, as it did in Fig. 3 *D*, appears to only transiently block nuclear vesicle fusion, but permanently blocks the assembly of the XFXFG pore proteins and of pores themselves into a double nuclear membrane.

GTPγS Blocks Nuclear Pore Assembly at Two Steps

Lastly, Intermediate II was diluted into egg cytosol containing 2 mM GTPγS. In this instance, after incubation the intermediate did not bind mAb 414 (Fig. 4 *C*). We also did not observe any nuclear pores in the fused membrane patches when the samples were examined by electron microscopy (not shown). Thus, unlike NEM, nuclear envelope assembly is inhibited at two distinct steps by GTPγS.

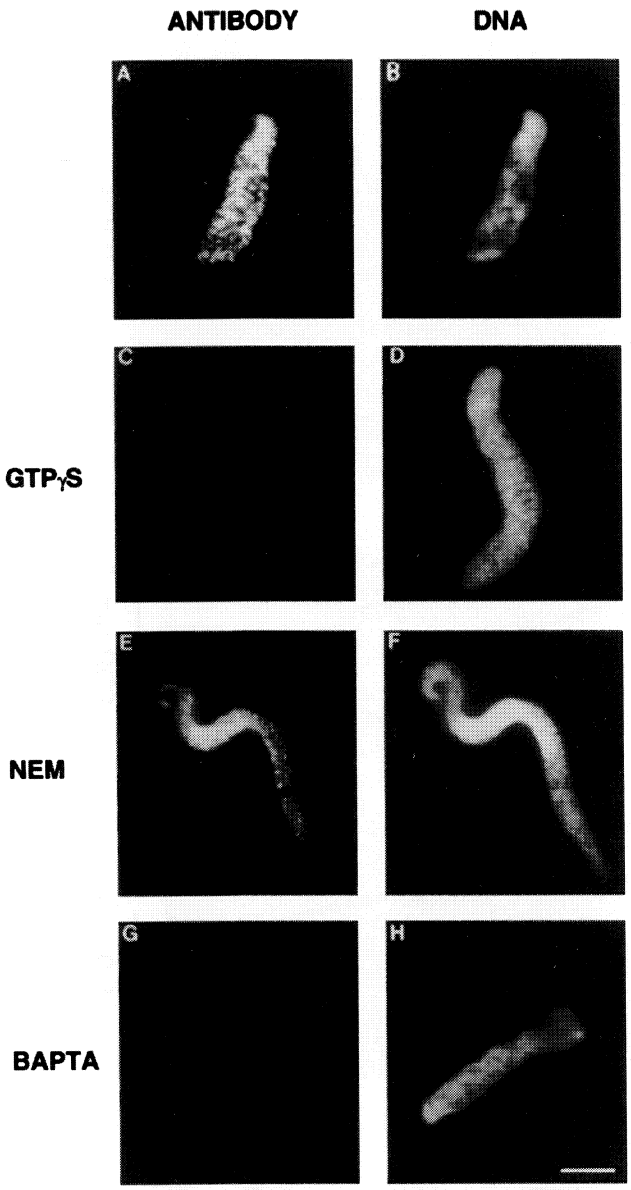


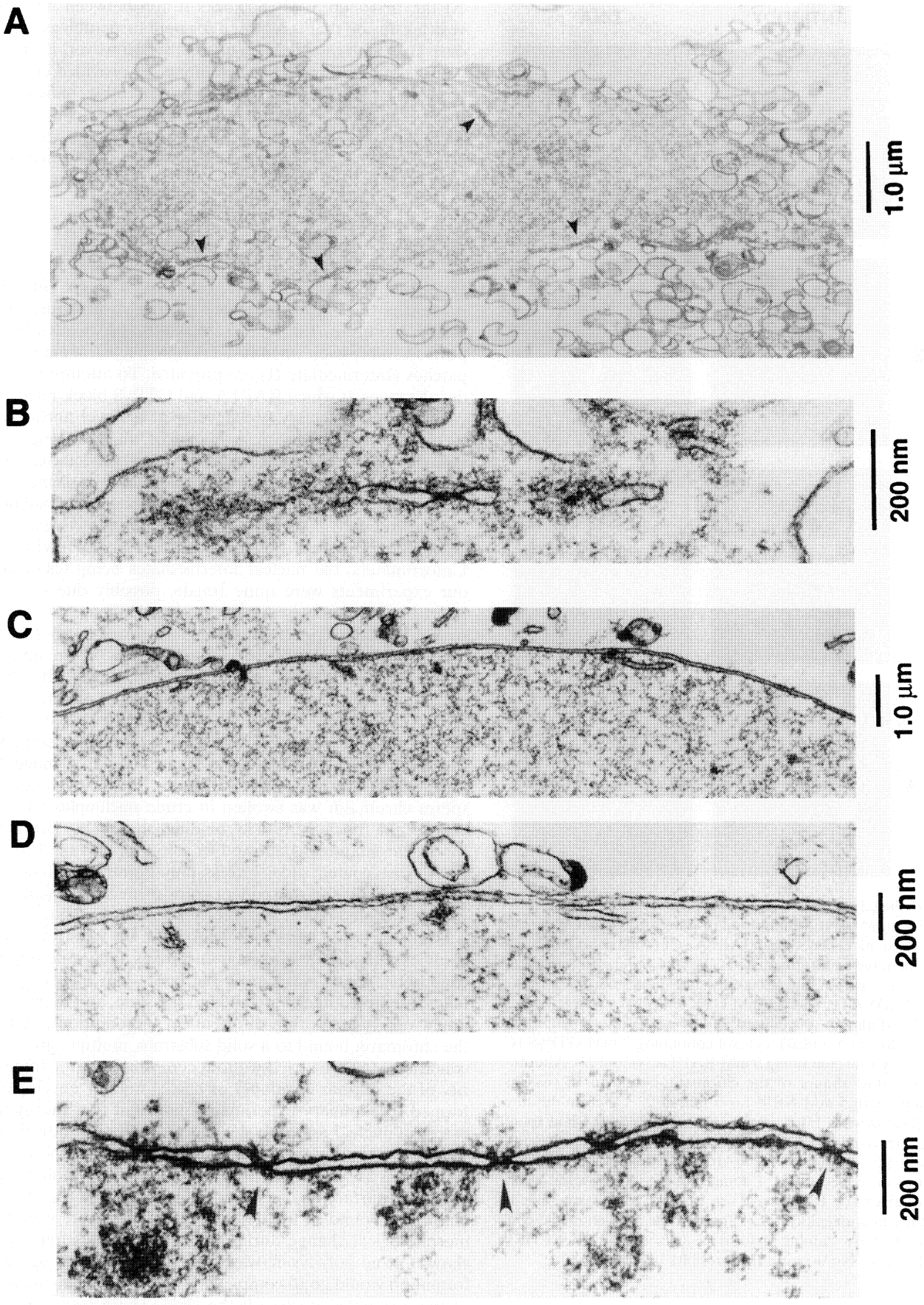
Figure 4. Fused nuclear vesicles that form in the absence of cytosol are resistant to NEM inhibition of pore protein assembly, but are sensitive to GTPγS and BAPTA. Chromatin-bound fused vesicles, denoted as Intermediate II (Fig. 2 *C*), were assembled by mixing sperm chromatin (5,000/μl), nucleoplasmin, membrane vesicles, 1 mM ATP, and 0.1 mM GTP for 1 h at room temperature. A 10-μl aliquot was diluted into 40 μl of either membrane-free egg cytosol (*A* and *B*), cytosol containing 2 mM GTPγS (*C* and *D*), or cytosol containing 5 mM BAPTA (*G* and *H*). Alternatively, the 10-μl aliquot of the chromatin-bound fused vesicles was treated with 5 mM NEM for 5 min at 23°C before diluting into 40 μl of cytosol. Each sample was incubated for 1 h at room temperature, fixed with EGS, pelleted onto a poly-lysine–coated glass coverslip, and assayed for mAb 414 binding by immunofluorescence as described in Fig. 1. Panels on the left show the localization of mAb 414. Each image was exposed to the same extent and at the same magnification. Panels on the right show the corresponding DNA staining pattern visualized with HOECHST 33258. The scale bar in panel *H* represents 10 μm.

At the first step, GTPγS blocks fusion between nuclear vesicles, fusion that is required for pore formation as demonstrated with the NEM results above. However, if fused patches of double nuclear membrane are provided (Intermediate II), GTPγS also blocks pore assembly, indicating GTPγS blocks a second step in pore assembly (Fig. 4 *C*). The latter block is not simply due to inhibition of further vesicle fusion since NEM does not inhibit pore assembly onto Intermediate II (Fig. 4 *E*).

A Novel Anchored Assay for Stepwise Nuclear Assembly

From the experiments above, both BAPTA and GTPγS prevent pore assembly at a step (or steps) subsequent to membrane fusion, that is, these reagents specifically block pore assembly even when fused double nuclear membrane patches (Intermediate II) are provided. To attempt to order the steps at which BAPTA and GTPγS block nuclear pore protein assembly, we developed a novel anchored method for the stepwise assembly of nuclei. Typically, with the Xenopus nuclear assembly system used above, order of addition experiments are performed by assembling nuclear intermediates in solution in the presence of one inhibitor, isolating and washing the nuclei by centrifugation, then adding fresh cytosol plus or minus a second inhibitor. Unfortunately, the nuclear intermediates being tested in our experiments were quite fragile, possibly due to the lack of import and assembly of nuclear lamina, matrix, or scaffold proteins. As a result, the intermediates often failed to survive repetitive centrifugations in an intact state and order of addition analysis proved difficult. The nuclei formed in the presence of 5 mM BAPTA were particularly fragile (Macaulay, C., unpublished results). To overcome these problems, we reasoned that intermediates in nuclear assembly would be less susceptible to damage if they were fastened to a rigid substrate. To accomplish this, sperm chromatin was swollen in crude nucleoplasmin to expose the nuclear vesicle binding sites. The swollen sperm chromatin was allowed to settle on and bind to a poly-lysine–coated slide. Excess poly-lysine was blocked with BSA, and the subsequent steps of nuclear assembly were performed on the anchored chromatin template.

Using anchored chromatin substrates, we found it relatively easy to assemble nuclear envelopes under one set of conditions, gently wash away the lysate leaving the partially assembled nuclei attached to the slide, and then add a new egg lysate with a different set of conditions. With the chromatin bound to a solid substrate, multiple growth conditions could be tried without compromising the integrity of the nuclear envelopes or partial nuclear envelopes formed. Preliminary experiments indicated that the final extent of nuclear assembly depended on the concentration of poly-lysine used on the slide. At high poly-lysine concentrations (1 mg/ml), a nuclear envelope assembled on the upper surface of the swollen chromatin, but the chromatin was bound so tightly to the slide that there was no morphological change during the assembly reaction (not shown). If no poly-lysine was used, the process of nuclear formation would go to completion, ultimately lifting many of the assembled nuclei off the slide. These nuclei were lost during subsequent washing steps. At a low concentra-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tion of poly-lysine (0.2 mg/ml), however, enough attachment of chromatin occurred to keep the majority of the assembling nuclei tethered to the slide, while still allowing for substantial nuclear envelope assembly, growth, and chromatin decondensation.

To characterize the extent to which nuclear assembly took place, a mixture of egg cytosol and membranes was incubated with the anchored chromatin for 60 min at room temperature. The excess lysate was removed, a new mixture of cytosol, membranes, and transport substrate (TRITC-SS-HSA; Newmeyer and Forbes, 1988) was added, and the nuclei were incubated for an additional 60 min. After this time, nuclei of a variety of sizes were observed, but the chromatin of all showed greater decondensation after the assembly reaction than before the assembly reaction (compare Fig. 6 *A* to 6 *B*). When transport substrate was included in the second assembly reaction, all nuclei were observed to have transport substrate bound to the nuclear envelope, i.e., they appeared to carry out the binding step of nuclear transport (Fig. 6 *C*; Newmeyer and Forbes, 1988). Only a small number of the nuclei (~1%) also showed accumulation of transport substrate (not shown). Immunofluorescence staining with mAb 414 showed a nuclear rim stain on the anchored nuclei (not shown; see Fig. 7 *A*). Taken together, these data are consistent with a normal nuclear envelope containing nuclear pores forming on the surface of the chromatin, but being prevented from completely encircling the chromatin by the attachment to poly-lysine on the slide.

Nuclei assembled using anchored chromatin substrates were also tested for incorporation of Bio-16-dUTP. If after 60 min of formation the excess lysate was removed and the nuclei were incubated with egg cytosol and membranes containing Bio-16-dUTP (0.5 nm) for an additional hour, followed by incubation with Texas red–streptavidin, all of the nuclei were seen to have incorporated Bio-16-dUTP (Fig. 6 *E*). This incorporation was sensitive to the DNA polymerase inhibitor, aphidicolin (Fig. 6 *G*), but we have not yet established whether the incorporation is due to bone fide semiconservative DNA replication or to DNA repair. Nevertheless, the results indicate that the anchored nuclear assembly assay may be useful for the study, not only of nuclear envelope assembly, but also of other nuclear functions.

BAPTA Blocks Pore Protein Assembly at a Step Downstream from the GTPγS-Sensitive Steps

Theoretically, the above anchored assay should allow the ordering of the positions at which BAPTA and GTPγS block pore assembly. A mixture of cytosol, membranes, and 5 mM BAPTA was thus added to poly-lysine bound swollen sperm chromatin to allow a pore-free double nuclear membrane to assemble on the sperm chromatin (Intermediate III). After 1 h, the incubation mix was removed, and the anchored BAPTA nuclei were gently washed with buffer. Egg cytosol devoid of membrane vesicles, but containing differing reagents, was then added for a second 1-h incubation. The anchored nuclei were then washed, fixed, and assayed for the presence of pore proteins by immunofluorescence using mAb 414. When 5 mM BAPTA was present in the second cytosol, we found that the nuclei neither grew nor bound mAb 414 (Fig. 7, *C* and *D*). Thus, the anchored nuclei showed an inhibition of pore protein assembly by BAPTA identical to that of nuclei assembled in solution (Fig. 1 *G*). When the second cytosol did not contain BAPTA, the anchored nuclei further decondensed their chromatin, rounded up, and now showed binding of mAb 414 (Fig. 7, *A* and *B*). This indicates that the inhibitory effect of BAPTA on pore assembly is completely reversible, such that when BAPTA is no longer present, pore assembly resumes. In a separate experiment, the BAPTA nuclei resulting from the first incubation were treated with NEM, then fresh cytosol was added for a 1-h incubation. The resulting nuclei now bound mAb 414 (Fig. 7, *E* and *F*). This agrees with our previous demonstration that NEM blocks pore assembly by preventing membrane vesicle fusion, but has no effect on subsequent steps of pore assembly. Here, on BAPTA nuclei, where an extensively fused double nuclear membrane has already formed, NEM treatment did not inhibit pore protein assembly.

Lastly, BAPTA pore-free nuclei were formed and GTPγS was added to the second cytosol. We found that the resulting nuclei did bind mAb 414 (Fig. 7, *G* and *H*), i.e., BAPTA nuclei were completely insensitive to GTPγS inhibition of pore protein assembly. From this, we conclude that the BAPTA-sensitive step in the pore assembly pathway must occur downstream of both the first and second GTPγS-sensitive steps. The data above is compiled in Fig. 8 *A*.

Discussion

In this study, we have examined the process of nuclear pore assembly, focusing on the role of the double nuclear membrane. Through use of three inhibitors of nuclear envelope formation, NEM, GTPγS, and BAPTA, intermediates were created containing different amounts of fused double nuclear membrane and examined for the presence of pore proteins and for individual nuclear pore complexes themselves. When membrane vesicles were pretreated with NEM to prevent vesicle–vesicle fusion, pore complex assembly was inhibited. On the other hand, when intermediates containing already fused nuclear membranes were treated with NEM, pore complex assembly was not inhibited. From these results we conclude that NEM does not

Figure 5. Nuclear pore complexes can assemble into fused nuclear vesicles that were treated with NEM, but not those incubated in the presence of BAPTA. Chromatin-bound fused vesicles (Intermediate II, Fig. 2 *C*) were assembled as described in Fig. 4. In one case, a 10-μl aliquot was treated with 5 mM NEM for 5 min at 23°C. The NEM treated nuclear intermediates were diluted into 40 μl of membrane-free cytosol and incubated for 1 h at 23°C (*A* and *B*). In a second case, a 10-μl aliquot of the nuclear intermediates that was not treated with NEM, was diluted into 40 μl of egg cytosol containing 5 mM BAPTA (*C* and *D*). In a third case, nuclei were assembled in the presence of 5 mM EGTA (*E*). The samples were then processed for electron microscopy as described in Fig. 3. The arrowheads (panels *A* and *E*) indicate the position of representative pore complexes located in the fused double nuclear membranes.

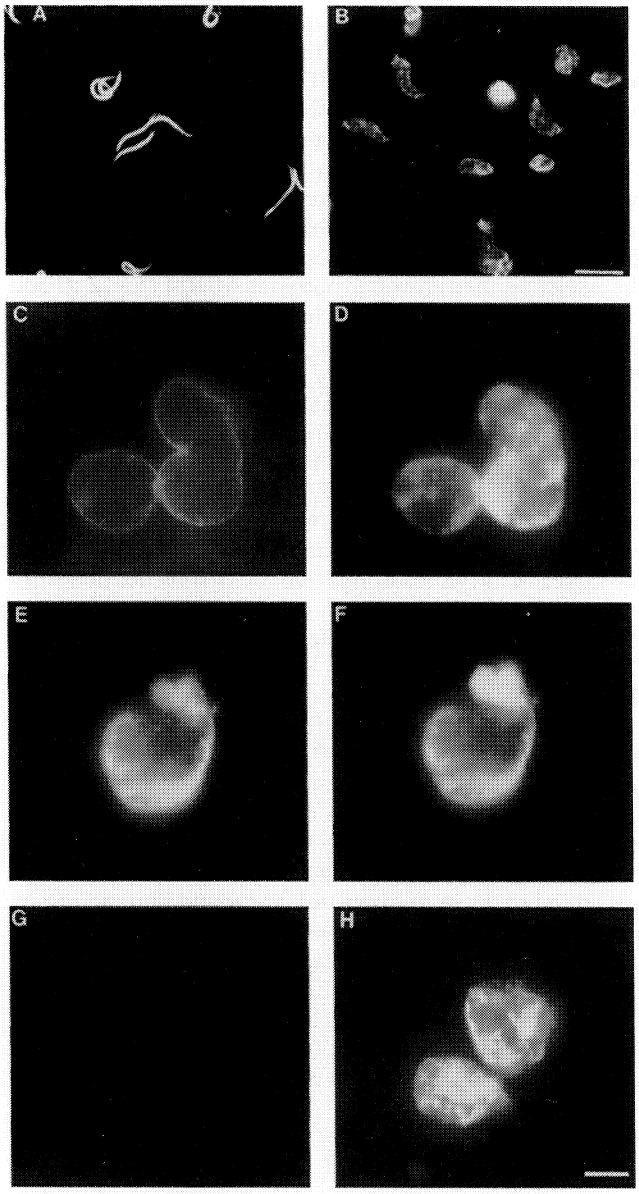

Figure 6. An anchored nuclear assembly assay. Sperm chromatin that were swollen by incubation in nucleoplasmin were attached to a poly-lysine–coated glass slide, as described in Materials and Methods. After blocking the excess poly-lysine with 5% BSA, the anchored chromatin was incubated with either egg lysis buffer (*A*), or with a 25-μl mixture of egg cytosol and membranes (*B–H*). After a 60-min incubation, the excess egg lysate was removed and a fresh 25-μl aliquot of egg cytosol and membranes containing either a TRITC-labeled nuclear transport substrate (*C* and *D*), 0.5 nM Bio-16-dUTP (*E* and *F*), or 0.5 nM Bio-16-dUTP plus 50 μg/ml aphidicolin (*G* and *H*) was added for an additional 60 min incubation. The excess lysate was removed, the nuclei gently rinsed and fixed with paraformaldehyde. To visualize incorporated Bio-16-dUTP (*E* and *G*), the nuclei were processed for immunofluorescence using Texas red–conjugated streptavidin. The DNA staining pattern was visualized by including HOECHST 33258 in the mounting medium (*A*, *B*, *D*, *F*, and *H*). The scale bar in panel *B* represents 30 μm, and illustrates the magnification of the nuclei in panels *A* and *B*. The scale bar in panel *H* represents 10 μm and illustrates the magnification of the nuclei in panels *C–H*.

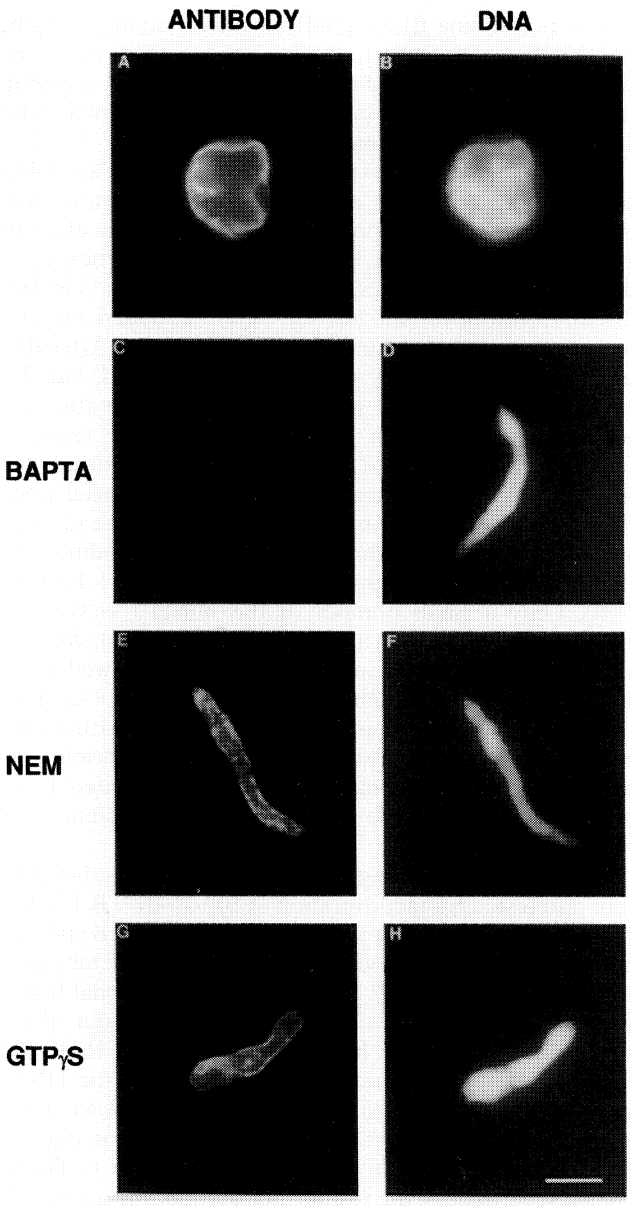

Figure 7. BAPTA reversibly blocks a step in nuclear assembly that is downstream of the steps that are sensitive to either NEM or GTPγS. Anchored nuclei with fused pore-free double membranes (Intermediate III, Fig. 2 *D*) were assembled by placing a mixture of membranes, cytosol, and 5 mM BAPTA on swollen sperm chromatin previously bound to a poly-lysine–coated glass slide, as described in Fig. 6. After 1 h, the cytosol-membrane-BAPTA mixture was removed and the slides were rinsed with buffer. In one case, the chromatin-bound pore-free double nuclear membranes were treated with 5 mM NEM before membrane-free egg cytosol was added for a second 1 h incubation (*E* and *F*). Otherwise, membrane-free egg cytosol containing either no inhibitors (*A* and *B*), 5 mM BAPTA (*C* and *D*), or 2 mM GTPγS (*G* and *H*) was added to the chromatin-bound pore-free double nuclear membranes for a second 1-h incubation. The resulting nuclei were rinsed with buffer, fixed, and assayed for mAb 414 binding by immunofluorescence. Panels on the left show the localization of mAb 414. Each image was exposed to the same extent and at the same magnification. Panels on the right show the corresponding DNA staining pattern visualized with HOECHST 33258. The scale bar (panel *H*) represents 10 μm.

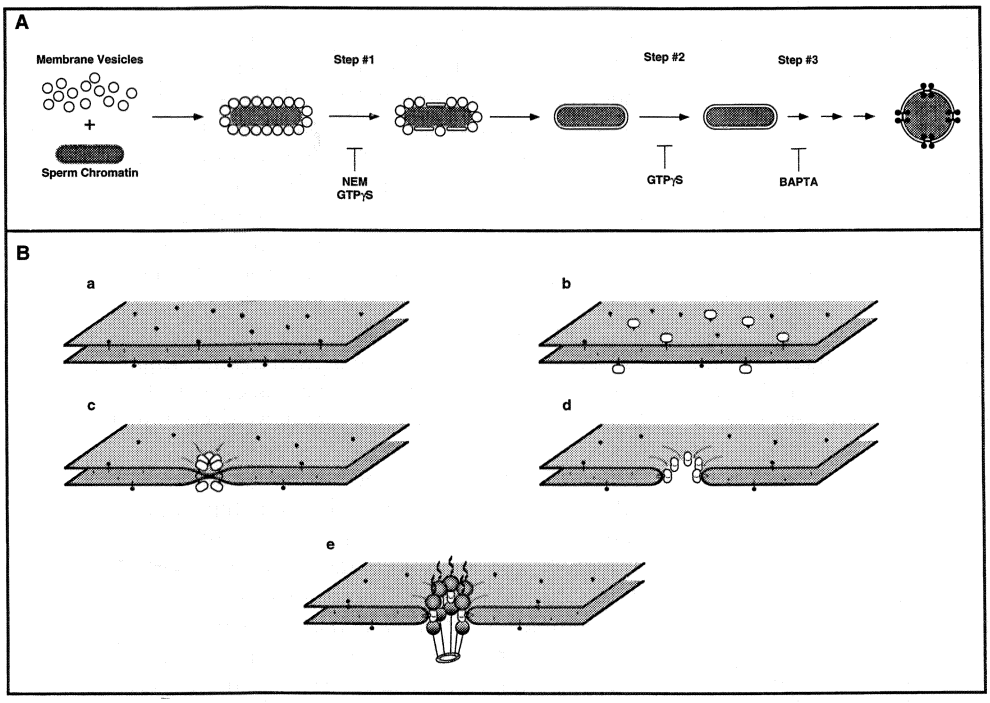

Figure 8. (*A*) Multiple steps in pore assembly were identified and ordered by their distinct sensitivities to the inhibitors NEM, GTPγS, and BAPTA. A first step in pore assembly (Step 1) proved to be nuclear vesicle fusion. This step was inhibited by NEM or GTPγS. In contrast, if nuclear vesicle fusion was allowed to occur, pore assembly could be blocked with either GTPγS or BAPTA. Analysis with an anchored nuclear assembly assay allowed ordering of these steps, indicating that Step 2, as defined by sensitivity to GTPγS, occurs before Step 3, as defined by sensitivity to BAPTA. (*B*) A model for nuclear pore complex assembly onto the double nuclear membrane. The fused double nuclear membrane contains integral membrane (POM) pore proteins (*a*), which likely act as receptors for the binding of soluble pore proteins (*b*). Binding of the soluble pore proteins to the membrane proteins could initiate their lateral clustering within the plane of the membrane (*c*). Oligomerization of the POM protein complexes might then trigger fusion between the inner and outer nuclear membranes (*d*). The resulting circular fenestrations in the envelope that are the product of the fusion process would then act as targets for the assembly of complete nuclear pore structures (*e*).

prevent pore complex assembly directly, but does so indirectly by blocking nuclear vesicle fusion. This indicates that a fused double nuclear membrane is a prerequisite for assembly of the nuclear pore.

Nonhydrolyzable analogs of GTP have previously been used to inhibit fusion between nuclear membrane vesicles (Boman et al., 1992*a*,*b*; Newport and Dunphy, 1992). We found that the addition of GTPγS to a nuclear assembly reaction also blocked pore complex assembly. However, we found that GTPγS blocked pore complex assembly both onto Intermediate I and onto the patches of fused nuclear membranes of Intermediate II (Fig. 4 *C*). This indicates that GTPγS can inhibit pore complex assembly at more than one step in the assembly pathway, at the initial vesicle–vesicle fusion step, and also at a second step in pore assembly which is subsequent to vesicle fusion.

In contrast to NEM and GTPγS, BAPTA had a transient effect on fusion between chromatin-bound vesicles (Sullivan et al., 1993). However, BAPTA completely blocked the assembly of nuclear pores. The block to pore assembly continued as long as BAPTA was present in the extract. Once BAPTA was removed and new cytosol was added, however, pore assembly took place. This indicates a direct interference in a process required for pore assembly, but not a permanent disabling of the proteins involved.

The nuclear intermediate formed in the presence of BAPTA at late times contains a fully fused double nuclear membrane devoid of nuclear pores, which we term Intermediate III. NEM treatment of Intermediate III did not block pore protein assembly when fresh cytosol was added. Again, this demonstrates that once a fused double nuclear membrane assembles, subsequent nuclear pore assembly can occur and, moreover, is insensitive to NEM treatment of the membranes. Thus, the membrane-associated proteins which contribute to pore assembly must be NEM-resistant.

Interestingly, using the anchored nuclear assembly assay we found that GTPγS does not block pore assembly onto Intermediate III (Fig. 7 *G*), whereas GTPγS had blocked pore protein assembly onto Intermediate II (Fig. 4 *C*). Why would this be so? Both intermediates seemingly have fused double nuclear membranes that should be a target for pore protein assembly. Sullivan et al. (1993) suggested that BAPTA treatment might make membranes refractory to the subsequent inhibitory effects of GTPγS, perhaps by preventing the binding of ARF. However, when we incubated membrane vesicles with cytosol and BAPTA for 30 min, and then washed and used the vesicles in a nuclear assembly reaction, the BAPTA-treated vesicles were identical to untreated vesicles in that both were sensitive to GTPγS inhibition of fusion in a nuclear assembly reaction (Macaulay, C., and D. Forbes, unpublished results). If it is not BAPTA treatment itself, some other difference between the double nuclear membrane of Intermediate III and the fused membrane patches of Intermediate II must account for their differing susceptibility to GTPγS inhibition of pore protein assembly. One of the major differences is that Intermediate II is assembled in a crude nucleoplasmin preparation which lacks most cytosolic proteins, whereas Intermediate III is assembled in complete cytosol. It seems most probable that there are proteins in cytosol that allow BAPTA-nuclei to assemble to a stage past the point at which pore protein assembly is sensitive to GTPγS inhibition. Thus, the fused nuclear membranes that form in

the presence of complete cytosol containing BAPTA (Intermediate III) are blocked at a step in pore assembly downstream from the fused nuclear vesicles that form in the absence of cytosol (Intermediate II).

How Do Nuclear Pores Assemble?

All of the above results are consistent with the conclusion that the nuclear pore complex assembles onto a preexisting double nuclear membrane. Thus, for pore assembly to occur the inner and outer nuclear membranes must fuse together at the site where the future pore will assemble. Unlike the fusion between adjacent chromatin-bound membrane vesicles, fusion between the inner and outer nuclear membranes to form the pore is not sensitive to NEM in our experiments. Given that the two membrane fusion events are topologically quite distinct, however, they likely involve different sets of proteins with different sensitivity to NEM. With respect to fusion between chromatin-bound vesicles, the NEM-sensitive protein has not yet been identified. Although NSF, the NEM-sensitive ATPase involved in membrane fusion in the secretory pathway, and p97, an NSF-related ATPase also involved in membrane fusion, are abundant in egg cytosol (Peters et al., 1990; Acharya et al., 1995; Macaulay, C., unpublished), we found that cytosol could not rescue fusion once nuclear membrane vesicles were NEM treated (Fig. 1 *E*). Either the NEM-sensitive protein involved in the fusion between nuclear vesicles is distinct from NSF and p97, or the protein may be NSF or p97, but be unexchangeable with its cytosolic counterparts.

The fusion that we conclude takes place between the inner and outer nuclear membranes is likely to be an early step in assembly of the nuclear pore (Rothman, 1994; Dabauvalle and Scheer, 1991). When modeling a mechanism for pore assembly, the intermembrane fusion event could conceivably itself be separated into distinct steps (Fig. 8 *B*). Certainly, an initiating event must trigger fusion. One would expect the integral membrane pore proteins to hold the key to such a trigger. The only two known vertebrate pore membrane (POM) proteins are gp210 and POM121 (Gerace et al., 1982; Filson et al., 1985; Wozniak et al., 1989; Greber et al., 1990; Hallberg et al., 1993). A direct physical modification of a pore membrane protein or, alternatively, the binding of a soluble pore protein to a pore membrane protein could well be the triggering event. It is not known how many integral membrane pore proteins on each nuclear membrane would be required to initiate intermembrane fusion. The influenza hemagluttinin protein, which mediates viral-host membrane fusion, consists of trimer of subunits, each of which contains a fusion peptide (for review see White, 1992). Perhaps a set of soluble pore proteins binds to multiple copies of a pore membrane protein and initiates lateral clustering of the membrane proteins into an oligomer (Fig. 8 *B*). The clustering of a sufficient number of the proteins (perhaps eight, given the symmetry of the nuclear pore) would then generate the fusion event. Interaction between the lumenal domains of pore protein multimers in the inner and outer nuclear membranes could logically initiate fusion of the lipid bilayers, yielding a "donut hole" in the double membranes. After fusion and formation of the membrane hole, the many soluble proteins of the pore would then coalesce to build the large and elaborate pore structure.

Why in this model would a preexisting double nuclear membrane be required for the above steps to take place? First, a single nuclear vesicle may not contain enough integral membrane pore proteins to generate the oligomerization required for pore formation. The side-to-side fusion between chromatin-bound vesicles would be needed to create a flattened nuclear membrane cisternae containing enough pore membrane proteins for oligomerization and pore assembly to occur. Alternatively, initial vesicle–vesicle fusion and flattening might be required either to place the inner and outer nuclear membranes topographically close enough for fusion to take place, or to form a membrane vesicle that is large enough to contain the membrane hole created during pore assembly.

Considering these models, our electron microscopy results suggest that BAPTA blocks a step in pore assembly before the fusion of the inner and outer nuclear membranes. It may block binding of soluble pore proteins to the POM proteins, the oligomerization of the POM-protein complexes, or the fusion event between the inner and outer nuclear membranes (Fig. 8 *B*). Whichever step that BAPTA inhibits, it must occur after the steps that are sensitive to GTPγS. The molecular mechanism by which BAPTA acts to inhibit pore assembly remains an interesting question. BAPTA may dissipate a Ca^{2+} gradient, created either by the stimulation of IP_3 receptors or by a different gradient-producing channel. IP_3 receptors have been localized to nuclear membranes (Malviya, 1994), and are thought to be involved in the vesicle–vesicle fusion events that form the double nuclear membrane (Sullivan et al., 1993). Interestingly, BAPTA, in addition to dissipating Ca^{2+} gradients, has also been reported to be a competitive antagonist of IP_3 binding to its receptor, so it may directly block the stimulation of IP_3 receptors and the induction of local Ca^{2+} gradients (Richardson and Taylor, 1993). Gradients of Ca^{2+}, which have also been reported to be important in the process of vesicular transport from the endoplasmic reticulum and in synaptic vesicle fusion (Rexach and Schekman, 1991; Schwaninger et al., 1991; Kelly, 1995), could well be important for the intermembrane fusion which forms the nuclear pore.

Sullivan et al. (1993) saw a slowing of nuclear vesicle fusion in the presence of BAPTA. Both our results and those of Sullivan et al. (1993) indicate that BAPTA inhibits nuclear envelope growth and we now believe that this is due to a complete block in pore assembly. However, we found that BAPTA had an additional effect on nuclei assembled in vitro. When BAPTA (5 mM) was added to a nuclear assembly reaction after the nuclear envelopes had been assembled, the nuclei (which contained pores) became very fragile (Macaulay, C., unpublished observations). In all cases, the effects of BAPTA depended on its concentration. At least 4–5 mM BAPTA was required to see an effect; 1–2 mM BAPTA had no effect. Greber and Gerace (1995) found that microinjection of 0.1–1 mM BAPTA into NRK cells had no effect on nuclear transport (and therefore presumably on nuclear membrane integrity), but higher concentrations could not be achieved in vivo. Thus, we conclude that while BAPTA is a powerful reagent for looking at specific events in nuclear envelope as-

sembly, it should not be presumed to have no other effects on nuclear structure or function. These effects may prove useful in analyzing other steps in nuclear assembly.

In a previous study which examined pore assembly, circular, membrane-free structures were observed on the surface of decondensed chromatin under conditions of limiting membrane and were hypothesized to be "prepores" which would act to target membrane vesicle binding to chromatin (Sheehan et al., 1988). In this model, a mature pore would form when membrane vesicles bound to the chromatin-bound prepores in a circle and fused to each other at the position of the prepores. We did not observe prepore-like structures on any of the nuclear intermediates formed here, although our electron microscopy conditions were not identical to Sheehan et al. (1988). Our BAPTA data indicates that a double nuclear membrane can form in the absence of pore complexes. Indeed, we found in our experiments that formation of the double nuclear membrane was required before the assembly of the pore complex.

A model where pore assembly requires the prior formation of a double nuclear membrane is particularly attractive, since such a model can be used not only for pore assembly at the end of mitosis, but also to explain the formation of pores in the preexisting double nuclear membranes of the interphase cell. At S phase in vertebrates, pore number doubles in the intact nucleus in preparation for division (Maul et al., 1972). Similarly, the model can explain pore assembly in yeast, where the nuclei undergo a closed mitosis, and in the cytosolic fused membrane cisternae of annulate lamellae (Kessel, 1992). With such a model, the mechanism of pore assembly would be the same in each of the above biological situations. The development of the anchored nuclear assembly assay and the identification of inhibitors of steps in nuclear pore assembly should allow us to further probe this model. In addition, it should be possible to analyze the molecular mechanisms by which the different inhibitors act to prevent pore assembly and to further analyze the hierarchical nature of pore assembly.

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