

A Trypsin-sensitive Receptor on Membrane Vesicles Is Required for Nuclear Envelope Formation In Vitro

Katherine L. Wilson and John Newport

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

Abstract. The reformation of functioning organelles at the end of mitosis presents a problem in vesicle targeting. Using extracts made from *Xenopus laevis* frog eggs, we have studied in vitro the vesicles that reform the nuclear envelope. In the in vitro assay, nuclear envelope growth is linear with time. Furthermore, the final surface area of the nuclear envelopes formed is directly dependent upon the amount of membrane vesicles added to the assay. Egg membrane vesicles could be fractionated into two populations, only one of which was competent for nuclear envelope assembly. We found that vesicles active in nuclear envelope assembly contained markers (BiP and α -glucosidase II) characteristic of the endoplasmic reticulum (ER), but that the majority of ER-derived vesicles do not contrib-

ute to nuclear envelope size. This functional distinction between nuclear vesicles and ER-derived vesicles implies that nuclear vesicles are unique and possess at least one factor required for envelope assembly that is lacking in other vesicles. Consistent with this, treatment of vesicles with trypsin destroyed their ability to form a nuclear envelope; electron microscopic studies indicate that the trypsin-sensitive protein is required for vesicles to bind to chromatin. However, the protease-sensitive component(s) is resistant to treatments that disrupt protein-protein interactions, such as high salt, EDTA, or low ionic strength solutions. We propose that an integral membrane protein, or protein tightly associated with the membrane, is critical for nuclear vesicle targeting or function.

THE organelles of eukaryotic cells contain specific sets of proteins that determine organelle identity and function. Membrane vesicles shuttle between organelles during cell growth by budding from and fusing with designated organelle membranes. This process must involve specific signals that determine the appropriate targets for vesicle fusion, but no such signaling molecules have yet been identified. A dramatic example of vesicle targeting occurs at the end of each mitotic cycle in higher eukaryotes when disassembled elements of the nucleus, endoplasmic reticulum (ER),¹ and Golgi apparatus accurately reassemble into their interphase structures (Colman et al., 1985; Warren, 1985; Lucocq et al., 1987). The assembly of the nuclear envelope is particularly interesting since vesicles interact with chromosomes as well as other vesicles to determine the location and structure of the reforming nucleus.

The nuclear envelope is composed of two membrane bilayers, the inner and outer membranes, which are separated by a 50-nm cisternal space (for reviews see Franke et al., 1981; Newport and Forbes, 1987). Both nuclear membranes are perforated by pore complexes, which regulate the passage of nuclear proteins and RNA into and out of the nucleus. The outer nuclear membrane is continuous at multiple points with the ER and is capable of performing many ER

functions such as the translocation and glycosylation of secreted proteins (Puddington et al., 1985). The inner membrane, on the other hand, is closely apposed to an electron-dense proteinaceous network, the nuclear lamina. The proteins of the lamina are related to intermediate filaments, and are thought to provide structural integrity to the nuclear envelope. The lamin proteins have also been proposed to serve as the anchor for chromatin attachment to the nuclear periphery (reviewed by Franke, 1987; Gerace, 1986; Newport and Forbes, 1987). Early in mitosis the entire nucleus disassembles—the chromosomes condense, the lamina depolymerizes, the pores disassemble, and the membranes vesicularize.

The reassembly of the nucleus at the end of mitosis involves, in the simplest sense, enclosure of the DNA by membrane vesicles which then fuse to one another. Despite a general knowledge of nuclear structure, the mechanisms by which membrane vesicles are specifically targeted to reform nuclei are unknown. A necessary early step in nuclear envelope formation is likely to be vesicle binding to decondensing chromatin. It has been proposed that the lamin proteins in Chinese hamster ovary (CHO) cells are directly responsible for vesicle binding to chromosomes; in this model the lamin proteins A and C serve as chromatin-bound receptors for the lamin B protein, 50% of which remains membrane associated during mitosis (Gerace and Blobel, 1980; Gerace et al., 1984; Gerace, 1986). In sum, two types of lamin are pro-

1. *Abbreviations used in this paper:* DHCC, 3, 3'-dihexyloxacarbocyanine; ER, endoplasmic reticulum; MWB, membrane wash buffer.

posed to mediate the interaction between membranes and chromatin. However, in other organisms only one type of lamin has been found. The single identified lamin of mouse embryos is of the lamin B type (Stewart and Burke, 1987), whereas the lamin of *Xenopus* embryos resembles lamin C (Stick and Hausen, 1985; see Wolin et al., 1987) and does not associate with membranes at mitosis (Benavente et al., 1985; Krohne and Benavente, 1986). The evidence suggests that the lamin protein of *Xenopus* eggs cannot by itself mediate the interaction between membranes and chromatin, implying that proteins as yet unidentified are important for this chromatin-membrane interaction.

We have studied nuclear envelope assembly in vitro using extracts from the eggs of the frog *Xenopus laevis*. Each *Xenopus* egg contains enough stored nuclear membrane components and chromatin proteins to assemble over 4,000 nuclei (Laskey et al., 1977; Woodland and Adamson, 1977; Lohka and Masui, 1983; Forbes et al., 1983; Newport and Forbes, 1985); these stores are normally depleted during the rapid cell divisions of early embryogenesis. DNA or chromosomes incubated in extracts of activated *Xenopus* eggs form "synthetic" nuclei that are identical to normal nuclei in structure and function: the nuclear envelope is composed of both inner and outer membranes (Lohka and Masui, 1984; Newport and Forbes, 1985; Newport et al., 1985; Newport, 1987), the envelope contains pore complexes that import nuclear proteins in a signal sequence-dependent manner (Newmeyer et al., 1986a, b; Newmeyer and Forbes, 1988), there is a peripheral lamina layer, and the membrane-enclosed DNA replicates (Lohka and Masui, 1984; Newport and Forbes, 1985; Blow and Laskey, 1986; Blow and Watson, 1987; Newport, 1987; Sheehan et al., 1988). Furthermore, nuclei formed in vitro respond appropriately to cell cycle regulation and undergo complete nuclear disassembly when exposed to *Xenopus* egg extracts that contain mitotic factors (Miake-Lye and Kirschner, 1985; Lohka and Maller, 1985; Lohka and Maller, 1987; Newport and Spann, 1987; Dunphy and Newport, 1988). Because nuclei assembled in these extracts are so like natural nuclei by several different criteria, results pertaining to the assembly of the nuclear envelope should accurately reflect envelope assembly in vivo.

In this study, we demonstrate that vesicles that reform a nuclear envelope carry at least one component that targets them uniquely to the reforming nucleus. We show that a trypsin-sensitive integral membrane protein, or protein tightly associated with membranes, is necessary for vesicle binding to chromatin. By altering the amount of membranes added to our in vitro nuclear assembly assay, we established conditions where nuclear envelope size could be regulated by the availability of nuclear-specific vesicles. Furthermore, we present evidence that nuclear-targeted vesicles are functionally distinct from vesicles that reform the ER.

Materials and Methods

Buffers

Buffer X: 200 mM sucrose, 7 mM MgCl₂, 80 mM KCl, 15 mM NaCl, 5 mM EDTA, 15 mM Pipes. Egg lysis buffer: 250 mM sucrose, 2.5 mM MgCl₂, 50 mM KCl, 1 mM dithiothreitol (DTT), 50 µg/ml cycloheximide, 5 µg/ml cytochalasin B (prevents actin gelation), 10 µg/ml each aprotinin and leupeptin (Sigma Chemical Co., St. Louis, MO). Membrane wash buffer (MWB): 250 mM sucrose, 50 mM KCl, 2.5 mM MgCl₂, 50 mM

Hepes, pH 7.5, 1 mM DTT, 1 mM ATP, 1 µg/ml aprotinin, 1 µg/ml leupeptin. Hoechst buffer: 20 µg/ml bisbenzimidazole DNA dye (Hoechst 33258; Calbiochem-Behring Corp., La Jolla, CA), 200 mM sucrose, 5 mM MgCl₂, 80 mM KCl, 15 mM NaCl, 5 mM EDTA, 15 mM Pipes, pH 7.4, 3.7% formaldehyde.

Preparation of Sperm Chromatin

Demembrated sperm chromatin consists of *Xenopus* sperm treated with lyssolecithin to remove both the plasma and nuclear membranes without affecting the highly condensed chromatin. It was prepared as described by Lohka and Masui (1983) with minor modifications. Testes were obtained from mature *Xenopus* males (not treated with hormone). Demembrated sperm were stored at -70°C in Buffer X.

Obtaining *Xenopus* Eggs

Xenopus eggs were obtained and dejellied as described by Newport (1987). For nucleus assembly extracts, eggs were synchronously activated by exposure to Ca²⁺ and the Ca²⁺ ionophore A23187 (2.0 µg/ml; Newport, 1987). After activation, eggs complete meiosis and initiate DNA synthesis, leading to a cytoplasm active for nuclear assembly. Mitotic extracts were obtained from unactivated eggs, which remain in second meiotic metaphase (Newport and Spann, 1987).

Preparation of Nuclear Assembly Extracts

Crude nuclear assembly extracts were prepared from lysed activated *Xenopus* eggs, as previously described (Newport, 1987), except that the packed eggs were lysed in a centrifuge (Sorvall Instruments Div., Newton, CT) at 12,000 g (HB-4 rotor, 10,000 rpm, 4°C, 12 min). The crude cytoplasmic extract was further fractionated by centrifugation (model No. TL100; Beckman Instruments, Inc., Palo Alto, CA) at 200,000 g (TLS-55 rotor, 55,000 rpm, 1 h, 4°C). This ultracentrifugation step separated the membrane components of the nuclear assembly extract from the soluble components. The soluble fraction was centrifuged again at 200,000 g for 25 min to remove residual membranes. The resulting membrane-free nuclear assembly extract was then supplemented with an ATP-regenerating system (10 mM phosphocreatine, 1 mM ATP, pH 7, 50 µg/ml creatine kinase); aliquots were frozen in liquid nitrogen and stored at -70°C. Soluble extracts prepared this way were stable for months. The resulting "interphase" membrane fraction was diluted in MWB and pelleted at 26,600 g (model No. TL100; Beckman Instruments, Inc.; 20,000 rpm, 10 min, 4°C) onto a 1.3-M sucrose cushion. Membranes were resuspended in MWB and used fresh, or resuspended in MWB containing 0.5 M sucrose, frozen in liquid nitrogen, and stored at -70°C.

Mitotic Membranes

Crude mitotic extracts were prepared from unactivated *Xenopus* eggs as previously described (Newport and Spann, 1987), except that 10 µg/ml (each) aprotinin and leupeptin were included in the final lysis buffer rinse. Crude extracts were centrifuged at 200,000 g (as above) to separate soluble mitotic components from the mitotic membranes. Membranes were washed in MWB and pelleted (as above), then resuspended in MWB at a protein concentration of 5–20 mg/ml. Although mitotic membranes are stable to freezing, freshly prepared unfrozen mitotic membranes were used. In all experiments performed, identical results could be obtained using either interphase or mitotic membranes, with the exception of the sucrose gradient fractionation experiment shown in Fig. 4.

Preparation of Embryonic Membranes

Eggs were synchronously fertilized in vitro and dejellied as described (Newport and Kirschner, 1982) and incubated for 12 h in MMR (100 mM NaCl, 2 mM KCl, 1 mM MgSO₄, 2 mM CaCl₂, 5 mM Hepes [pH 7.8], 0.1 mM EDTA) plus 10 µg/ml gentamycin. Incubation in MMR, which causes embryos to exogastrulate, was done deliberately to prevent blastocoel formation and thereby prevent dilution of the crude extract. Approximately 5% of cells at the gastrula stage are in mitosis at any given time (Graham and Morgan, 1966). Gastrulae were washed in egg lysis buffer, lysed using a model No. P-1000 Pipetman (Gilson Co., Inc., Worthington, OH), and then centrifuged as described for the egg extracts (Newport, 1987) to obtain a postnuclear, crude cytoplasmic extract. The crude extract was further fractionated by centrifugation at 200,000 g as described above to obtain the em-

bryonic membrane fraction. Membranes were washed in MWB and used fresh.

Trypsin Digestions

Protease pretreatment of membranes was done at 0°C in a final volume of 300–500 μ l of MWB (initially) lacking protease inhibitors. TPCK-treated trypsin (Sigma Chemical Co., St. Louis, MO) was added to each tube (final concentration 10–20 μ g/ml), plus extra DTT (+1 mM final). The control tube was then supplemented with protease inhibitors (aprotinin and leupeptin; final concentration 100 μ g/ml) and incubated for 5 min at 0°C to inactivate the trypsin before membrane addition. Membranes washed and resuspended in MWB (minus protease inhibitors) were added and incubated 20–30 min at 0°C. Final membrane protein concentration in the protease digest mixture was 0.4–0.6 mg/ml. To stop the protease digestion, protease inhibitors were added to the digest tube, mixed, and incubated an additional 10 min at 0°C. All tubes were then diluted to 2.4 ml final volume with MWB, vesicles were pelleted at 26,600 g for 10 min (model No. TL100; 20,000 rpm, 4°C), resuspended in a small volume of MWB (plus inhibitors), and assayed for nuclear envelope-forming activity.

High salt extractions and other membrane treatments were done in final volumes of 0.5–1.0 ml, for 20–30 min at 0°C before dilution and pelleting as described above and in Results.

Nuclear Assembly Assays

Frozen membrane-free nuclear assembly extracts were rapidly thawed by hand and mixed with the appropriate membranes. A typical reaction consisted of 20 μ l nucleus assembly extract (membrane free, containing an ATP-regenerating system), 2–4- μ l membranes (at 5–20 mg protein/ml), and 1,500 sperm/ μ l extract. Reactions were incubated at room temperature (22–24°C) for up to 5 h, and nuclear envelope assembly was monitored by light microscopy of aliquots diluted 1:1 with Hoechst buffer. The amount of membranes added to nuclear assembly extracts to reconstitute nuclear membrane formation varied from 1 to 6 egg equivalents of membrane per volume of extract. One egg equivalent is approximately the concentration of membranes found in the crude nucleus assembly extract, assuming that we recover 100% of the membranes after fractionation at 200,000 g.

Membrane Fractionation on Sucrose Step Gradients

Freshly prepared membranes isolated from mitotic (unactivated) eggs were supplemented with 2.3 M sucrose to a final concentration of 1.3 M sucrose, mixed well, and overlaid with sucrose solutions containing 1.1, 0.9, and 0.7 M sucrose in 10 mM Tris (pH 8.1) and 1 mM DTT. (Finer step increments between 1.1 and 0.7 M sucrose did not significantly resolve the vesicles with nuclear envelope-forming activity.) Membranes from \sim 30 ml of starting eggs were loaded into two 50.1 ultracentrifuge tubes (Beckman Instruments, Inc.) (5.5-ml capacity) and centrifuged at 200,000 g (47,000 rpm, 2 h, 4°C) to separate membranes into distinct subfractions. We used mitotic membranes because we expected them to be in a naturally disassembled state which might facilitate fractionation. In contrast to the fractionation behavior of mitotic membranes, interphase membranes did not separate cleanly with respect to nuclear envelope assembly activity. Instead, both heavy and light interphase membrane fractions were active.

Depletion of Nuclear Vesicles from the Extracts

To deplete extracts of nuclear vesicles, all available nuclear vesicles were incorporated into a saturating amount of nuclei, as follows. Nuclear assembly extract and membranes were mixed, prespun briefly (30 s; Eppendorf) to remove particulate matter, and then distributed among a series of parallel reaction tubes which also contained either sperm chromatin (5,000 sperm/ μ l assembly extract) or buffer. Each final reaction tube contained 22 μ l assembly extract (with ATP-regenerating system), 1.1 μ l membranes (\sim 1 egg equivalent concentration of membranes), and either no sperm chromatin or 1.1 \times 10⁵ sperm (5,000 sperm/ μ l assembly extract). Nucleus assembly was initiated by transferring tubes from ice to incubation at 22–24°C, and allowed to proceed for 0–4 h. At a given time, each tube was diluted 1:1 with MWB and centrifuged for 30 s in an Eppendorf to pellet the chromatin or nuclei. (Observations by light microscopy confirmed that nuclei were efficiently pelleted under these conditions; longer spins [even 60 s] began to pellet vesicles in addition to nuclei.) This centrifugation step separated the nuclear (or chromatin) pellet from the free (unincorporated) vesicles in the supernatant. The supernatant and washed pellet from each tube

were then assayed for either BiP or α -glucosidase II activity as described below. Parallel samples were photographed by light microscopy. The average nuclear envelope surface area at each time point was calculated from measurements of the diameters of 18 to 42 individual nuclei, taken from photographic negatives.

Assays for BiP and α -Glucosidase II

α -Glucosidase II enzyme activity was measured by the hydrolysis of *p*-nitrophenyl α -D-glucopyranoside (Burns and Touster, 1982; Hino and Rothman, 1985). Samples (20 μ l) were added to 500 μ l assay buffer (4 mM substrate [Sigma Chemical Co.], 50 mM Hepes, pH 6.8, 1% sodium cholate). Reactions were incubated at 30°C for 1–2 h, and terminated by addition of 1 ml of 0.64% ethylene-diamine (pH 10.7). The absorbance OD₄₀₀ of the reaction product was compared to a reference curve generated from pure *p*-nitrophenol (Mallinckrodt Inc., St. Louis, MO).

We assayed for BiP by immune decoration of nitrocellulose filters. Samples were resuspended in buffer (4% SDS, 10% glycerol, 125 mM Tris, pH 6.8, 5% β -mercaptoethanol), boiled, and subjected to electrophoresis on a 10% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose filters, and the filters were incubated with anti-BiP antibody, followed by iodinated protein A, as described (Reymond et al., 1984). D. Bole generously provided a rat mAb raised against mouse BiP (Bole et al., 1986), which cross reacts with the *Xenopus* BiP. Autoradiograms were scanned using an Ultrosan XL Laser Densitometer (LKB Instruments, Inc., Gaithersburg, MD), and the amount of BiP in each lane was quantitated by cutting out and weighing the peaks.

Protein concentration was measured using the BCA Protein Assay Reagent (Pierce Chemical Co., Rockford, IL) after diluting samples in 50 μ l 0.2% Triton X-100. BSA was used as the standard.

Microscopy

Samples for electron microscopy were diluted 20-fold with cacodylate (0.2 M cacodylate, pH 7.4), immediately made 3% in glutaraldehyde and 1.5% in formaldehyde, and fixed on ice for 2 h. Nuclei and chromatin were then centrifuged 45 s in an Eppendorf centrifuge (4°C) and rinsed in cacodylate. The pellet was stirred into 4% agarose/cacodylate (Sigma ultra-low gelling agarose, type IX), chilled, cut into pieces, and washed three times in cold cacodylate. Samples were postfixed in 2% osmium tetroxide, embedded in Spurr's medium, sectioned (80–120 nm thick), and poststained in uranyl acetate followed by lead citrate. A Philips EM 300 operated at 80 kV was used with a 50- μ m objective lens aperture.

For light microscopy we used a Photomicroscope III (Carl Zeiss, Inc., Thornwood, NY) fitted for fluorescence microscopy with exciter-barrier reflector combinations suitable for 3,3'-dihexyloxycarbocyanine (DHCC; fluorescein channel) and bisbenzimidazole.

Results

An Assay for Nuclear Membrane Assembly

Crude extracts were prepared essentially as described (Newport, 1987) from *Xenopus* eggs, which contain all of the molecular components necessary to assemble nuclei around metaphase chromosomes, sperm chromatin, or protein-free DNA. The membrane and soluble components of such extracts can be separated from each other, and from other cellular components, by sedimentation at 200,000 g for 1 h (Lohka and Masui, 1984). The crude membrane fraction from our 200,000 g centrifugation step was washed free of residual soluble components by dilution in MWB followed by centrifugation at 26,600 g (see Materials and Methods). Addition of a washed membrane fraction to the soluble extract components reconstitutes the ability to form an intact nuclear envelope around sperm chromatin (Fig. 1, insets a and b; Lohka and Maller, 1987; Newport, 1987). Nuclear envelopes were detectable both by phase-contrast microscopy, which reveals the distinct phase-dense structure surrounding the DNA, and by fluorescence microscopy in the

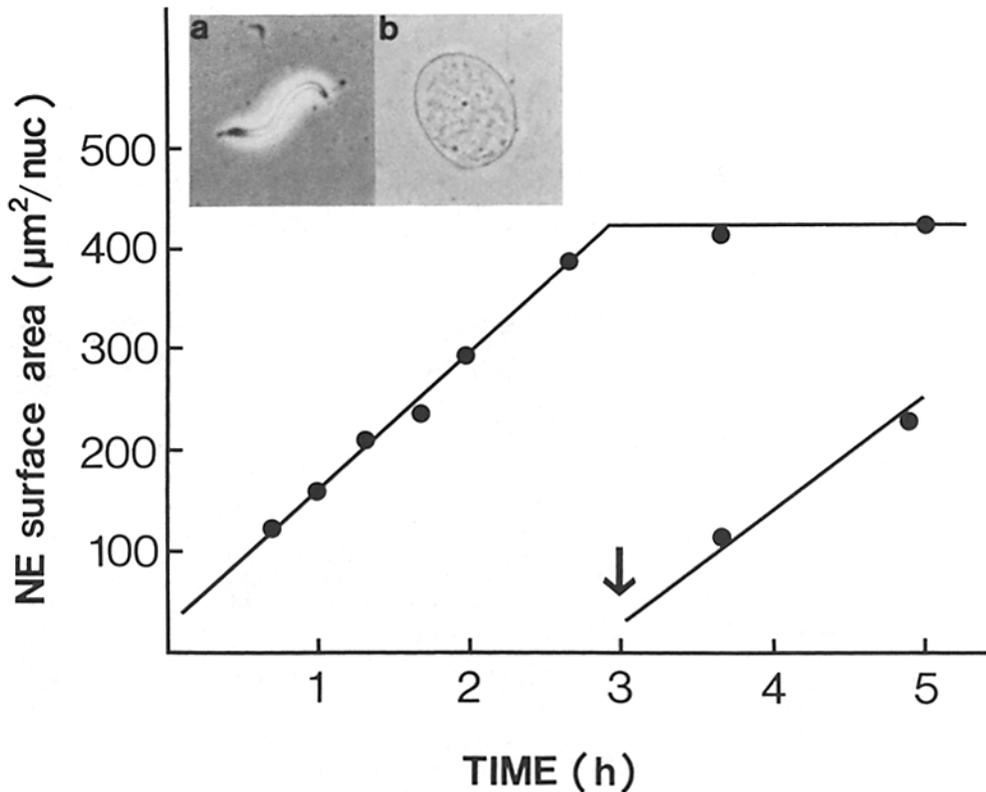


Figure 1. Nuclear envelope surface area ($\mu\text{m}^2/\text{nuc}$) as a function of incubation time in nuclear-assembly extract. Sperm chromatin (100 sperm/ μl extract) was incubated ($22\text{--}24^\circ\text{C}$) in nuclear assembly extract with a 1-egg equivalent concentration of interphase membranes. Aliquots were removed at different times to photograph nuclei by light microscopy; measurements of nuclear diameter taken from photographic negatives were used to calculate nuclear envelope surface area. After 3 h of incubation (arrow), additional sperm chromatin was added and monitored by light microscopy. New sperm nuclei grew at the same rate as the original nuclei. (Inset) Phase-contrast micrographs of sperm chromatin after (a) 10 min and (b) 3 h of incubation in nucleus-assembly extract and mitotic membranes.

presence of the lipophilic dye DHCC, which binds to the nuclear membrane. As an alternative source of membranes for nuclear assembly, we used membranes isolated from unfertilized ("mitotic") *Xenopus* eggs. When used in the nuclear assembly extract, these mitotic (vesicularized) membranes were also active in envelope formation. Membranes isolated from mitotic eggs and activated eggs are referred to as mitotic membranes and interphase membranes, respectively.

Nuclear Envelope Growth Is Linear with Time

To determine the stability of our extracts with respect to envelope assembly, we monitored the kinetics of envelope growth in reconstituted extracts. For these experiments the use of sperm chromatin as a template for nuclear envelope assembly (Lohka and Masui, 1983) ensured that all nuclei contained the same amount of DNA. Increasing concentrations of sperm chromatin (100–800 sperm/ μl extract) were added to a reconstituted nuclear assembly extract. Nuclear envelope growth was monitored by light microscopy and photography. Measurements of nuclear diameters from photographs were used to calculate the average nuclear envelope surface area at a given time. As shown in Fig. 1, average nuclear envelope surface area increased linearly for 2.5 h, then maintained a stable size ($\sim 420 \mu\text{m}^2$) for an additional 2 h. To determine whether the plateau in nuclear envelope size was due to inactivation or depletion of a necessary component, more sperm chromatin was added to the same reactions after 3 h of incubation (Fig. 1, arrow). The additional sperm chromatin acquired a nuclear envelope at the same rate as did the original chromatin. We conclude that the extract is stable for at least 5 h, and that neither soluble nor membrane components are limiting when there are $<1,000$ sperm/ μl assem-

bly extract. Because nuclei provided with an excess of membranes and soluble components ultimately stopped growing when they achieved an average surface area of $300\text{--}400 \mu\text{m}^2$, we conclude that some other factor (perhaps chromatin) may limit further growth (see Discussion).

Trypsin Digestion of Vesicles Destroys Nuclear Membrane-forming Activity

To determine whether a vesicle-associated protein is required for nuclear envelope formation, vesicles isolated from mitotic extracts were first treated with trypsin and subsequently tested for the ability to form a nuclear envelope around sperm chromatin. Specifically, vesicles were incubated with trypsin (10–20 $\mu\text{g}/\text{ml}$) for 30 min on ice, and then proteolysis was terminated by the addition of protease inhibitors. Control membranes were pretreated with trypsin in the presence of the protease inhibitors, aprotinin and leupeptin. After this treatment, protease-treated or control vesicles were concentrated by centrifugation and added to the soluble extract components and sperm chromatin. Control membranes produced a nuclear envelope as visualized by nuclear rim staining with DHCC (Fig. 2 C), whereas the trypsinized membranes did not (Fig. 2 A). We observed hundreds of sperm chromatin units incubated with trypsinized membranes; none formed a nuclear envelope. Binding of protease-treated vesicles to chromatin could not be distinguished from samples that contained no added vesicles (compare Fig. 2 A with B). Therefore at least one protein on the vesicle surface is required for nuclear assembly function.

To determine if the vesicle protein(s) involved in nuclear envelope assembly is weakly or tightly associated with the vesicle membrane, we subjected vesicles to treatments that

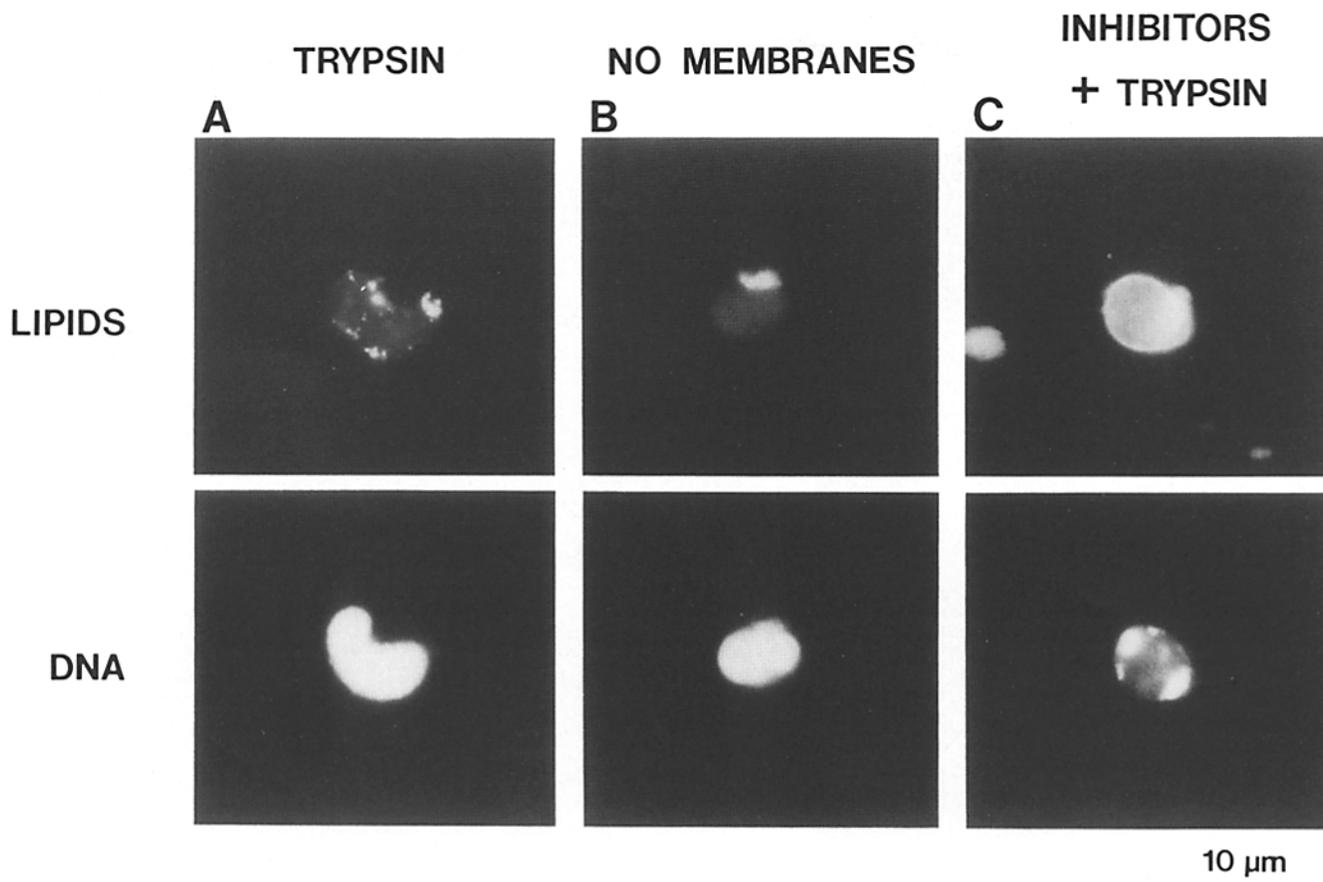


Figure 2. Trypsin treatment of vesicles prevents formation of a nuclear envelope. Sperm chromatin and membrane-free assembly extract (with ATP-regenerating system) were incubated with either trypsin-digested vesicles, no vesicles, or control vesicles. Samples were photographed after 2 h of incubation at room temperature. (A) Mitotic membranes treated with trypsin (20 $\mu\text{g}/\text{ml}$) and washed before use; (B) MWB only; (C) mitotic membranes treated with trypsin (20 $\mu\text{g}/\text{ml}$) in the presence of protease inhibitors, then washed before use. *Top*, membranes visualized through fluorescence of the lipophilic dye, DHCC. Occasional nonspecific sticking of DHCC precipitates was observed. *Bottom*, DNA fluorescence of the same structures as visualized with bisbenzamide.

disrupt protein-protein interactions and weak protein-membrane interactions. Specifically, we exposed vesicles for 20 min on ice to high salt (0.9 M KCl/MWB), EDTA (10 mM EDTA in MWB), or low ionic strength (10 mM Tris, pH 8, 1 mM DTT, 1 mM ATP, 250 mM sucrose) solutions. Vesicles were then pelleted and assayed for function (not shown). In contrast to the protease result, none of these treatments inhibited nuclear envelope assembly. The protease-sensitive factor(s) is therefore tightly associated with the vesicle and may be an integral membrane protein.

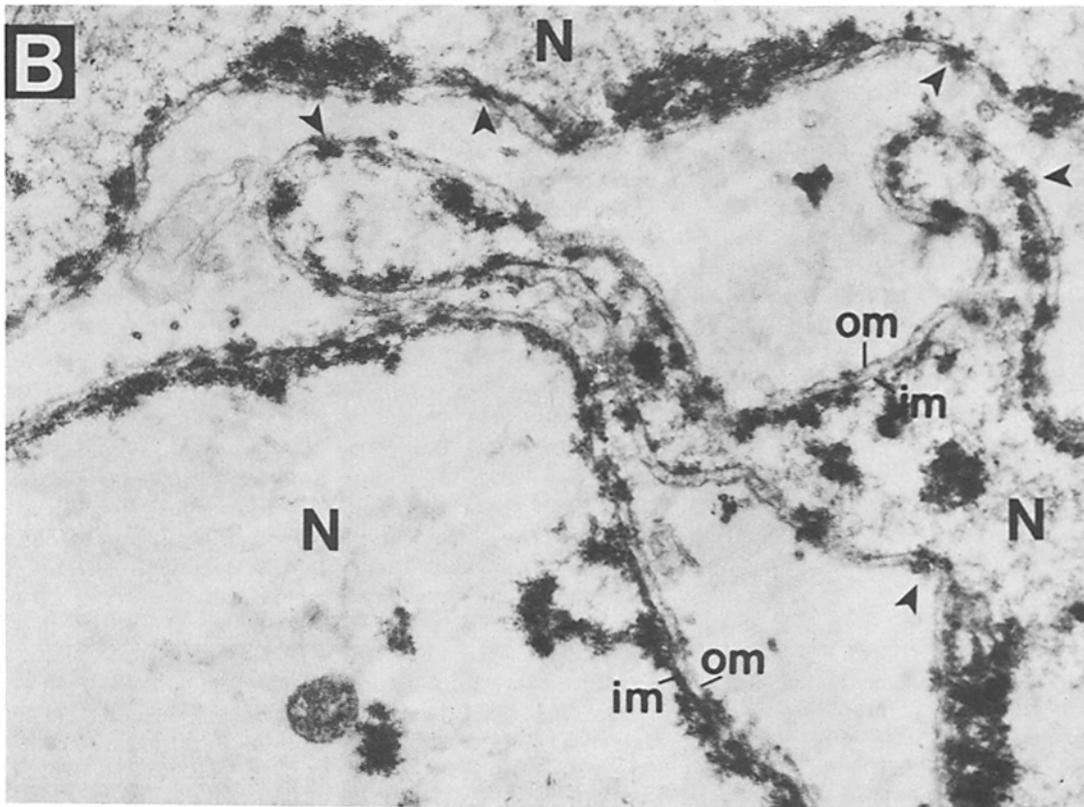
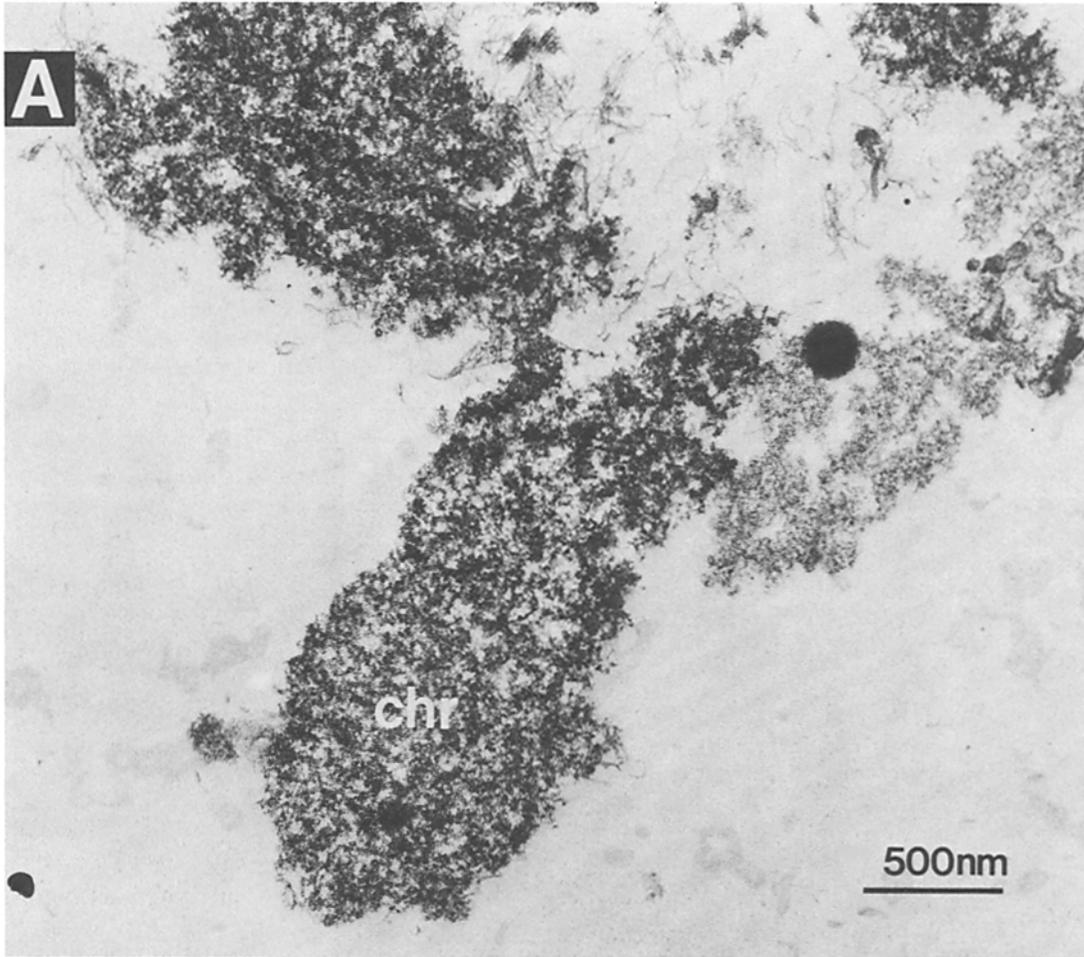
What Step in Nuclear Envelope Formation Requires the Trypsin-sensitive Factor?

A simple pathway for envelope assembly around sperm chromatin or metaphase chromosomes would require at least two steps: (a) binding of vesicles to chromatin, and (b) fusion of adjacent vesicles. The previous experiment suggested that protease-treated vesicles were blocked at the binding step. To test this, we examined the protease-treated and control samples by electron microscopy. As shown in Fig. 3 B, electron micrographs of chromatin incubated with control vesicles (treated with trypsin plus inhibitors) revealed decondensed

chromatin associated with typical nuclear envelopes and pore complexes. In contrast, Fig. 3 A shows that chromatin incubated with protease-treated vesicles exhibited uneven edges, no membrane structure, and no bound vesicles. These results demonstrate that protease-treated vesicles do not bind to chromatin, and therefore indicate that trypsin treatment removes at least one protein necessary for vesicle binding.

Fractionation of Mitotic Vesicles

The crude membrane fraction used above contained widely differing types of membranes, including vesicles derived from the breakdown of the oocyte nucleus, ER, and Golgi apparatus (Colman et al., 1985) and vesicles destined for insertion into embryonic plasma membranes (Byers and Armstrong, 1986). To attempt to isolate the vesicles responsible for nuclear envelope assembly, we fractionated crude mitotic membranes by sedimentation on discontinuous sucrose gradients. Specifically, the membrane fraction from a mitotic extract was made 1.3 M in sucrose and overlaid with 1.1-, 0.9-, and 0.7-M sucrose solutions (see Materials and Methods). After centrifugation at 200,000 g, the membranes located at each interface were recovered, washed in MWB, and



tested for function by addition to a membrane-free nuclear assembly extract containing sperm chromatin. We found that the light membranes (recovered from the 1.1/0.9-M and 0.9/0.7-M interfaces) formed a nuclear envelope around sperm chromatin (not shown). In contrast, no nuclear envelope formed when membranes isolated from the 1.3/1.1-M interface ("heavy" membranes) were used. Chromatin incubated with heavy and light fractions combined did acquire nuclear envelopes, indicating that the heavy fraction did not contain an inhibitor of nuclear envelope formation, but rather lacked vesicles capable of forming a nuclear envelope.

Characterization of light (1.1/0.7-M) and heavy (1.3/1.1-M) membrane fractions by electron microscopy revealed that the heavy layer contained abundant mitochondria and large granular vesicles, 400–900 nm in diameter, and fewer small vesicles. The nuclear envelope-forming light membrane fraction consisted primarily of heterogeneous small vesicles, 100–400 nm in diameter (not shown). The light fraction therefore contained vesicles of the expected size for envelope assembly (see Lohka and Masui, 1984) in addition to those derived from the breakdown of other organelles.

Since the outer nuclear envelope is continuous with the ER and appears to carry out many ER functions in protein secretion (Franke, 1974; Puddington et al., 1985), we asked whether protein markers characteristic of the ER (and outer nuclear envelope) were present in the light vesicle fraction. For this we assayed the above membrane fractions for two proteins: BiP (a luminal ER protein; Bole et al., 1986; Munro and Pelham, 1987) and α -glucosidase II (a membrane-associated ER protein; Brands et al., 1985; Lucocq et al., 1986). We found that the specific activity of α -glucosidase II was 4–10 times higher in light vesicles than in the heavy membrane fraction (Fig. 4). The distribution of BiP mirrored that of α -glucosidase (not shown). We concluded that the light vesicle fraction, which contains vesicles active in nuclear envelope formation, is enriched in vesicles derived from the ER and outer nuclear envelope.

The cofractionation of BiP and α -glucosidase II with the nuclear envelope-forming vesicles suggested a way to test the hypothesis (based on EM observations; see Franke, 1974; Longo, 1976) that vesicles derived from the breakdown of the ER can reassemble a nuclear envelope. Alternatively, we proposed that "nuclear-specific" vesicles might be similar to ER vesicles in composition but would be uniquely identified by receptors that allow specific interactions with chromatin.

Envelope Growth Ceases When the Supply of Nuclear Vesicles Has Been Incorporated into Nuclei

If a class of nucleus-specific vesicles exists, we predict that there would be competition between growing nuclei for the available pool of nuclear vesicles. Nuclear vesicles would become limiting for nuclear envelope growth, and the final size of individual nuclei would be directly proportional to the

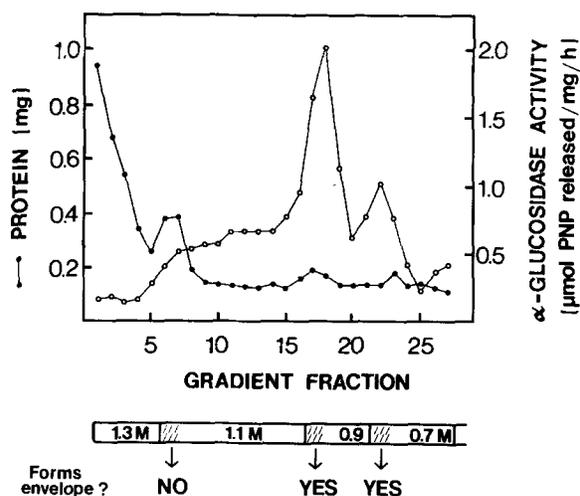


Figure 4. Mitotic membranes fractionated on discontinuous sucrose gradient. The membrane fraction of a mitotic extract was made 1.3 M in sucrose and overlaid with the sucrose concentrations shown in lower part of the figure. After centrifugation at 200,000 g for 2 h, gradient fractions ($\sim 200 \mu$ l) were collected from the bottom and assayed for total protein (mg/fraction [●]) and α -glucosidase II activity (expressed as nmol PNP released/mg protein per hour [○]). Pooled membrane fractions were tested in vitro for vesicles active (YES) or inactive (NO) in nuclear envelope assembly.

input vesicle concentration. This was indeed what we found, as shown in Fig. 5. At a fixed concentration of nuclei (2,000/ μ l extract), low concentrations of membrane vesicles produced small nuclei whereas high membrane concentrations resulted in much larger nuclei. To quantitate these differences, the final nuclear envelope surface area was measured as a function of the input membrane concentration (Fig. 6). We find that below a specific membrane concentration, envelope size is limited by and directly proportional to the amount of membrane present (see also Lohka and Masui, 1984). Above this membrane concentration, nuclear vesicles appear to be in excess and some other component may limit further growth. It is important to note that even when nucleus-forming vesicles were limiting, the extract was observed to contain a large population of free membrane vesicles, indicating that only a subpopulation of vesicles is capable of participating in nucleus formation.

A second way to determine whether there is a limited pool of nuclear vesicles would be to hold constant the amount of membranes and nucleus-forming extract, but vary the amount of sperm chromatin. The results of such an experiment are shown in Fig. 7. With low numbers of nuclei, nuclear surface area was maximal ($\sim 355 \mu\text{m}^2$). However, as the number of sperm nuclei increased from 1,000 to 13,000/ μ l of extract, the final size of the envelope surrounding each

Figure 3. Trypsin treatment of vesicles abolishes vesicle binding to chromatin. Electron micrographs of sperm chromatin incubated for 2 h in nucleus assembly extract containing (A) mitotic membranes pretreated with trypsin (20 μ g/ml) and washed before use or (B) mitotic membranes pretreated with trypsin (20 μ g/ml) in the presence of protease inhibitors, then washed before use. (A) Trypsin treatment prevents vesicle attachment to chromatin (*chr*); note the rough chromatin edges, lack of bound vesicles or membranes, and lack of pores. (B) Sections of three sperm nuclei (*N*) formed using vesicles pretreated with trypsin plus protease inhibitors. Both inner (*im*) and outer (*om*) nuclear membranes are visible, and numerous pore complexes can be seen in cross section (arrowheads). Bar, 500 nm.

LOW

HIGH

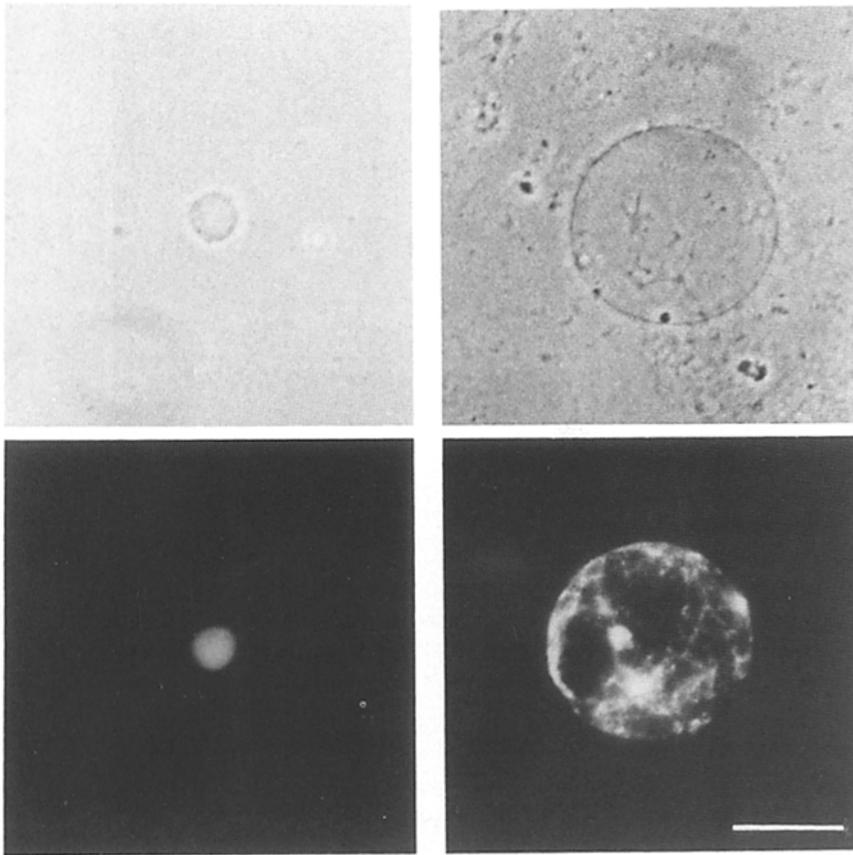


Figure 5. Final nuclear envelope size is regulated by membrane concentration. Sperm chromatin (2,000/ μ l extract) was incubated in nuclear-assembly extracts containing low or high concentrations of interphase membranes. *LOW*, 0.1-egg equivalent concentration of membranes; *HIGH*, 1.0-egg equivalent concentration of membranes. 1 egg equivalent is the membrane concentration of the crude nucleus assembly extract before fractionation, assuming 100% recovery after fractionation at 200,000 g. (Top) Nuclear envelopes as visualized by phase-contrast microscopy. (Bottom) DNA fluorescence of the same nuclei as visualized by bisbenzamide. Bar, 5 μ m.

individual nucleus decreased. We found that the total surface area of nuclear envelopes formed was constant for four different concentrations of nuclei: the number of sperm nuclei multiplied by their average surface area yielded the same value ($4.7 \times 10^5 \mu\text{m}^2 \pm 15\%$). This constant value represents the total nuclear envelope surface area that the membranes in this experiment could assemble. Therefore, different numbers of nuclei were competing to bind a limited pool of vesicles active in nuclear envelope formation.

Most Vesicles with ER Markers Do Not Contribute to Nuclear Envelope Size

The above experiments provided a way to deplete nucleus-forming vesicles from an extract through the affinity of these vesicles for chromatin. After nucleus-forming vesicles were quantitatively incorporated into nuclear envelopes, we could use differential centrifugation to separate the nuclei from vesicles that lacked envelope-forming activity. Thus we could determine whether the nuclear envelope was composed of bulk ER membrane or formed by a unique class of vesicles. For this, saturating amounts of sperm chromatin (5,000/ μ l extract) were incubated with nuclear-assembly extract and a limiting amount of membranes (\sim 1 egg equivalent membrane concentration). After nucleus assembly occurred, the nuclei were pelleted by a brief centrifugation (see Materials and Methods). The resulting supernatant and nuclear pellet were then assayed for the markers BiP and α -glu-

cosidase II. As shown in Fig. 8, 80% of the BiP and 80% of the α -glucosidase activity remained in the supernatant at a time when the nuclear envelopes had reached a stable size, whereas only 20% of these markers pelleted with the nuclei. Assuming that these markers are equally distributed (per membrane mass) between ER-derived vesicles and nuclear-derived vesicles, this result indicates that the majority of ER vesicles do not incorporate into the nuclear envelope.

We determined that the nuclei had indeed depleted the extract of nuclear vesicles: when additional membranes were added to an extract containing nuclei that had remained constant in size for 2 h, the nuclei grew larger, approximately

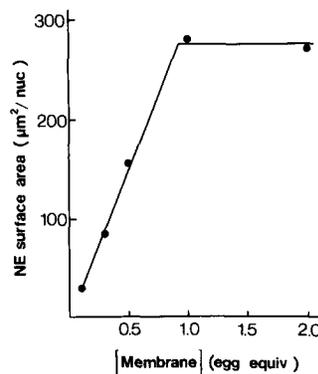


Figure 6. Final nuclear envelope size as a function of membrane concentration. Sperm chromatin (2,000/ μ l extract) was incubated at 22–24°C in nuclear assembly extracts containing different concentrations of interphase membranes. Nuclear growth was monitored by light microscopy; nuclei were photographed after growth ended (3–4 h) to calculate nuclear envelope surface area (see legend to Fig. 1).

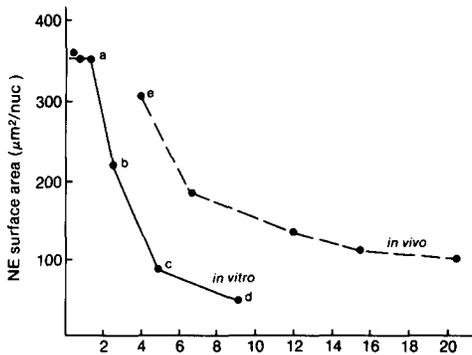


Figure 7. Final nuclear envelope size as a function of nucleus concentration. 1,000–13,000 sperm chromatin/ μl extract were incubated for 3–4 h in nuclear assembly extract containing a 1-egg equivalent concentration of interphase membranes. When nuclei ceased growth, envelope size was calculated from photographic negatives and plotted. The solid line shows that as nuclear concentration increased, the average surface area of each nucleus decreased (abscissa: sperm $\times 10^{-3}$ per embryo equivalent). For samples marked *a*, *b*, *c*, and *d*, the total number of sperm was multiplied by the average nuclear envelope surface area (μm^2) to obtain the total nuclear surface area formed in that reaction. When twice as much membrane was added initially, nuclear envelope size began to decrease after $\sim 4,000$ sperm/ μl (rather than 2,000), demonstrating that a limitation for membranes caused the observed decrease in envelope size. The experiments reported in Figs. 1, 6, and 7 used frozen aliquots of a single nuclear assembly extract and interphase membrane preparation. The “intrinsic maximum” size obtained by nuclei in these experiments ranged from ~ 300 to $400 \mu\text{m}^2$. The plotted values for sperm number were obtained by multiplying the number of sperm per microliter of extract by 0.7 to give the number of sperm per embryo equivalent of extract (Newport and Spann, 1987). The dotted line is a graph of the average nuclear envelope surface area in embryos at different stages of development (abscissa: nuclei $\times 10^{-3}$ per embryo; see Discussion). Total surface area ($\mu\text{m}^2 \times 10^{-5}$): *a*, 4.5; *b*, 5.4; *c*, 4.3; *d*, 4.6; *e*, 12.1.

doubling in surface area within 1 h (Fig. 9). Control nuclei, supplemented with buffer instead of membranes, did not change in size. This result demonstrates that the nuclei were still capable of further envelope growth, which the available ER vesicles had been unable to satisfy.

We showed in Fig. 1 that nucleus-forming vesicles were functional after a 4-h incubation in the extracts, ruling out the possibility that ER-derived vesicles are selectively inactivated and prevented from contributing to nuclear size. To ascertain that the ER markers in the supernatant were still vesicle-associated and had not leaked out of vesicles, the following fractionation was performed. The postnuclear supernatant was diluted twofold with MWB, remaining vesicles were pelleted at 26,600 g, and the vesicle pellet and new supernatant were assayed for BiP protein. BiP, a soluble protein of the ER lumen, would be released from damaged vesicles. We found that virtually all of the BiP protein remaining in the postnuclear supernatant was pelleted with the vesicles in the second centrifugation. Thus the ER vesicles which did not contribute to nuclear envelope growth were intact.

Our results demonstrated that 80% of the ER-derived vesicles were intact but unable to contribute to nuclear envelope formation. One possible explanation for these findings is that the nuclear membrane is normally derived from bulk ER but that during isolation of these membranes we inactivated

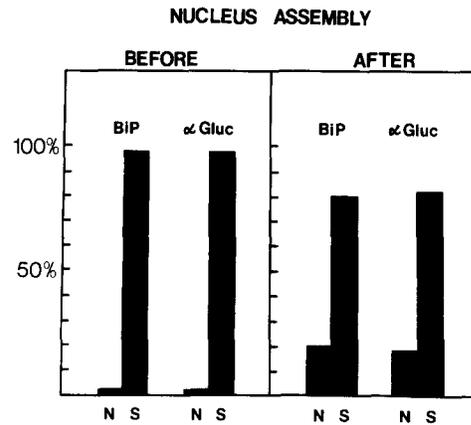


Figure 8. Nuclear vesicle depletion: the majority of BiP protein and α -glucosidase II activity do not pellet with nuclear envelopes. Chromatin (5,000 sperm/ μl extract) was added to nuclear assembly extracts containing membranes (a 1-egg equivalent concentration) and either pelleted immediately (before nucleus assembly occurred) or 2–3.5 h later (after nucleus assembly occurred). The pelleting conditions used did not pellet free vesicles. Chromatin/nuclear pellets (*N*) and postnuclear supernatants (*S*) were assayed for each marker: α -glucosidase II activity was measured by hydrolysis of PNPG, and BiP protein was quantitated from immunoblots (see Materials and Methods). Amounts are given as a percentage of the total (*N* + *S*) for each tube and each time point. The amount of each marker that pelleted in controls lacking chromatin was 2–6% of the BiP, and 5–11% of the α -glucosidase activity; these background amounts were subtracted from the nuclear pellets. We graphed the average value of three samples. Individual values for the amount of BiP or α -glucosidase pelleted after nucleus formation rarely exceeded 20% of the total and were often as low as 10%.

(in 80% of the membranes) a component required for envelope growth. An alternative explanation is that the nuclear envelope is composed of a unique subpopulation of vesicles distinct from the bulk ER membranes. If the second possibility were correct we would expect that, as fertilized eggs divide and form embryonic nuclei, the pool of nuclear-specific vesicles would be depleted from the cytoplasm through incorporation into embryonic nuclei. Therefore, the depletion of nuclear-specific vesicles we observed *in vitro* should also occur *in vivo* as cell division proceeds. To test this prediction, eggs were fertilized and allowed to develop to the 20,000

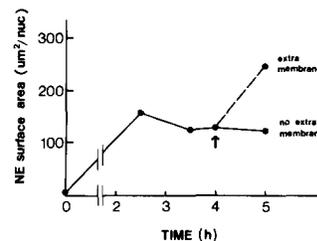


Figure 9. Nuclear envelope surface area measured in the nuclear vesicle depletion experiment. The surface area of the nuclei formed in the experiment of Fig. 8 is plotted vs. time in the extract. The nuclei grew to an average surface area of $125 \mu\text{m}^2$ and then remained stable in size for hours in the absence of additional membranes (*no extra membrane*). To determine that nuclei were capable of further growth, extra membranes (equal to the amount originally present) were added to a 4-h-old reaction (arrow). The new membranes caused a doubling of nuclear envelope size within one hour (*extra membrane*).

cell stage (12 h, gastrulation; see Kirschner et al., 1985). Embryos were then lysed, and the nuclei removed by low-speed centrifugation. The extract was then centrifuged at 200,000 *g* to obtain a total membrane fraction. As with a typical membrane preparation from eggs, the washed embryonic membranes were over fivefold enriched in α -glucosidase activity relative to the crude extract (per mg protein; data not shown). The washed embryonic membranes were then assayed *in vitro* for nuclear envelope formation around sperm chromatin. In the presence of embryonic membranes, ~30% of the chromatin units acquired small patches of membrane but none were enclosed by membrane (not shown). The chromatin remained in the elongated shape typical of sperm chromatin that has undergone limited decondensation but has not formed a nuclear envelope (Lohka and Masui, 1984). Mixing experiments showed that the embryonic membranes did not inhibit the assembly activity of control (egg-derived) vesicles. The amounts of embryonic membrane used in these experiments (0.8–1.6 μ g membrane protein/ μ l extract) equaled or exceeded that of egg-derived membranes that supported robust nuclear envelope growth. These results show that bulk ER-containing membranes, which were isolated from embryos under conditions identical to those used to obtain membranes from eggs, are unable to form nuclear envelopes. Therefore, the nuclear envelope is formed from a nucleus-specific set of vesicles, and our *in vitro* observation that 80% of the presumptive ER membranes remained unassociated with nuclear envelopes cannot be attributed to inactivation of the ER membranes during isolation.

Discussion

We have shown that a functionally distinct population of membrane vesicles is involved in nuclear envelope assembly. The final size attained by nuclear envelopes *in vitro* can be modulated by limiting the pool of these envelope-specific vesicles. In vesicle fractionation experiments, we found that vesicles active for nuclear envelope formation cofractionate at light sucrose densities with BiP and α -glucosidase II, two markers characteristic of the ER and the outer nuclear membrane. In experiments that removed all available nuclear envelope-forming vesicles from the extracts by their affinity for an assembling nucleus, only 20% of each marker was incorporated into nuclear envelopes. We deduce that the remaining 80% of these markers, which is unassociated with the nuclear envelope, represents ER vesicles. We conclude that bulk ER vesicles are not the major source of membrane material involved in nuclear envelope assembly at the end of mitosis. Instead, the functional distinction between nuclear vesicles and the majority of the ER vesicles suggests that nuclear vesicles possess an activity or factor responsible for nuclear envelope formation that is lacking in other vesicle types. In support of this, we have shown that proteolysis of the vesicles destroys their ability to form a nuclear envelope; indeed, EM studies of proteased samples reveal a complete lack of vesicle binding to chromatin. Furthermore we observe that the protease-sensitive component(s) required for envelope assembly is resistant to treatments that disrupt protein-protein interactions and weak protein-membrane interactions. These results are consistent with the proposal that an integral membrane protein or protein tightly associated with the vesicle membrane is required for targeting of a

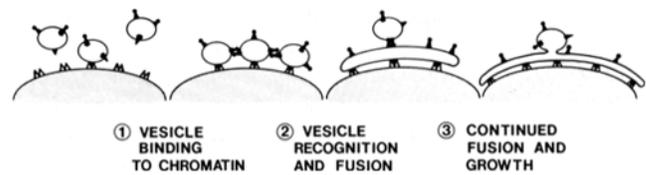


Figure 10. Model for nuclear envelope formation. The following activities are proposed to be required for envelope formation: (1) a membrane-bound receptor for chromatin (depicted as a sharp cone); (2) a chromatin-bound ligand (depicted as a double cone); and (3) a fusogenic activity that allows recognition and fusion of adjacent vesicles (depicted as a ball-tipped cone). Although the two proposed vesicle activities are drawn as separate proteins, it is possible that both activities could be performed by one bifunctional protein. The model is not meant to constitute a dependent pathway, since vesicle-vesicle recognition and fusion may occur independently of vesicle binding to chromatin (see Discussion).

specific class of vesicles to the chromosomes at the end of mitosis. We could not determine from this analysis whether protease treatment destroyed proteins required for other vesicle functions in addition to chromatin binding.

Electron micrographic studies indicate that the initial stages of nuclear envelope assembly involve at least three steps (for reviews see Lohka and Maller, 1987; Newport and Forbes, 1987): (a) binding of vesicles to chromatin, (b) flattening of the vesicle on the chromatin surface, and (c) fusion of these bound vesicles to one another. Once chromatin is fully enclosed within an envelope, further growth must occur by fusion of vesicles to the outer nuclear envelope. These events are illustrated in Fig. 10. From such a scheme, we predict that envelope formation involves the following molecular activities: (a) a membrane-bound receptor that mediates the interaction between vesicles and chromatin, (b) a chromatin-bound ligand that is recognized by the membrane receptor, and (c) a fusogenic activity that allows adjacent compatible vesicles to fuse. Once the chromatin is fully enclosed within an envelope, further growth would presumably involve the fusogen and the compatibility component, which would allow additional nuclear vesicles to recognize and fuse with the outer nuclear membrane.

What is the nature of the putative membrane-bound receptor for chromatin? The protease-sensitive membrane protein that we have shown is essential for vesicle binding to chromatin is not a known lamin protein, since the only known lamin in *Xenopus* eggs, lamin L_{III}, does not associate with membranes at mitosis (Stick and Hausen, 1985; Benavente et al., 1985). Furthermore, depletion of lamin L_{III} from the nucleus-forming extracts does not interfere with envelope formation (Newport, Wilson, and Dunphy, manuscript in preparation). Therefore, we think that the initial interaction between membrane vesicles and chromatin is not mediated by a vesicle-bound lamin but instead involves a nonlamin membrane protein. Whether lamin L_{III} participates in the assembly of the nuclear envelope or simply stabilizes the assembled structure will remain in question until the molecular identities of the membrane-bound receptor and its chromatin ligand are established.

In considering the nature of the chromatin ligand to which the membrane protein binds, it is formally possible that vesicles bind to DNA sites within the chromatin. However, from

nuclear assembly experiments described here and elsewhere we think it is more likely that the vesicle receptor interacts with a chromatin protein. In support of this, naked phage DNA incubated in nucleus assembly extracts does not associate with membranes until after assuming a highly ordered chromatin structure (Forbes et al., 1983; Newport, 1987). This chromatin ligand may be an integral component of the metaphase chromosome, since metaphase chromosomes incubated in nucleus assembly extracts immediately acquire a membrane (Burke and Gerace, 1986; Newport, 1987). The highly condensed sperm chromatin used in our experiments may already contain vesicle-binding sites or may acquire the necessary factor(s) from the soluble extract.

After binding of the vesicles to the chromatin, fusion between vesicles must occur. Precedent for a vesicle-associated fusogenic activity comes from studies of enveloped viruses, in which proteins such as hemagglutinin have been shown to contain a domain for recognizing the plasma membrane and an activity that induces membrane fusion (Doms et al., 1985; White et al., 1986). Alternatively, a soluble cytoplasmic fusogen, synexin, is proposed to stimulate fusion in neural cells (Pollard et al., 1987). At present we cannot distinguish whether the fusogen required for nuclear envelope formation *in vitro* is bound to vesicles or located in the soluble fraction of the nuclear assembly extracts.

Once the chromatin becomes completely enclosed by a double nuclear membrane, further envelope growth appears to occur by vesicle-vesicle recognition and fusion (see Lohka and Maller, 1987). The existence of annulate lamellae within the cytoplasm of many cells argues that nuclear envelope-like structures may form independently of chromatin. Annulate lamellae, which consist of stacks of double-membraned structures containing numerous pore complexes, have been observed in amphibian oocytes, *Drosophila* embryos, certain tissue culture cell lines, and in transformed cells (Franke, 1974; Maul, 1977; Kessel, 1983). There is no lamina structure associated with the annulate lamellae, nor is there any DNA. Although the function of annulate lamellae is unknown, their formation in cells that contain an excess of nuclear assembly components (Stafstrom and Staehelin, 1984; Kessel, 1983) and in our nucleus-assembly extracts (Newport, unpublished observations) suggests that nuclear vesicles may be able to fuse and assemble pore complexes independently of any interaction with either chromatin or the nuclear lamins.

Our observations indicate that the size of the nuclear envelope *in vivo* can, in principle, be regulated by limiting the number of nucleus-specific targeting proteins. We have shown (Fig. 7) that when the number of nuclei exceeds a critical concentration ($\sim 3,000$ nuclei/ μl extract or $\sim 2,100$ nuclei/egg equivalent of extract) these nuclei compete with each other for the limited pool of nucleus-specific vesicles present; i.e., those vesicles that carry a membrane-bound receptor that targets them to the nucleus. As a result of this competition, the final size of the average nuclear envelope becomes smaller as the number of nuclei increases. A similar competition may occur *in vivo* as cell division increases the number of nuclei within each embryo. When the size of nuclear envelopes at different stages of development is measured, one finds that envelope size is constant until each embryo reaches the 4,000-cell stage. After the 4,000-cell stage, nuclear envelope size decreases with each increase in cell number (Ger-

hart, 1980; Newport, unpublished observations). Thus, when envelope size is graphed as a function of the number of nuclei in each embryo, a curve very similar to that of *in vitro* nuclear size is produced (see Fig. 7; Gerhart, 1980). The quantitative similarity of the *in vivo* and *in vitro* systems argues that envelope size in both cases can be limited by the same mechanism; i.e., a limited pool of vesicles carrying the appropriate targeting signal. Whether changes in nuclear envelope size can affect nuclear functions such as DNA replication is currently under investigation.

Our evidence suggests that most ER vesicles do not contribute to nuclear envelope growth. To confirm our *in vitro* results, we isolated ER-containing membranes from embryos under the same conditions used to isolate membranes from eggs; in this case, embryonic nucleus formation during development was exploited as an *in vivo* method of depleting the cytoplasm of nucleus-specific membranes. We demonstrated that embryonic membranes, prepared after the majority of nuclear-specific membranes were incorporated into embryonic nuclei, were enriched in ER but did not support nuclear envelope growth. These *in vivo* results thus verified our *in vitro* depletion result that the majority of ER vesicles do not form nuclear envelopes. In addition, we have quantitated our recovery of vesicles active in nuclear envelope assembly. By conservative estimates, we recover a significant proportion of the egg's nuclear envelope-forming membrane in the active state. By measuring the average nuclear size and number of nuclei at different stages of development, it has been calculated that the total nuclear envelope surface area per embryo at the midblastula transition (4,000 cells) is $12.1 \times 10^5 \mu\text{m}^2$ (Gerhart, 1980; Newport, unpublished observations; see Fig. 7). If we assume 100% recovery of membranes during our fractionation procedures, and assemble a known number of nuclei in an extract reconstituted with membranes to a 1-egg equivalent concentration, then the average total surface area that assembles *in vitro* is $4.7 \times 10^5 \mu\text{m}^2$, or $>38\%$ of that in an embryo. (Our actual recovery of membranes is probably $<100\%$.) A minimum recovery of 38% of the nuclear envelope-forming capacity of an embryo is inconsistent with the hypothesis that 80% of the ER vesicles could have formed nuclear envelopes but were inactivated. The proposed distinction between nuclear- and ER-derived vesicles may be surprising in view of the biochemical similarity between the outer membrane and ER (Franke, 1974; Puddington et al., 1985; Pathak et al., 1986). However, despite the presence of shared proteins, Richardson and Maddy (1980) have demonstrated significant differences in the polypeptide compositions of the nuclear envelope and ER, leading them to conclude, as we have, that the nuclear envelope is a specialized membrane system functionally distinct from the ER. How the cell maintains the separation between nuclear-specific membrane proteins and ER membrane proteins is an intriguing question.

In conclusion, our experiments have shown that the targeting of functionally distinct vesicles to the reforming nucleus can be achieved using *Xenopus* egg extracts. We can now use this *in vitro* system for the biochemical identification of molecules such as the proposed protease-sensitive receptor that targets vesicles to nuclei. The identification of proteins required for nuclear envelope formation should provide insight into the general problem of vesicle recognition and sorting within cells.

We thank Todd Price for the electron microscopic studies and David Bole for kindly providing antisera to BiP. We are grateful to Douglass Forbes, Bill Harris, Rob Jensen, Bill Dunphy, Don Newmeyer, Tim Spann, Deborah Finlay, Roy Parker, and our other colleagues for helpful discussion and editing of this manuscript.

This work was supported by National Institutes of Health grant GM 33523-04 to J. Newport. J. Newport is the recipient of a Searle Fellowship. K. L. Wilson is a Damon Runyon-Walter Winchell Postdoctoral Fellow.

Received for publication 28 December 1987, and in revised form 21 March 1988.

References

- Benavente, R., G. Krohne, and W. W. Franke. 1985. Cell type-specific expression of nuclear lamina proteins during development of *Xenopus laevis*. *Cell* 41:177-190.
- Bole, D. G., L. M. Hendershot, and J. F. Kearney. 1986. Posttranslational association of immunoglobulin heavy chain binding protein with nascent heavy chains in nonsecreting and secreting hybridomas. *J. Cell Biol.* 102:1558-1566.
- Blow, J. J., and R. A. Laskey. 1986. Initiation of DNA replication in nuclei and naked DNA by a cell-free extract of *Xenopus* eggs. *Cell* 47:577-587.
- Blow, J. J., and J. V. Watson. 1987. Nuclei act as independent and integrated units of replication in a *Xenopus* cell free DNA replication system. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:1997-2002.
- Brands, R., M. D. Snider, Y. Hino, S. S. Park, H. V. Gelboin, and J. E. Rothman. 1985. Retention of membrane proteins by the endoplasmic reticulum. *J. Cell Biol.* 101:1724-1732.
- Burke, B., and L. Gerace. 1986. A cell free system to study reassembly of the nuclear envelope at the end of mitosis. *Cell* 44:639-652.
- Burns, D. M., and O. Touster. 1982. Purification and characterization of glucosidase II, an endoplasmic reticulum hydrolase involved in glycoprotein biosynthesis. *J. Biol. Chem.* 257:9991-10000.
- Byers, T. J., and P. B. Armstrong. 1986. Membrane protein redistribution during *Xenopus* first cleavage. *J. Cell Biol.* 102:2176-2184.
- Colman, A., E. A. Jones, and J. Heasman. 1985. Meiotic maturation in *Xenopus* oocytes: a link between the cessation of protein secretion and the polarized disappearance of Golgi apparatus. *J. Cell Biol.* 101:313-318.
- Doms, R. W., A. Helenius, and J. White. 1985. Membrane fusion activity of the influenza virus hemagglutinin. *J. Biol. Chem.* 260:2973-2981.
- Dunphy, W. G., and J. W. Newport. 1988. Mitosis-inducing factors are present in a latent form during interphase in the *Xenopus* embryo. *J. Cell Biol.* 106:2047-2056.
- Forbes, D. J., M. W. Kirschner, and J. W. Newport. 1983. Spontaneous formation of nucleus-like structures around bacteriophage DNA microinjected into *Xenopus* eggs. *Cell* 34:13-23.
- Franke, W. 1974. Structure, biochemistry, and functions of the nuclear envelope. *Int. Rev. Cytol.* 4(Suppl.):71-236.
- Franke, W. 1987. Nuclear lamins and cytoplasmic intermediate filament proteins: a growing multigene family. *Cell* 48:3-4.
- Franke, W. W., U. Scheer, G. Krohne, and E.-D. Jarasch. 1981. The nuclear envelope and the architecture of the nuclear periphery. *J. Cell Biol.* 91 (Suppl.):39s-50s.
- Gerace, L. 1986. Nuclear lamina and organization of nuclear architecture. *Trends Biochem. Sci.* 11:443-446.
- Gerace, L., and G. Blobel. 1980. The nuclear envelope lamina is reversibly depolymerized during mitosis. *Cell* 19:277-287.
- Gerace, L., C. Comeau, and M. Benson. 1984. Organization and modulation of nuclear lamina structure. *J. Cell Sci.* 1(Suppl.):137-160.
- Gerhart, J. C. 1980. Mechanisms regulating pattern formation in the amphibian egg and early embryo. In *Biological Regulation and Development*. Vol. 2. R. F. Goldberger, editor. Plenum Publishing Corp., New York. 113-315.
- Graham, C. F., and R. W. Morgan. 1966. Changes in the cell cycle during early amphibian development. *Dev. Biol.* 14:439-460.
- Hino, Y., and J. E. Rothman. 1985. Glucosidase II, a glycoprotein of the endoplasmic reticulum membrane. Proteolytic cleavage into enzymatically active fragments. *Biochemistry*. 24:800-805.
- Kessel, R. G. 1983. The structure and function of annulate lamellae: porous cytoplasmic and intranuclear membranes. *Int. Rev. Cytol.* 82:181-303.
- Kirschner, M., J. Newport, and J. Gerhart. 1985. The timing of early developmental events in *Xenopus*. *Trends Genet.* 1:41-47.
- Krohne, G., and R. Benavente. 1986. The nuclear lamins: a multigene family of proteins in evolution and differentiation. *Exp. Cell Res.* 162:1-10.
- Laskey, R. A., A. D. Mills, and N. R. Morris. 1977. Assembly of SV40 chromatin in a cell-free system from *Xenopus* eggs. *Cell* 10:237-243.
- Lohka, M. J., and J. L. Maller. 1985. Induction of nuclear envelope breakdown, chromosome condensation, and spindle formation in cell-free extracts. *J. Cell Biol.* 101:518-523.
- Lohka, M. J., and J. L. Maller. 1987. Regulation of nuclear formation and breakdown in cell-free extracts of amphibian eggs. In *Molecular Regulation of Nuclear Events in Mitosis and Meiosis*. R. A. Schlegel, M. S. Halleck, and P. N. Rao, editors. Academic Press Inc., New York. 67-109.
- Lohka, M. J., and Y. Masui. 1983. Formation in vitro of sperm pronuclei and mitotic chromosomes induced by amphibian ooplasmic components. *Science (Wash. DC)*. 220:719-721.
- Lohka, M. J., and Y. Masui. 1984. Roles of cytosol and cytoplasmic particles in nuclear envelope assembly and sperm pronuclear formation in cell-free preparations from amphibian eggs. *J. Cell Biol.* 98:1222-1230.
- Longo, F. J. 1976. Derivation of the membrane comprising the male pronuclear envelope in inseminated sea urchin eggs. *Dev. Biol.* 49:347-368.
- Lucocq, J. M., D. Brada, and J. Roth. 1986. Immunolocalization of the oligosaccharide trimming enzyme glucosidase II. *J. Cell Biol.* 102:2137-2146.
- Lucocq, J. M., J. G. Pryde, E. G. Berger, and G. Warren. 1987. A mitotic form of the Golgi apparatus in HeLa cells. *J. Cell Biol.* 104:865-874.
- Maul, G. G. 1977. The nuclear and cytoplasmic pore complex: structure, dynamics, distribution, and evolution. *Int. Rev. Cytol.* 6(Suppl.):75-186.
- Miake-Lye, R., and M. W. Kirschner. 1985. Induction of early mitotic events in a cell-free system. *Cell* 41:165-175.
- Munro, S., and H. R. B. Pelham. 1987. A C-terminal signal prevents secretion of luminal ER proteins. *Cell* 48:899-907.
- Newmeyer, D. D., and D. J. Forbes. 1988. Nuclear import can be separated into distinct steps in vitro: nuclear pore binding and translocation. *Cell* 52:641-653.
- Newmeyer, D. D., D. R. Finlay, and D. J. Forbes. 1986a. In vitro transport of a fluorescent nuclear protein and exclusion of non-nuclear proteins. *J. Cell Biol.* 103:2091-2102.
- Newmeyer, D. D., J. M. Lucocq, T. R. Bürglin, and E. M. De Robertis. 1986b. Assembly in vitro of nuclei active in nuclear protein transport: ATP is required for nucleoplasmic accumulation. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:501-510.
- Newport, J. 1987. Nuclear reconstitution in vitro: stages of assembly around protein-free DNA. *Cell* 48:205-217.
- Newport, J., and D. J. Forbes. 1985. Fate of DNA injected into *Xenopus* eggs and in egg extracts: assembly into nuclei. In *Banbury Report 20: Genetic Manipulation of the Early Mammalian Embryo*. F. Constantini and R. Jaenisch, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. 243-250.
- Newport, J., and D. J. Forbes. 1987. The nucleus: structure, function, and dynamics. *Annu. Rev. Biochem.* 56:535-565.
- Newport, J., and M. Kirschner. 1982. A major developmental transition in early *Xenopus* embryos. I. Characterization and timing of cellular changes at the midblastula stage. *Cell* 30:675-686.
- Newport, J., and T. Spann. 1987. Disassembly of the nucleus in mitotic extracts: membrane vesicularization, lamin disassembly, and chromosome condensation are independent processes. *Cell* 48:219-230.
- Newport, J., T. Spann, J. Kanki, and D. Forbes. 1985. The role of mitotic factors in regulating the timing of the midblastula transition in *Xenopus*. *Cold Spring Harbor Symp. Quant. Biol.* 50:651-656.
- Pathak, R. K., K. L. Luskey, and R. G. W. Anderson. 1986. Biogenesis of the crystalloid endoplasmic reticulum in UT-1 cells: evidence that newly formed endoplasmic reticulum emerges from the nuclear envelope. *J. Cell Biol.* 102:2158-2168.
- Pollard, H. B., E. Rojas, and A. L. Burns. 1987. Synexin and chromaffin granule membrane fusion. *Ann. N. Y. Acad. Sci.* 493:524-541.
- Puddington, L., M. O. Lively, and D. S. Lyles. 1985. Role of the nuclear envelope in synthesis, processing, and transport of membrane glycoproteins. *J. Biol. Chem.* 260:5641-5647.
- Reymond, C. D., R. H. Gomer, M. C. Mehdy, and R. A. Firtel. 1984. Developmental regulation of a Dictyostelium gene encoding a protein homologous to mammalian *ras* protein. *Cell* 39:141-148.
- Richardson, J. C. W., and A. H. Maddy. 1980. The polypeptides of rat liver nuclear envelope. II. Comparison of rat liver nuclear membrane polypeptides with those of the rough endoplasmic reticulum. *J. Cell Sci.* 43:269-277.
- Sheehan, M. A., A. D. Mills, A. M. Sleeman, R. A. Laskey, and J. J. Blow. 1988. Steps in the assembly of replication-competent nuclei in a cell-free system from *Xenopus* eggs. *J. Cell Biol.* 106:1-12.
- Stafstrom, J. P., and L. A. Staehelin. 1984. Are annulate lamellae in the *Drosophila* embryo the result of overproduction of nuclear pore components? *J. Cell Biol.* 98:699-708.
- Stewart, C., and B. Burke. 1987. Teratocarcinoma stem cells and early mouse embryos contain only a single major lamin polypeptide closely resembling lamin B. *Cell* 51:383-392.
- Stick, R., and P. Hausen. 1985. Changes in the nuclear lamina composition during early development of *Xenopus laevis*. *Cell* 41:191-200.
- Warren, G. 1985. Membrane traffic and organelle division. *Trends Biochem. Sci.* 10:439-443.
- White, J., R. Doms, M.-J. Gething, M. Kielian, and A. Helenius. 1986. Viral Membrane Fusion Proteins. In *Virus Attachment and Entry into Cells*. R. L. Crowell and K. Lomberg-Holm, editors. American Society for Microbiology, Washington DC. 54-59.
- Wolin, S. L., G. Krohne, and M. W. Kirschner. 1987. A new lamin in *Xenopus* somatic tissues displays strong homology to human lamin A. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:3809-3818.
- Woodland, H. R., and E. D. Adamson. 1977. The synthesis and storage of histones during the oogenesis of *Xenopus* oocytes. *Dev. Biol.* 57:118-135.