Eukaryotic cells contain a large number of distinct compartments delimited by membranes. Cytoplasmic vesicular transport contributes to modification and turnover of these membranous boundaries. Such transport processes must involve specific recognition elements for vesicle targeting and binding, and mechanisms to regulate initiation and timing of membrane fusion events.

The cell nucleus is delimited by a nuclear envelope (NE), consisting of an inner and outer membrane, periodically interrupted by nuclear pores, and an underlying lamina (reviewed by Dingwall and Laskey, 1992; Wiese and Wilson, 1993). The outer membrane often appears continuous with the endoplasmic reticulum (ER). The NE undergoes cycles of breakdown and reassembly at each cell division, processes involving vesicularization and membrane vesicle (MV) fusion, respectively.

Nuclear membrane vesicularization and fusion events also occur shortly after fertilization in the oocyte or egg cytoplasm. In the sea urchin, eggs are fertilized in G1 after completion of both meiotic divisions, and thus already contain a fully formed female pronucleus (Longo and Anderson, 1968; Longo, 1976). The sperm NE vesiculates rapidly and a new pronuclear envelope reforms as the sperm chromatin decondenses (Longo and Anderson, 1968; Longo, 1976). The male pronuclear envelope is derived from pre-existent egg ER and to a limited extent from de novo membrane synthesis (Longo, 1976, 1991). In addition, polar remnants of the sperm NE located at the base (centriolar fossa region) and tip (acrosomal fossa region) of the nucleus resist disassembly at fertilization, are incorporated into the pronuclear envelope (Longo and Anderson, 1968). The male pronuclear envelope apparently assembles by fusion of NE precursor vesicles along the chromatin surface (Longo, 1976). In vitro, lipophilic detergent-resistant structures at the poles of the sperm nucleus target membrane vesicle binding to the chromatin and fuse with these vesicles, suggesting an organizing role for the lipophilic structures (LSs) (Collas and Poccia, 1995). Taken together, these observations indicate that in the sea urchin, both gametes contribute to the male pronuclear envelope.

Pronuclear envelope assembly has been achieved in cell-free systems derived from eggs of *Xenopus* (Lohka and Masui, 1984; Wilson and Newport, 1988; Newport and Dunphy, 1992), *Drosophila* (Ulitzur and Gruenbaum, 1989; Berrios and Avilion, 1990), surf clams (Longo et al., 1994) and sea urchins (Cameron and Poccia, 1994; Collas and Poccia, 1995). Various sets of membrane vesicles contributing to the NE have been identified. They include vesicles containing integral membrane proteins of the inner nuclear membrane (lamin B receptor;
MATERIALS AND METHODS

Reagents and buffers
Unless indicated otherwise, all reagents were from Sigma (St Louis, MO). The lipophilic dye octadecyl indocarbocyanine (DiIC<sub>18</sub>) was from Molecular Probes (Eugene, OR). Nuclear buffer Tris-NaCl (TN) and membrane wash buffer (MWB) were as described (Collas and Poccia, 1995).

Gametes
Gametes of *Lytechinus pictus* (Marinus, Inc., Long Beach, CA) were used and fertilization performed according to the method of Poccia and Green (1986).

Sperm nuclei
Permeabilized sperm nuclei were prepared as described (Collas and Poccia, 1995). Sperm heads were isolated after sonication of sperm and centrifugation at 1,500 g. Membranes were solubilized in 0.1% TX-100 on ice for 10 minutes, the nuclei spun as above and the pellet resuspended in TN. The suspension (10<sup>6</sup> nuclei/ml) was diluted 25-fold before being added to egg extracts at a ratio of ca. 1 nucleus/egg equivalent.

Egg extracts
Sea urchin egg extracts were prepared as previously reported (Cameron and Poccia 1994; Collas and Poccia, 1995). Briefly, fertilized eggs were washed in sea water and egg lysis buffer, resuspended in an equal volume of lysis buffer and homogenized. The lysate was centrifuged at 10,000 g for 10 minutes at 4°C. The crude cytoplasmic extract (S10) was withdrawn and further fractionated at 100,000 g for 2 hours at 4°C in a Ti50 rotor (Beckman Instruments, Carlsbad, CA). The clear cytosolic fraction (S100) was recentrifuged at 100,000 g for 1 hour to pellet residual membranes and frozen at -80°C.

Membrane vesicle isolation and fractionation
All solutions contained 1 mM of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF). The membrane fraction pellets at 100,000 g was washed twice by resuspension in MWB and centrifugation at 150,000 g for 45 minutes through 0.25 M sucrose. This fraction is referred to as MV0. MV0 was fractionated by centrifugation at 150,000 g for >20 hours to density equilibrium through a 0.2-2.0 M linear sucrose gradient. The fractions were recovered by side puncture with an 18 gauge needle, washed in 4 volumes of MWB and recentrifuged at 150,000 g through 0.25 M sucrose for 30 minutes. Each fraction was resuspended in a minimal volume of MWB and stored frozen.

Membrane vesicle concentration was determined by light absorbance at 340 nm (A<sub>340</sub>). Zero was set by measuring the A<sub>340</sub> of MWB. The A<sub>340</sub> of a reference membrane vesicle fraction (see Results) was set to 100%. Vesicle concentration in the fractions was expressed as a percentage of the reference fraction.

Fluorescent labeling of membranes
Sperm LSs were fluorescently labelled green on ice for 10-15 minutes with 10 µg/ml of the lipophilic dye dihexyloxacarbocyanine (DHCC) in TN, and nuclei washed three times in TN by centrifugation at 1,200 g (Collas and Poccia, 1995). Labelled nuclei were kept in TN in the dark until use. Concentrated vesicles (20 µl) were fluorescently labelled green or red as indicated with 10 µg/ml DHCC or DiIC<sub>18</sub>, respectively, in 100 µl MWB for 1 hour. Vesicles were washed by centrifugation through 0.25 M sucrose in TN, resuspended in and recentrifuged through MWB, and resuspended in 20 µl MWB. Because DHCC fluoresces green and DiIC<sub>18</sub> red, DHCC and DiIC<sub>18</sub>-labelled objects were referred to as green and red objects, respectively, in the text.

Treatment of vesicles
All treatments were done for 20 minutes on ice, using 10 µl of concentrated vesicles.

Trypsinization
Vesicles were resuspended in 250 µl MWB containing 20 µg/ml trypsin. Trypsin digestion was terminated by adding 100 µg/ml soybean trypsin inhibitor, 10 µg/ml aprotinin and 10 µg/ml leupeptin. After an additional 10 minutes on ice, vesicles were washed through 0.25 M sucrose and resuspended in MWB to their original volume. Control vesicles were incubated for 30 minutes in a cocktail of trypsin, trypsin inhibitor, aprotinin and leupeptin at the above concentrations.

High salt
Vesicles were incubated in 500 µl lysis buffer adjusted to 0.9 M KCl and washed as above.

N-ethyl maleimide (NEM)
Vesicles were incubated in 500 µl lysis buffer with 1 mM NEM for 20 minutes, 10 mM dithiothreitol (DTT) added, and vesicles incubated for another 10 minutes before being washed as above. As a control, vesicles were treated simultaneously with 1 mM NEM and 10 mM DTT for 30 minutes. For each treatment, controls for possible carryover effects from treated vesicles were performed. Red-labelled treated vesicles were added to a separate group of demembranated nuclei with an equal amount of untreated, green-labelled vesicles and binding of each set of vesicles to chromatin monitored.

Chromatin decondensation assay
Decondensation of TX-100-permeabilized sperm chromatin was performed for 1 hour at room temperature (RT) in S100 containing an ATP-generating system (ATP-GS: 2 mM ATP, 20 mM creatine phosphate, 50 µg/ml creatine kinase) (Cameron and Poccia, 1994). Chromatin was considered decondensed when it appeared spherical under fluorescence after staining with 0.1 µg/ml Hoechst (Cameron and Poccia, 1994).
Membrane vesicle binding and fusion assays

Green- or red-labelled vesicles were incubated with decondensed chromatin in S100 containing ATP-GS and binding to chromatin permeabilized for 40 minutes (Collas and Poccia, 1995). Vesicle binding was determined by fluorescence after centrifugation of the chromatin at 500 g for 20 minutes through 1 M sucrose to separate chromatin-bound from unbound vesicles. Emission of green- and red-labelled bound, but not fused, vesicles remained separate in the green and red channels, respectively. Fusion of red-labelled vesicles with green-labelled LSs or of red- with green-labelled vesicles, was promoted with 100 μM GTP (Collas and Poccia, 1995). Membrane fusion resulted in mixing of the two dyes and was assessed by orange fluorescence in the green channel. This is due to green fluorescence of DHCC and fluorescence energy transfer from DHCC to DiIC18 due to the proximity of the two fluorophores within the same membranes resulting in red emission (Collas and Poccia, 1995). In all experiments a minimum of 200 nuclei per treatment were examined on 5-10 different slides. Experiments were replicated at least twice.

Isolation of chromatin-bound vesicles

To isolate vesicles bound to chromatin, nuclei were separated from unbound membranes by centrifugation through 1 M sucrose as described and resuspended carefully in 100 μl MWB containing 0.1% TX-100. Membranes were solubilized for 15 minutes on ice and chromatin briefly pelleted at 2,000 g. Extracted vesicles were used for enzyme assays as described.

Measurement of α-glucosidase and α-D-mannosidase II activities

Levels of the ER marker α-glucosidase were measured according to the method of Burns and Touster (1982). Samples were diluted to 250 μl in MWB and 10 μl removed for determination of protein concentration. The remainder was diluted 1:1 with 100 mM Hepes, pH 6.8, 2% sodium cholate and 8 mM p-nitrophenyl-α-D-glucopyranoside, and incubated at RT for 1 hour. Then 1 ml of 1% ethylenediamine was added to quench α-glucosidase activity and Aαs measured. Enzyme activity was standardized using purified α-glucosidase from yeast and p-nitrophenol. One unit of α-glucosidase corresponds to the amount of enzyme required to release 1 µmol p-nitrophenol per hour. Protein concentration was determined with a bicinchoninic acid protein assay kit (Pierce, Rockford, IL) using bovine serum albumin as a standard.

Activity of α-D-mannosidase II, a membrane protein residing in the medial Golgi, was assayed by measuring hydrolysis of p-nitrophenyl-α-D-mannopyranoside as described previously (Tulsiani et al., 1977) except that 6 mM substrate was used. On ice, membrane samples were diluted to 50 μl in MWB and added to 100 μl of a cocktail consisting of 0.1 M sodium acetate buffer, pH 5.5, 1% TX-100, 1.5 mM EDTA and 6 mM p-nitrophenyl-α-D-mannopyranoside. Samples were incubated at 37°C for 3 hours and the reaction stopped by the addition of 100 μl of an alkaliine buffer (130 mM glycine, 67 mM NaCl and 83 mM Na2CO3, pH 10.7). The relative amounts of released p-nitrophenol were determined by measuring A405, using p-nitrophenol as a standard. One unit of α-D-mannosidase II corresponds to the amount of enzyme required to release 1 µmol of p-nitrophenol per hour (Tulsiani et al., 1977).

Microscopy and photography

Fluorescence observations were made under a Zeiss epifluorescence microscope using a Zeiss Neofluar ×100, 1.3 mm NA objective. Black and white images in the blue (Hoechst), green (fluorescein isothiocyanate, FITC) and red (rhodamine) channels were captured with a Hamamatsu Photonics C2400 SITE camera and Argus-10 Image Analyzer using a software from Digital Vision, Inc. (Dedham, MA). Images were processed and colored using the Aldus Photostyler software (Aldus Co., Seattle, WA) and photographs printed on a Tektronix Phaser IISDX dye sublimation printer (Tektronix, Inc., Wilsonville, OR).

RESULTS

Five membrane vesicle fractions of different densities derive from egg cytoplasmic lysates

Membrane vesicles were prepared from a 10,000 g supernatant of a fertilized sea urchin egg homogenate by centrifugation at 100,000 g. The membrane pellet, designated MV0, was resuspended and further fractionated at 150,000 g through a 0.2-2 M sucrose linear gradient. Four fractions formed, designated MV1, MV2, MV3 and MV4 of respective densities (ρ) 1.02, 1.04-1.08, 1.13 and 1.18. The fractions were collected by side puncture, washed as described and resuspended. The most abundant fraction, MV2, was further fractionated in a 0.5-0.8 M sucrose step gradient into two populations, MV2α (ρ=1.04) and MV2β (ρ=1.07). The proportions (mean from three separate preparations) of each fraction relative to total egg cytoplasmic membranes (MV0) were determined by A340 (Table 1).

Chromatin binding patterns of membrane vesicle fractions differ

Since initial binding of total membrane vesicles to chromatin occurs at the poles and spreads equatorially prior to GTP-triggered fusion (Collas and Poccia, 1985), we determined which individual vesicle fractions were capable of binding to chromatin and whether binding occurred in preferred regions. Target sperm chromatin was washed with 0.1% Triton X-100 and so contained LSs at each pole, but no lateral membranes or lamins (Collas and Poccia 1995; Collas et al., 1995). Concentrations of MV1, MV2α, MV2β, MV3 and MV4 were normalized. MV2β, the most abundant fraction, was used at its initial concentration in S10, and concentrations of MV1, MV2α, MV3 and MV4 were increased before incubation with chromatin by 7-, 15-, 15- and 19-fold, respectively (see Table 1). Vesicles were fluorescently labelled red and binding to chromatin containing green fluorescently labelled LSs marking the poles was monitored after centrifugation of nuclei through sucrose. The fraction of nuclei exhibiting different vesicle binding patterns is indicated in Fig. 1 and the localization of vesicle binding on chromatin shown in Fig. 2.

MV1 bound exclusively in the LS regions (polar binding), and binding was never seen along lateral aspects of the chromatin. MV2α also bound specifically in the LS regions. In

Table 1. Proportions of membrane vesicle fractions formed after centrifugation to density equilibrium through linear sucrose gradients

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Proportion (% of MV0)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MV1</td>
<td>11±1.4</td>
</tr>
<tr>
<td>MV2α</td>
<td>5±1.0</td>
</tr>
<tr>
<td>MV2β</td>
<td>75±2.0</td>
</tr>
<tr>
<td>MV3</td>
<td>5±0.3</td>
</tr>
<tr>
<td>MV4</td>
<td>4±0.2</td>
</tr>
</tbody>
</table>

*Determined by A340. Mean ± standard deviation of 3 measurements of 3 different samples from 3 separate preparations.
Factors for vesicle binding (Collas and Poccia, 1995). (not shown), confirming the requirement for egg cytosolic thermost, none of the vesicle fractions bound to chromatin in vesicle binding to chromatin (Collas and Poccia, 1995). Further, detergent-extractable chromatin components are required for previous results that LSs (as well as possible additional binding of all vesicle fractions (not shown), supporting our observations (Collas and Poccia, 1995). Consequently, removal of LSs from chromatin with 1% TX-100 prevented binding of all vesicle fractions (not shown), supporting our previous results that LSs (as well as possible additional detergent-extractable chromatin components) are required for vesicle binding to chromatin (Collas and Poccia, 1995). Furthermore, none of the vesicle fractions bound to chromatin in lysis buffer containing the ATP-GS in the absence of cytosol (not shown), confirming the requirement for egg cytosolic factors for vesicle binding (Collas and Poccia, 1995).

Not all vesicles of each fraction were capable of binding to chromatin. Each vesicle fraction was incubated with an excess of chromatin (4,000 demembranated sperm nuclei/µl S100) to ensure that not all chromatin contained bound membranes (P. Collas and D. L. Poccia, unpublished data). After 1 hour, the chromatin was spun down, remaining unbound vesicles recovered after centrifugation at 150,000 g through 0.25 M sucrose and vesicle concentrations determined by measuring A340. The concentration of the input vesicle fraction was determined after similar incubations in S100 without chromatin and centrifugation through sucrose as above. Proportions of bound vesicles were calculated as [1 - (A340 remaining vesicles/A340 input vesicles)]×100. Over 60% of MV1 and MV2α vesicles were found to bind chromatin whereas only 29% of MV2β bound chromatin (Table 2). These results indicate that the majority of MV1 and MV2α vesicles, but only a small subset of MV2β, are capable of binding to chromatin.

In summary, we have isolated by density differences five egg membrane vesicle populations with different abilities to interact with sperm chromatin. MV1 and MV2α exhibit polar binding, MV2β peripheral binding, and MV3 and MV4 do not bind to chromatin. Most but not all vesicles of MV1 and MV2α, and only one third of MV2β vesicles are capable of chromatin binding.

**Membrane vesicle fractions differ by several criteria**

The sensitivities to various treatments of MV1, MV2α and MV2β binding were evaluated. Vesicles were treated with trypsin, high salt or NEM as described in Materials and Methods and labelled red before monitoring binding to chromatin containing green LSs (Table 3). Trypsin at 20 µg/ml abolished binding of MV1, MV2α and MV2β, indicating that binding to chromatin requires proteins on all three vesicle fractions. High salt (0.9 M KCl) washes prevented binding of MV1 and MV2α, but not of MV2β vesicles, which were detected over the entire chromatin surface (not shown). This suggests that binding of MV1 and MV2α is mediated by loosely associated membrane proteins whereas MV2β binding may depend on integral membrane proteins. NEM (1 mM) treatment prevented binding of MV1, but not of MV2α and MV2β. Inhibition of binding in these experiments was not due to carry over effects of treated vesicles since green-labelled, untreated MV1, MV2α or MV2β mixed with red-labelled, treated counterparts bound to chromatin in the usual fashion (not shown). Therefore, in addition to buoyant density and target sites on chromatin, MV1, MV2α and MV2β can be distinguished by whether their binding is inhibited by high salt and NEM treatments.

To determine whether vesicles were derived from the endoplasmic reticulum (ER), the activity of α-glucosidase, an
enzyme marker for the ER, was assayed (Burns and Touster, 1982). Table 4 shows that the specific activity of α-glucosidase in total vesicle fractions is highest in MV2β, MV3 and MV4. MV1 and MV2α show background level activities. In addition, levels of α-glucosidase activity in the subset of MV1, MV2α and MV2β vesicles bound to chromatin and reextracted with 0.1% TX-100 were similar to those of the total fractions (Table 4), indicating clearly that the chromatin-binding subset of MV2β contains the ER marker. Since MV2β constitutes 75% of total egg vesicles, most of the egg ER appears concentrated in MV2β. MV1 and MV2α could represent a special subset of ER vesicles lacking the enzyme, or could be vesicles derived from a different organelle.

The latter possibility was examined by assessing the activity of α-D-mannosidase II, a membrane protein of the Golgi, using p-nitrophenyl α-D-mannopyranoside as a substrate (Tulsiani et al., 1977, 1982). As shown in Table 5, specific mannosidase II activities in MV2α and MV2β were 13 and 3.5 times greater than that of cytosol, respectively; MV1 exhibited near-background level activity, suggesting that most of the Golgi is concentrated in MV2α and perhaps some in MV2β. When the chromatin-binding subset of MV2β was analyzed no substantial enzyme activity was found. In contrast, high specific mannosidase II activity was detected in the chromatin-binding fraction of MV2α (Table 5). Therefore to our surprise, it appears from these results that vesicles derived from the medial Golgi, and concentrated in MV2α, participate in NE formation. The chromatin-binding fraction of MV1 may originate from non-medial Golgi vesicles or non-Golgi-derived vesicles.

**Only MV1 fuses directly with LSs**

Formation of the sea urchin male pronuclear envelope in vitro involves fusion of vesicles with the LSs (Collas and Poccia, 1995). We therefore examined whether MV1, MV2α and MV2β would fuse with the LSs. Red-labelled vesicles were allowed to bind to chromatin containing green LSs, and membrane fusion induced with 100 µM GTP. LS-vesicle fusion was assessed by the mixing of the two fluorophores (DHCC and DiIC18), producing orange fluorescence in the green channel (Collas and Poccia, 1995). The results are represented in Fig. 3. MV1 bound to LSs appeared orange in the green channel (Fig. 3A), indicating fusion with the LSs. In contrast, emission of MV2α (Fig. 3B) or MV2β (Fig. 3C) and of LSs remained separate in the green and red channels, indicating the lack of fusion of these vesicles with LSs. The results indicate that MV1 appears to be fusigenic, whereas MV2α and MV2β do not. GTP did not trigger MV1-LS fusion in sucrose-washed nuclei containing bound MV1 and resuspended in egg lysis buffer (not shown), indicating a cytosolic requirement for LS-vesicle fusion (Collas and Poccia, 1995). LS-vesicle fusion results are summarized in Table 6.

**Fusion of MV2α requires MV1; fusion of MV2β requires MV2α and MV1**

The fusion of MV1, MV2α and MV2β with each other was examined in pairwise binding double-labelling experiments. First, fusion of MV1 with MV2α was examined. MV1 was labelled green and MV2α red. LSs were not labelled. After vesicle binding to chromatin, addition of GTP resulted in orange fluorescence in the polar regions indicating fusion of MV1 with MV2α (Fig. 3D). In a separate experiment, labelling LSs green and MV2α red (leaving MV1 unlabelled) revealed the fusion of MV2α with the LSs (not shown). Thus, MV1 fused with MV2α and permitted fusion of MV2α with LSs.

**Table 2. Proportion of membrane vesicles exhibiting binding to chromatin**

<table>
<thead>
<tr>
<th>Vesicle fraction</th>
<th>% Fraction bound to chromatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>MV1</td>
<td>67±0.8</td>
</tr>
<tr>
<td>MV2α</td>
<td>62±0.9</td>
</tr>
<tr>
<td>MV2β</td>
<td>29±3.0</td>
</tr>
</tbody>
</table>

*Mean ± standard deviation of 3 A340 measurements from 3 samples of each fraction in two separate experiments.

**Table 3. Partial characterization of binding properties of MV1, MV2α and MV2β to chromatin**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MV1</th>
<th>MV2α</th>
<th>MV2β</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysis buffer</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>20 µg/ml Trypsin</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>0.9 M KCl</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>1 mM NEM</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*Binding was assessed after incubation of treated vesicles with chromatin for 40-60 minutes and centrifugation of chromatin through 1 M sucrose. +, MV binding in 70-90% of nuclei; –, binding in 0-30% of nuclei examined.

**Fig. 3. Fusion of vesicles with LSs and with each other.** Vesicles were bound to chromatin and MV-LS and/or vesicle-vesicle fusion promoted with GTP. (A) Fusion of red MV1 with green LSs. Membrane fusion results in mixing of both fluorophores, producing orange fluorescence in the green channel. (B) Lack of fusion of green LSs with red MV2α. The labels remain separate. (C) Lack of fusion of green LSs with red MV2β. (D) Fusion of green MV1 with red MV2α. (E) Lack of fusion of green MV1 with red MV2β. (F) Lack of fusion of green MV1 with red MV2α in the presence of unlabelled MV2β. (G) Fusion of green MV1 with red MV2β in the presence of unlabelled MV2α. Bar, 5 µm.
In contrast, red-labelled MV2β did not fuse with green MV1 under the same conditions, the dyes remaining separate (Fig. 3E). Increasing MV1 concentration by 10-fold also resulted in no fusion (not shown), making it unlikely that lack of fusion was due to limiting amounts of MV1 vesicles. In the third combination, green-labelled MV2α did not fuse with red-labelled MV2β in the absence of MV1 (Fig. 3F).

Fusion under conditions where all three populations were bound was tested. MV1 was labelled green, MV2β red, and MV2α not labelled. Vesicles were bound to chromatin by their original proportions (11% MV1, 5% MV2α, 75% MV2β). Adding GTP produced orange fluorescence over the nuclear surface, indicating fusion of MV1 with MV2β and of MV2β vesicles with one another along the nuclear surface (Fig. 3G). In control incubations without MV2α, no fusion occurred (not shown; see Fig. 3F). To confirm the fusion of all three fractions with each other, green MV2α, red MV2β and unlabelled MV1 were bound to chromatin. GTP produced orange fluorescence over the nuclear surface indicating fusion of MV2α with MV2β and of MV2β vesicles with one another (not shown). In addition, fusion of MV1, MV2α and MV2β with LSs was demonstrated by the use of green LSs, red MV2β, and unlabelled MV1 and MV2α (not shown). In any fusion event, spreading of the orange fluorescence among all vesicles indicates that fusion occurs not only between two different vesicle fractions but also between all vesicles within these fractions. No fusion events occurred in egg lysis buffer alone; all required egg cytosol (data not shown).

The results of this series of experiments are summarized in Table 6. The data illustrate: (1) fusion of MV1 with MV2α in the LS regions; (2) lack of fusion of MV1 with MV2β in the absence of MV2α; (3) lack of fusion of MV2α with MV2β in the absence of MV1; and (4) the fusion of the three fractions with each other. They show that fusibility is associated with MV1 (which can fuse with LSs or MV2α), and that MV2α is needed to pass the fusion signal to MV2β.

**All three fractions are required for nuclear envelope formation**

The requirement of MV2α for the formation of a continuous envelope was verified by assessing exclusion of 150 kDa FITC-dextran from nuclei. Dextran exclusion has previously been used to assay the formation of a continuous envelope around chromatin in vitro (Newmeyer et al., 1986; Collas and Poccia, 1995). Unlabelled MV1 and MV2β were assembled on chromatin in the presence or absence of MV2α, and GTP was added to induce fusion as above. After 1 hour, an aliquot was stained with Hoechst and 2 mg/ml 150 kDa FITC-dextran. The dextran was excluded from nuclei only in the presence of MV2α (Fig. 4), indicating the formation of a continuous envelope only in the presence of MV1, MV2α and MV2β (Table 6).

**DISCUSSION**

Four membranous elements cooperate to form the sea urchin male pronuclear envelope in vitro: LSs (structures derived from the sperm NE remnants in vivo and resistant to detergent extraction in vitro) and three populations of vesicles derived from egg cytoplasm. The three maternal vesicle populations differ from one another in several characteristics. MV1 binds to nuclear polar regions, is NEM-sensitive, requires peripheral proteins to bind, lacks an ER marker enzyme and a medial Golgi marker enzyme, and fuses directly with LSs and MV2α. MV2α binds to polar regions, requires peripheral membrane proteins to bind, lacks an ER marker enzyme and a medial Golgi marker enzyme, and fuses directly with LSs and MV2α. MV2α binds to polar regions, requires peripheral membrane proteins to bind, lacks an ER marker enzyme and a medial Golgi marker enzyme, and fuses directly with LSs and MV2α.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (mg)*</th>
<th>Activity (units) ‡</th>
<th>Specific activity (units/mg protein)</th>
<th>Relative activity (-fold)</th>
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<tbody>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MV1</td>
<td>2.9</td>
<td>33 ± 7.5</td>
<td>11.4</td>
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</tr>
<tr>
<td>MV2α</td>
<td>3.7</td>
<td>49 ± 10.1</td>
<td>13.2</td>
<td>1.7</td>
</tr>
<tr>
<td>MV2β</td>
<td>4.2</td>
<td>64 ± 7.0</td>
<td>152.6</td>
<td>19.3</td>
</tr>
<tr>
<td>MV3</td>
<td>3.9</td>
<td>75 ± 47.8</td>
<td>193.8</td>
<td>24.5</td>
</tr>
<tr>
<td>MV4</td>
<td>3.2</td>
<td>36 ± 51.1</td>
<td>115.0</td>
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<td>Bound§</td>
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</tr>
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<td>MV1§</td>
<td>2.6</td>
<td>33 ± 6.2</td>
<td>12.7</td>
<td>1.6</td>
</tr>
<tr>
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<td>3.1</td>
<td>39 ± 3.6</td>
<td>12.6</td>
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</tr>
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<td>MV2β§</td>
<td>3.6</td>
<td>63 ± 69.2</td>
<td>176.1</td>
<td>22.3</td>
</tr>
</tbody>
</table>

*Number of mg protein in sample assayed (240 µl).

† One unit is the amount of enzyme required to release of 1 µmole of p-nitrophenol per hour. Samples and substrate were incubated for 1 hour at RT. Mean ± standard deviation of 3 measurements from 3 separate vesicle preparations.

‡ Background activity.

§ Vesicles bound to chromatin and re-extracted with 0.1% TX-100.
proteins, is NEM-insensitive, lacks the ER marker but contains the Golgi marker, and requires MV1 to fuse with LSs or MV2α. MV2β, derived from the bulk of the ER, binds to the entire nuclear surface, requires intrinsic membrane proteins to bind, is NEM insensitive, is rich in the ER, but not Golgi marker, and requires MV2α in order to fuse with LSs or MV1. All binding and fusion events require soluble factors from egg cytosol.

Membrane vesicle binding to sea urchin sperm chromatin, which we had previously described as a single process targeted by LSs (Collas and Poccia, 1995), now appears as an orchestrated series of events leading to the formation of an intact NE. Furthermore, it is now clear that LSs are not merely general adhesive sites for promiscuous binding of vesicles. MV3 and MV4 do not bind to chromatin, nor do egg plasma membranes or extracted membranes from sperm heads (Collas and Poccia, 1996). LSs alone cannot provide a general fusion signal to any bound vesicles. In the absence of MV1 no fusion is observed, suggesting that a critical part of the fusion machinery resides in MV1.

It is not known whether the three fractions MV1, MV2α and MV2β result from egg lysate homogenization or whether they also exist separately in the egg in vivo. Each fraction, however, contains vesicles with chromatin-binding activity and vesicles not capable of binding to chromatin. This finding was expected, since biochemically-distinct membranes can co-fractionate by density.

Of the set of vesicles bound to chromatin, each has unique properties. The ability of MV2β to bind entirely around the nuclear periphery suggests that it is a major contributor to the NE. MV2β is required for NE formation in vitro and is the only binding fraction with substantial α-glucosidase activity. Thus, in vitro, the bulk of NE precursor vesicles originates from the fraction with the ER marker. This is consistent with in vivo observations in the sea urchin egg, where the ER provides the major source of NE precursor vesicles (Longo, 1976, 1991). Yet, most of MV2β vesicles do not possess chromatin-binding activity, indicating that as in Xenopus (Wilson and Newport, 1988), the majority of ER-derived vesicles do not contribute to the NE. The in vivo observations of Longo (1976, 1991) do not exclude the participation of other membranes as well. It is clear that MV2β does not require MV1 or MV2α to bind to chromatin in vitro. However, MV2β vesicles will not fuse in the absence of MV1 and MV2α, thus the ER-derived fraction is not sufficient for NE assembly in vitro.

To support this view, our results indicate that Golgi-derived vesicles participate in NE formation in vitro. These vesicles appear concentrated in MV2α, since activity of α-D-mannosidase II was found almost exclusively in the chromatin-binding fraction of MV2α. The Golgi complex is usually held in the pericentriolar region by association with the microtubule organizing center (MTOC) (Rogalski and Singer, 1984; Cooper et al., 1990) suggesting that MV2α binds in the nuclear poles due to affinity for the MTOC. However, although the centriolar fossa region of demembranated sperm nuclei may still contain a centriole, the acrosomal fossa is devoid of this organelle (Schatten et al., 1986; see Longo, 1991). If MV2α binds to both centriolar and acrosomal LS regions, the specificity of MV2α for the nuclear poles therefore cannot be attributed to an affinity for the MTOC. However, it is formally possible that only a portion of MV2α enriched in the Golgi marker binds to the centriolar pole and the other pole binds non-Golgi MVs. Whether additional distinct Golgi-derived vesicles also contribute to the NE is at present not known. The development of

Table 5. α-D-Mannosidase II activity in S100 and total and chromatin-bound membrane fractions

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (mg)*</th>
<th>Activity (units)†</th>
<th>Specific activity (units/mg protein)</th>
<th>Relative activity (-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S100</td>
<td>3.0</td>
<td>3.12</td>
<td>1.04</td>
<td>1‡</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>3.36</td>
<td>2.24</td>
<td>2.1</td>
</tr>
<tr>
<td>MV1</td>
<td>1.5</td>
<td>29.46</td>
<td>13.39</td>
<td>13.1</td>
</tr>
<tr>
<td>MV2α</td>
<td>2.2</td>
<td>10.99</td>
<td>3.79</td>
<td>3.6</td>
</tr>
<tr>
<td>MV2β</td>
<td>2.9</td>
<td>4.31</td>
<td>1.23</td>
<td>1.2</td>
</tr>
<tr>
<td>MV3</td>
<td>3.5</td>
<td>4.74</td>
<td>1.58</td>
<td>1.5</td>
</tr>
<tr>
<td>MV4</td>
<td>3.0</td>
<td>3.18</td>
<td>2.27</td>
<td>2.2</td>
</tr>
<tr>
<td>Bound§</td>
<td></td>
<td>14.86</td>
<td>9.29</td>
<td>8.9</td>
</tr>
<tr>
<td>MV1§</td>
<td>1.4</td>
<td>5.44</td>
<td>2.47</td>
<td>2.4</td>
</tr>
<tr>
<td>MV2α§</td>
<td>1.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MV2β§</td>
<td>2.2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Number of mg protein in samples assayed (50 μl).
†One unit is the amount of enzyme which catalyzes the release of 1 μmol of p-nitrophenol per hour. Samples and substrate were incubated for 3 hours at 37°C.
‡Background activity.
§Bound to chromatin and extracted with 0.1% TX-100.

Table 6. Fusion of MV1, MV2α and MV2β with LSs and with one another

<table>
<thead>
<tr>
<th>MV fraction present</th>
<th>LSs</th>
<th>MV1</th>
<th>MV2α</th>
<th>MV2β</th>
<th>Fusion*</th>
<th>Dextran excl.†</th>
</tr>
</thead>
<tbody>
<tr>
<td>x x x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>x x x</td>
<td>x</td>
<td>x</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>x x x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>x x x</td>
<td>x</td>
<td>x</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>x x x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
</tbody>
</table>

* indicates fusion of all components with one another, between and within fractions.
†Exclusion of a 150 kDa FITC-conjugated dextran from nuclei.
antibodies cross-reacting with invertebrate Golgi compartments will help identify the origin of NE precursor vesicles not derived from the ER.

The contribution of Golgi-derived vesicles to the male pronuclear envelope in vitro is intriguing. Despite the absence of an MTOC in the acrosomal fossa region, it may still be hypothesized that participation of MV2α together with MV1 in NE formation may represent a tentative assembly of the Golgi complex: both MV1 (non-ER-derived) and MV2α (Golgi-derived) bind in the LS regions and fuse with each other. However, because MV1 does not contain the Golgi marker, another alternative may be that fusion of MV2α with MV1 and MV2β represents a tentative reconstitution of a retrograde fusion pathway (Lippincott-Schwartz et al., 1990). Golgi vesicles (MV2α) and vesicles of as yet unidentified (but non-ER origin (MV1)) would be required to supply components to permit fusion of ER vesicles (MV2β) to the chromatin. The inability of MV2β vesicles to fuse on their own could suggest that our in vitro conditions might support retrograde fusion rather than ‘organelle-assembly’ fusion. These alternatives remain to be investigated.

The participation of non-ER derived vesicles in NE assembly in vitro is not an unprecedented observation, or may not be excluded from previous reports. In a *Xenopus* egg lysate, two sets of vesicles isolated by sedimentation on a sucrose gradient were shown to participate in NE formation (Wilson and Newport, 1988). Both populations were enriched in the ER marker α-glucosidase, although one more so than the other. These authors concluded that NE precursor vesicles constituted a functionally-distinct subset of the ER marker-containing vesicles (Wilson and Newport, 1988). These findings, however, do not exclude the possibility of participation of a non-ER derived vesicle subset within the two populations identified. This hypothesis is substantiated by the identification of the Golgi-derived MV2α fraction within the abundant MV2 vesicle fraction in our study. In a similar *Xenopus* system, Vigers and Lohka (1991) identified two sets of vesicles (NEP-A and NEP-B) contributing to the NE. Vesicles of NEP-A constitute the bulk of the NE and originate from the ER, whereas vesicles of NEP-B bind to chromatin and lack the ER marker. These authors concluded that typical ER-derived vesicles are insufficient for NE assembly (Vigers and Lohka, 1991). The origin of these non-ER-derived vesicles was not identified in this study. The relationship between our vesicle fractions and NEP-A and NEP-B is uncertain. Because both MV2β and NEP-A contribute to most of the NE, exhibit high salt-resistant binding properties, and are enriched in the ER marker α-glucosidase, they are likely to contain similar sets of vesicles. Like NEP-B, MV1 and MV2α do not possess the ER marker but their relationship to NEP-B is uncertain. Clearly, however, they represent different sets of vesicles with distinct binding, fusion and biochemical characteristics.

The ability of MV2β to spread across the entire chromatin surface when binding is initiated by LSs at the poles is intriguing. Since maximal vesicle diameter is considerably smaller than the length of the nucleus, this implies that binding of the first vesicles facilitates binding of a subsequent set of vesicles. It is difficult to imagine mechanisms for this binding propagation step with a uniform set of vesicles. Since vesicles may contain several binding proteins, it is possible that MV-LS or MV-MV binding activates MV-chromatin binding elements facilitating spreading of MVs across the chromatin surface. That MV2β is contaminated with MV2α vesicles (which would provide binding sites for the bulk of the MV2β vesicles) is unlikely since fusion does not occur when MV1 is present. For similar reasons, it is unlikely that MV2β is contaminated with MV1.

Our results indicate that the signals for vesicle binding to demembranated nuclei involve protein elements on the vesicles. The properties of these binding elements appear specific for each fraction. Binding of MV1 to the LS regions involves NEM-sensitive and peripheral proteins; binding of MV2α or MV2β also in the LS regions also entails peripheral but NEM-insensitive molecules, whereas intrinsic and NEM-insensitive proteins are required for MV2β binding to chromatin. Several integral membrane proteins implicated in binding nuclear membranes to lamins have been described in other systems such as a lamin B receptor and several lamina-associated polypeptides (Worman et al., 1990; Chaudhary and Courvalin, 1993; Foisner and Gerace, 1993; Konstantinov et al., 1995). Thus lamins may be potential targets for MV1, MV2α or MV2β. However, we have previously shown by blotting and immunofluorescence using five different antibodies that decondensed chromatin does not contain lamins even in the LS regions, and that NE formation, under conditions used in the present study, occurs without lamin assembly (Collas et al., 1995). Barring the participation of an undiscovered sea urchin lamin, the involvement of lamins as receptors for the vesicles seems unlikely. Lamins are involved in nuclear swelling, the second step of pronuclear formation in vitro (Collas et al., 1995).

All fusion events appear to require participation of MV1 bound to LSs. LSs alone are incapable of providing fusion signals to other vesicle fractions, suggesting the fusion machinery is localized in MV1. Since fusion of MV1 vesicles does not occur in suspension (see Collas and Poccia, 1995; our unpublished observations), this machinery may either specifically require interaction with LSs, or may simply require proximity of MV1 vesicles which is brought about by docking to LSs.

Intracellular membrane fusion events are known to involve a number of molecules including G proteins (Bourne, 1988; Boman et al., 1992) and specific recognition elements such as those operating in the secretory pathway (Rothman, 1994). Such factors may operate in NE assembly as well. Vesicle targeting and fusion in the secretory pathway require a cytosolic component called NEM-sensitive factor (NSF). NSF binds to membranes via a set of soluble NSF attachment proteins (SNAPs). Binding of SNAP-containing vesicles to their target membranes is mediated by specific membrane receptors (SNAREs) and the NSF-SNAP-SNARE complex constitutes the fusion machinery (Rothman, 1994). SNAREs, although found from yeast to mammals, are specific for various vesicular processes (Bennett and Scheller, 1993). Components similar to general SNFs and SNAPs and vesicle-specific SNAREs might operate in NE assembly. The only vesicle fraction which appears to have fusogenic properties (MV1) is also the only one whose binding to the LSs is sensitive to NEM. Thus, NEM-sensitive elements may be essential for initiating membrane fusion whereas propagation of the fusion signal may involve different classes of molecules. Whether homologs of NSF, SNAPs and SNAREs are involved in vesicle targeting and/or fusion with LSs will need to be addressed. The isolation
of vesicle fractions with distinct properties should facilitate the isolation of the appropriate molecular agents of binding and fusion in this system.

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REFERENCES


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