# A Distinct Vesicle Population Targets Membranes and Pore Complexes to the Nuclear Envelope in *Xenopus* Eggs

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***Abstract.*** Extracts from *Xenopus* eggs capable of nuclear envelope assembly in vitro were fractionated by differential and density gradient centrifugation. Nuclear envelope assembly was found to require soluble components in the cytosol and two distinct particulate fractions, which we have called nuclear envelope precursor fractions A and B (NEP-A and NEP-B). Both NEP-A and NEP-B are sensitive to treatments with trypsin, sodium carbonate, and detergents, but can be distinguished from each other by their sensitivities to high salt and *N*-ethylmaleimide and by their levels of α-glucosidase activity. Vesicles in NEP-B bind to chromatin, whereas those in NEP-A do not. NEP-B may therefore be involved in the targeting of membranes to the surface of the chromatin, whereas NEP-A may provide a pool of vesicles that contributes many of the nuclear envelope membranes. NEP-B may also play a role in the assembly of nuclear pore complexes because the density of nuclear pores in the resulting envelope is dependent on the ratio of NEP-B to NEP-A in the reconstituted extract.

One of the distinctive properties of the cell nucleus in higher eukaryotes is that it undergoes dramatic changes in structure each time the cell divides. In interphase, nuclear constituents are separated from the rest of the cell by the nuclear envelope (NE),[1] which consists of two membranes: an outer membrane which is continuous with the ER (Franke, 1974; Franke et al., 1981), and an inner membrane which abuts the nuclear lamina that lies below it (Aebi et al., 1988). The nuclear lamina binds to chromatin, probably anchoring the interphase chromosomes to the NE (for review see Newport and Forbes, 1987; Gerace and Burke, 1988). The two membranes are traversed by a large number of pores. Associated with each pore is a nuclear pore complex, composed of many different proteins, that together control the passage of all large molecules to and from the nucleus (for review see Dingwall and Laskey, 1986; Newport and Forbes, 1987; Gerace and Burke, 1988). At mitosis, the nuclear envelope membranes of higher eukaryotes break up into small vesicles, the components of the pore complexes become dispersed throughout the cytoplasm, and the nuclear lamina is disassembled (Zeligs and Wollman, 1979; Gerace and Blobel, 1980). Most of these components are thought to be recycled to form new nuclear envelopes around the chromatin of both daughter cells without a need for new synthesis. Since the membranes of the ER and Golgi apparatus also become vesicular at mitosis (Zeligs and Wollman, 1979), the reformation of the NE at telophase poses interesting problems in the sorting and assembly of intracellular membranes.

Even though recent studies have revealed much about the structures and functions of the nuclear pore complexes and of the lamina in interphase nuclei (e.g., Unwin and Milligan, 1982; Benavente et al., 1985; Snow et al., 1987; Aebi et al., 1988; Newmeyer and Forbes, 1988; Stick et al., 1988; Reichelt et al., 1990), far less is known about NE assembly after mitosis. Gerace and co-workers have proposed that the assembly state of the NE membranes follows that of the nuclear lamins, which in turn are regulated by their levels of phosphorylation (Gerace et al., 1984; Miake-Lye and Kirschner, 1985; Ottaviano and Gerace, 1985). Both lamins A and C become soluble during mitosis, but lamin B remains bound to vesicles and at telophase is thought to target vesicles to the surface of the chromosomes for assembly into the NE (Gerace and Blobel, 1980; Burke and Gerace, 1986; Gerace and Burke, 1988; Stick et al., 1988). While this model for NE assembly is supported by observations in mammalian and chicken tissue culture cells, it may not be universally applicable. In *Xenopus* eggs only one lamin, called LIII, has been described, and it is soluble when the NE is disassembled (Benavente et al., 1985). The mechanism that directs vesicles to the chromosome surface is, therefore, not yet known in eggs. Similarly, it is unclear what distinguishes vesicles destined for the NE from those that will form the ER or the Golgi complex.

To study NE membrane assembly we have used a cell-free system from *Xenopus laevis* eggs that can assemble a NE around sperm chromatin, isolated chromosomes, or exogenously added DNA (Lohka and Masui, 1983; 1984; Newmeyer et al., 1986*a*,*b*; Newport, 1987; Sheehan et al., 1988; Wilson and Newport, 1988). The NEs assembled in this system are morphologically identical to those of intact

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[1] *Abbreviations used in this paper*: NE, nuclear envelope; NEP-A and NEP-B, nuclear envelope precursor fraction A and B, respectively.

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cells (Lohka and Masui, 1984) and can support normal nuclear transport (Dingwall and Laskey, 1986; Newmeyer et al., 1986*a*,*b*; Newmeyer and Forbes, 1988). Moreover, they can be disassembled in vitro in response to maturation promoting factor, an intracellular regulator of entry into mitosis (Lohka and Maller, 1985; Miake-Lye and Kirschner, 1985; Lohka et al., 1988).

A greater understanding of NE assembly would be gained if the components required for assembly were better defined. Previous studies have shown that the low-speed extract can be separated by centrifugation into two fractions that are both required for NE assembly. One of the fractions is rich in vesicles, while the other is composed largely of soluble proteins (Lohka and Masui, 1984; Sheehan et al., 1988; Wilson and Newport, 1988). We now report that NE assembly requires two distinct particulate fractions that can be separated from each other, and from the soluble components, by differential centrifugation. Both particulate fractions contain vesicles, but can be distinguished from each other by several criteria. One fraction appears to function in the targeting of membrane to the chromatin surface and in the assembly of the nuclear pore complexes, while the other provides the bulk of the membrane assembled into the NE.

## *Materials and Methods*

The cell-free extracts from *Xenopus* eggs were prepared and fractionated as described below. The procedure is summarized in Fig. 1.

### *Preparation of the Low-speed Extract from Xenopus Eggs*

Cell-free extracts were prepared from *Xenopus* eggs as described previously (Lohka and Masui, 1983; Lohka and Maller, 1985). Briefly, ovulated eggs were collected 12-13 h after the frogs had been injected with human chorionic gonadotropin, dejellied with 2% cysteine (pH 8.0), and washed with extraction buffer (100 mM KCl, 5 mM $MgCl_2$, and 20 mM Hepes, pH 7.5) containing 300 μM PMSF, 15 μg/ml leupeptin, and 1 mM DTT. The eggs were packed into 3-ml tubes and centrifuged at 10,000 *g* for 10 min. The resulting low-speed supernatant was collected from between the lipid cap and the pellet and was centrifuged again at 10,000 *g* for 10 min to remove some of the residual lipid, pigment, and yolk.

### *Fractionation of the Low-speed Extract by Differential Centrifugation*

*(a) First high-speed centrifugation. Step A*: 2-ml samples of freshly prepared low-speed extract were centrifuged at 200,000 *g* for 75 min in a TLS-55 rotor (Beckman Instruments, Inc., Palo Alto, CA) to yield a pellet and supernatant ($S_{200}$). The pellet was collected by side puncture of the tube with a 1-ml syringe and an 18G needle and the supernatant was collected with a cold Pasteur pipette. The supernatant fraction was used fresh or was frozen in liquid nitrogen in 200-μl volumes. The pellet fraction was used fresh or was diluted with an equal volume of 60% sucrose (wt/vol) in extraction buffer before freezing in liquid nitrogen.

*Step B*: The pellet fraction from step A was washed free of residual soluble components: 100 μl of the thawed pellet was diluted to 1.5 ml with extraction buffer and centrifuged for 10 min at 10,000 *g*. The pellet was resuspended in 100 μl of $S_{200}$ or cytosol (see step D below) as required. The washed pellet fraction was found to contain components essential for NE assembly and was therefore termed nuclear envelope precursor fraction-A (NEP-A).

*Step C*: In other experiments, the NEP-A was further fractionated by centrifugation through a discontinuous sucrose gradient: 1-ml samples of the thawed pellet from step A were layered onto a sucrose density step gradient consisting of 2 ml 35% sucrose and 2 ml 40% sucrose (wt/vol in extraction buffer) and centrifuged at 300,000 *g* for 4 h in an SW55 rotor (Beckman Instruments Inc., Palo Alto, CA). The material at the 35-40% interface, which we call sucrose-purified NEP-A, was collected and either used fresh or frozen in liquid nitrogen. Before use the sucrose-purified NEP-A was pelleted as described in step B above. NEP-A and sucrose-purified NEP-A could be used interchangeably in the reconstitution of NE assembly.

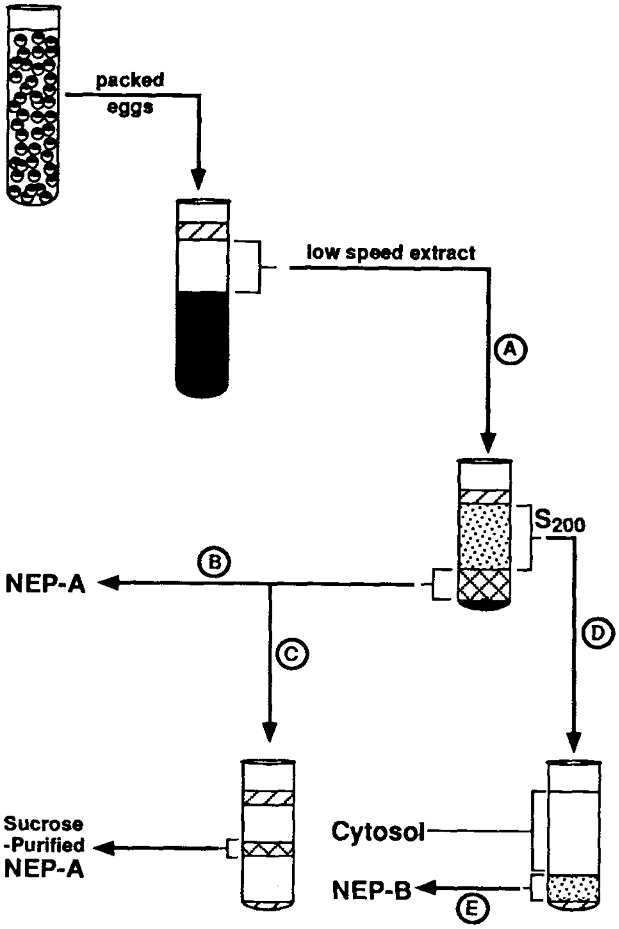


*Figure 1.* Fractionation scheme from the isolation of NE precursors from *Xenopus* eggs. *Xenopus* eggs were processed to yield fractions designated as low-speed extract ($S_{200}$), cytosol, and NEP-A and -B. Treatment conditions for steps A-E, including centrifugation times and speeds, are described in Materials and Methods.

***(b) Second high-speed centrifugation. Step D***: $S_{200}$ from step A was further fractionated by centrifugation at 200,000 *g* for 4 h in a TLS-55 rotor to yield a second supernatant (cytosol) and a second cloudy pellet. The supernatant and pellet were again collected by side puncture and used fresh or frozen in liquid nitrogen in 200-μl volumes. The cytosol fraction contained soluble components required for NE assembly.

*Step E*: The second high-speed pellet was washed free of residual cytosol: A 200-μl sample was diluted with 1.8 ml of extraction buffer containing 100 mM PMSF, 3 μg/ml leupeptin, and 0.5 mM DTT, and centrifuged at 10,000 *g* for 10 min to remove any residual NEP-A. In some experiments 2 mM ATP was also added to the extraction buffer, but this was later found to be unnecessary for the recovery of activity. The supernatant was transferred to TLS-55 tubes that had been treated with Sigma-cote (Sigma Chemical Co., St. Louis, MO) and centrifuged for 1 h at 25,000 *g*. The pellet was resuspended in 100 μl of cytosol and NEP-A to test for NE assembly activity. This pellet fraction was found to contain a second set of particulate components required for NE assembly, which we have called nuclear envelope precursor fraction-B (NEP-B). NEP-B could also be recovered in this manner directly from 200 μl of $S_{200}$ (as made in step A), rather than from the diluted second high-speed pellet.

### *Treatment of Precursor Fractions with Various Reagents*

The sensitivities of NEP-A and NEP-B to trypsin, three different detergents,

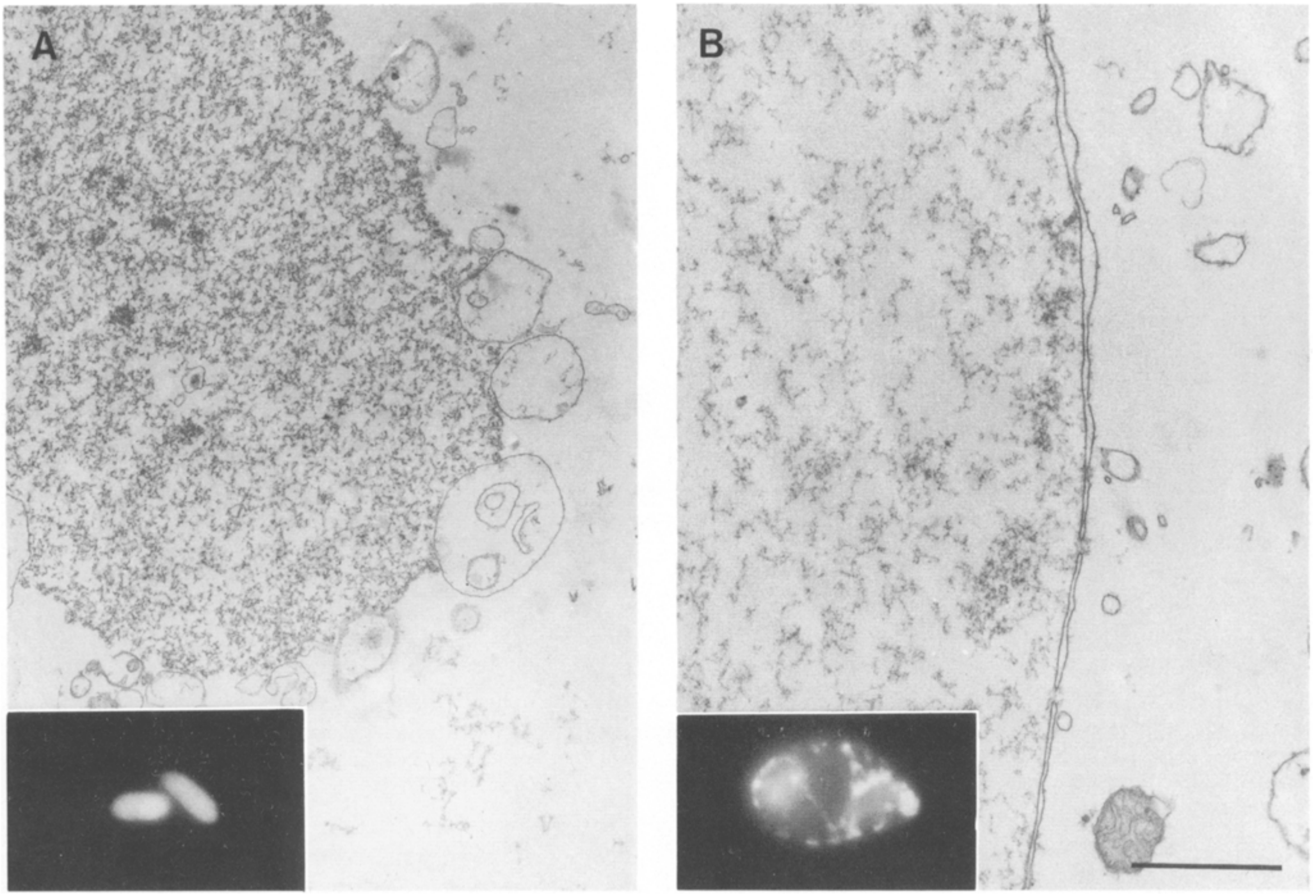


*Figure 2.* Incubation of demembranated sperm in fractions of *Xenopus* egg extracts. Sperm were incubated for 60 min at 18°C in $S_{200}$ alone (*A*) or $S_{200}$ plus NEP-A (*B*). In $S_{200}$ alone vesicles attach to the chromatin surface, but a NE does not form, whereas in $S_{200}$ plus NEP-A a NE forms. The NE appears morphologically normal by EM and is functionally competent, as indicated by enlargement due to the uptake of nuclear proteins from the extract. Bar, 1 $\mu$m. (*Insets*) Chromatin visualized by 4′,6-diamidine-2-phenylindole dihydrochloride staining. Both insets were photographed at the same magnification.

sodium carbonate, high salt, and *N*-ethylmaleimide were tested. *Trypsin treatment*: To treat NEP-A, a 100-$\mu$l sample of the pellet fraction from step A was diluted with 1.5 ml of extraction buffer containing 0.5 mM DTT and 15 $\mu$g/ml trypsin and incubated for 15 min on ice (Wilson and Newport, 1988). Proteolysis was halted by the addition of leupeptin and PMSF to final concentrations of 30 $\mu$g/ml and 300 mM, respectively. Both samples were recovered by centrifugation as described in step B. The NEP-B was treated in a similar manner except that a 200-$\mu$l sample of $S_{200}$ (from step A) was used and NEP-B was recovered as described in step E. In control experiments, leupeptin and PMSF were added at the start of the incubation. *Detergent treatment*: The detergent sensitivities of NEP-A and NEP-B were tested. The supernatant and pellet fractions from step A were incubated for 15 min on ice in extraction buffer containing 100 mM PMSF, 3 $\mu$g/ml leupeptin, 0.5 mM DTT, and one of three different detergents. All detergents were used at ~1.5 mM above the critical micelle concentrations, i.e., 1.8 mM for Triton X-100, 6.5 mM for CHAPS, and 26.5 mM for octyl-glucoside (see Helenius and Simons, 1975; Hjelmeland and Chrambach, 1984). After detergent treatment, NEP-A and NEP-B were recovered as described in steps B and E, respectively. After centrifugation the NEP-A was washed again in extraction buffer without detergent, while the NEP-B was overlaid with 200 $\mu$l of extraction buffer for 2 min and the supernatant was discarded. *Sodium carbonate treatment*: Treatments were carried out on both fractions as described for the detergent treatments, except that the extraction buffer and detergent were replaced with 0.1 M $Na_2CO_3$. *High salt treatment*: The fractions were washed as described above, except that the 0.1 M $Na_2CO_3$ was replaced with extraction buffer modified to contain 0.9 M KCl. *N-Ethylmaleimide treatment*: The fractions were treated with extraction buffer containing 100 mM PMSF, 3 $\mu$g/ml leupeptin, and 1 mM *N*-ethylmaleimide for 15 min on ice. The reaction was stopped by addition of 10 mM DTT and the particulate components were recovered by centrifugation as described in steps B and E. In control experiments 10 mM DTT was added at the same time as the *N*-ethylmaleimide.

In additional control experiments, untreated NEP-A or NEP-B were mixed with their treated counterparts before extracts were reconstituted. In no case was the ability of untreated fractions to participate in NE assembly inhibited by mixing with the treated fractions, indicating that the inhibition of NE assembly was not due to reagents carried over into the incubation mixture.

## Measurement of α-Glucosidase Activity

α-Glucosidase levels were measured according to Burns and Touster (1982). Samples were made up to 250 $\mu$l in extraction buffer and 5-$\mu$l volumes were removed for measurement of protein concentration. The remainder was diluted 1:1 with assay stock (100 mM Hepes, pH 6.8, 8 mM *p*-nitrophenol-α-D-glucopyranoside, and 2% sodium cholate) and incubated for 1 h at 18°C. α-Glucosidase activity was quenched by the addition of 1 ml 1% ethylenediamine and optical absorbance at 400 nm was measured for all samples. Enzyme activity was standardized using purified α-glucosidase from yeast (Boehringer Mannheim Biochemicals, Indianapolis, IN) and protein concentration was measured using the method of Lowry, as modified for membrane proteins by Goldin (1977), with BSA as a standard.

## Assay for NE Assembly

Detergent-treated *Xenopus* sperm (Lohka and Masui, 1983) were added to 100 $\mu$l of low-speed extract or the various mixtures of isolated components as described, to give a final concentration of $10^6$ to 4 × $10^6$ sperm/ml. An ATP-regenerating system was added to all samples, unless otherwise stated,

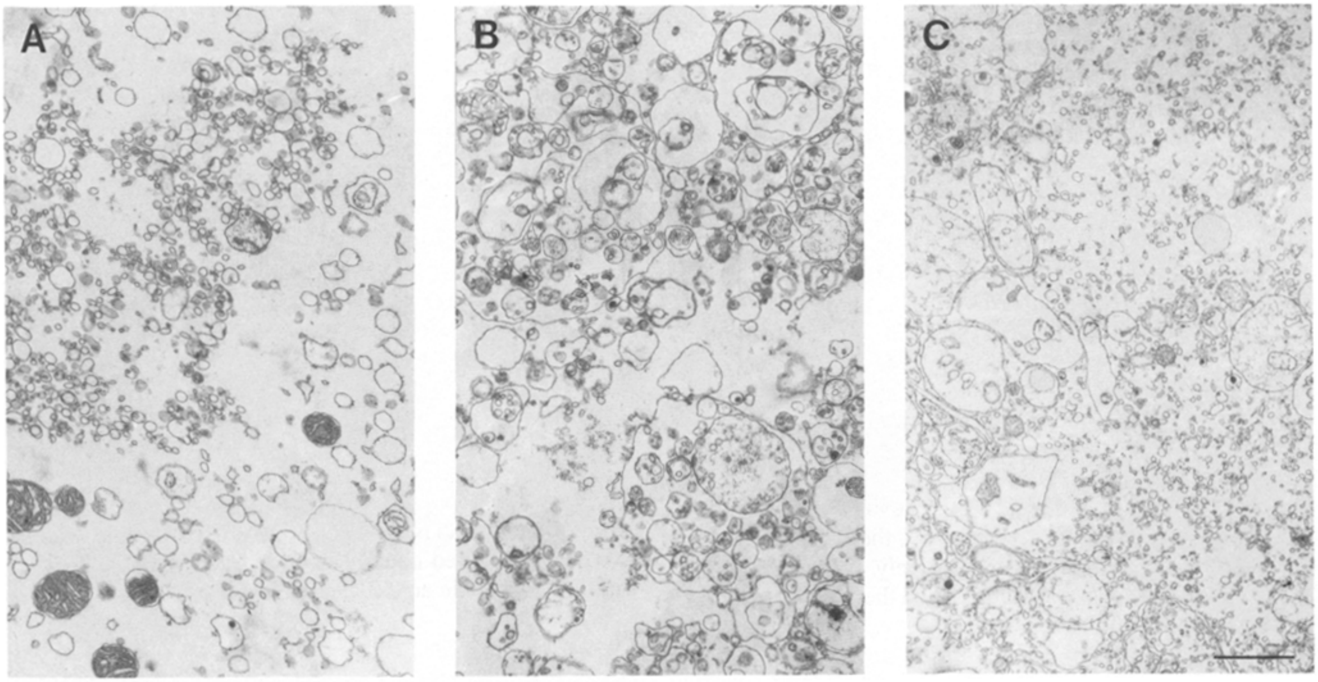


*Figure 3.* Electron micrographs of NEP-A (*A*), sucrose-purified NEP-A (*B*), and NEP-B (*C*). Both NEP-A and NEP-B appear highly heterogeneous and we have been unable to identify a morphologically distinct subpopulation of vesicles that might mediate the NE assembly activities of these fractions. Sucrose-density purification of NEP-A does not appear to reduce the heterogeneity of the fraction, but does remove most of the contaminating mitochondria. Bar, 1 μm.

to a final concentration of 6 mM phosphocreatine and 150 μg/ml creatine kinase (Boehringer Mannheim Biochemicals) and the samples were incubated for 1 h at 18°C. Unless otherwise stated, all components were present in the final reaction mixtures at approximately the same concentrations found in the low-speed extract, assuming 100% recovery during fractionation. Changes in nuclear morphology were monitored by mixing 5-μl samples of the incubation mixture with an equal volume of extraction buffer containing 10 μg/ml 4′,6-diamidine-2-phenylindole dihydrochloride and 10 μg/ml 3,3′-dihexyloxacarbocyanine iodide in extraction buffer. The samples were then examined by epifluorescent or phase contrast illumination. Photographs were taken on TMAX 400 film.

### Electron Microscopy

NEs assembled in extracts were examined ultrastructurally. After a 1-h incubation of sperm in the reconstituted system, 100-μl samples of the incubation mixture were mixed with 1.5 ml of 2% glutaraldehyde and 1.5 ml 1% $OsO_4$ (both from Electron Microscopy Sciences, Ft. Washington, PA) in 0.1 M phosphate buffer, pH 7.4. Fixation was carried out for 1 h on ice and the samples were centrifuged at 1,000 *g* for 10 min. The pellets were resuspended in phosphate buffer, transferred to BEEM capsules (Ted Pella, Inc., Redding, CA), and centrifuged again at 1,000 *g* for 5 min. The pellets were stained *en bloc* for 2 h with aqueous 2% uranyl acetate and dehydrated through a graded series of acetone and propylene oxide before embedding in Medcast (Ted Pella, Inc.). Thin sections were post-stained in uranyl acetate and lead citrate and viewed on a CM10 electron microscope (Philips Electronic Instruments, Inc., Mahwah, NJ) at 80 kV.

In experiments that examined the density of nuclear pores in newly assembled NEs, nuclei were randomly selected in each experimental sample and micrographs were taken at a magnification of 5,200×. These were printed and scored blind for the number of pores per micron of NE. Pores were identified by morphological criteria: only structures where the inner and outer membranes were clearly joined, leaving a gap of ~100 nm, were scored as pores (see Fig. 7).

## Results

### Soluble and Particulate Fractions Are Both Required for NE Assembly

As a first step toward fractionating the cell-free system, we determined the minimal centrifugation conditions that removed components necessary for nuclear envelope assembly. Centrifugation of low-speed extract for 75 min at 200,000 *g* (step A in Materials and Methods) resulted in the separation of the extract into a residual lipid cap, a slightly yellow supernatant ($S_{200}$), and a loose, cloudy pellet just above a dense amber plug (Fig. 1). The supernatant and pellet were collected and tested for their ability to support NE assembly. Since the freshly collected pellet fraction contained some residual $S_{200}$, it was washed as described in Materials and Methods (step B) before use. The nuclei of sperm incubated in $S_{200}$ alone changed from their characteristic highly condensed, elongated shape to a round or oval shape, but failed to acquire a NE (Fig. 2 *A*). Sperm nuclei incubated in the washed pellet resuspended in extraction buffer did not appear to change (data not shown), in agreement with previous observations (Lohka and Masui, 1984). However, sperm incubated in washed pellet resuspended in $S_{200}$ acquired a NE that was morphologically identical to those formed in the unfractionated low-speed extract (Fig. 2 *B*). These results show that both $S_{200}$ and the washed pellet fraction, which we called NEP-A, are required to make a NE.

The components in NEP-A required for NE assembly can also be recovered from the 35–40% interface of a sucrose step gradient (step C in Materials and Methods). This fraction, which we call sucrose-purified NEP-A, is largely free of contaminating mitochondria (compare Fig. 3, *A* and *B*) and can substitute for NEP-A in all of the reactions described above. Both NEP-A and sucrose-purified NEP-A appear by thin-section EM to contain vesicles of many different sizes (Fig. 3, *A* and *B*). However, we have been unable to distinguish morphologically any subpopulation of vesicles within the fraction that might be uniquely responsible for NE assembly.

### A Second Particulate Fraction Is Required for NE Assembly

Even though a NE was not formed around sperm nuclei incubated in $S_{200}$, ultrastructural examination showed that vesicles were bound to the chromatin (Fig. 2 *A*). However, these vesicles apparently did not fuse to give a continuous double membrane, even when they were closely apposed to each other. We therefore wished to determine whether these vesicles were essential for NE assembly, or whether the soluble components from $S_{200}$ were sufficient to complement NEP-A. To this end the $S_{200}$ fraction was centrifuged further at 200,000 *g* for 4 h (step D in Materials and Methods) to give a second supernatant (cytosol) and a second pellet. The second pellet was again recovered free of most contaminating soluble components and residual NEP-A by dilution and centrifugation as described in Materials and Methods (step E).

When sperm were incubated with cytosol alone (Fig. 4 *A*) or with cytosol and NEP-A (Fig. 4 *B*), the sperm enlarged as the chromatin decondensed extensively. However, in neither case was there NE assembly around the sperm, nor were vesicles bound to the chromatin, as judged either by EM or by labeling with the fluorescent membrane dye 3,3′-dihexyloxacarbocyanine iodide (Fig. 4, *A* and *B*). In cytosol plus the washed second pellet there was also no NE assembly around sperm (Fig. 4 *C*), which appeared almost identical to those incubated in $S_{200}$ (compare Figs. 2 *A* and 4 *C*). In contrast, when cytosol, NEP-A, and the washed second pellet were all present in the reaction mixture (Fig. 4 *D*), NE assembly was complete and virtually indistinguishable from that in the mixture of $S_{200}$ and NEP-A or in the original low-speed extract. Therefore, NE assembly requires not only the soluble components of $S_{200}$ but also a second particulate component, which we have designated NEP-B. NEP-B can be pelleted from $S_{200}$ by prolonged centrifugation, and the supernatant, which we have designated cytosol, is thus equivalent to $S_{200}$ minus NEP-B. As with NEP-A, NEP-B appears to contain a wide variety of vesicles (Fig. 3 *C*), and we found no morphological features that reliably distinguished the NEP-B vesicles from the NEP-A vesicles.

These results show that at least three different fractions required for NE assembly can be separated from the initial extract of *Xenopus* eggs by differential centrifugation: particulate components in NEP-A, particulate components in NEP-B, and soluble components in the cytosol.

### Partial Characterization of NEP-A and NEP-B

Since both NEP-A and NEP-B retained their ability to participate in NE assembly after they were washed and recovered by centrifugation, it was possible to assay the sensitivities of both fractions to different modifications of the washing buffer, as described in Materials and Methods. The results of these experiments are summarized in Table I. NEP-A and NEP-B were both inactivated by incubation with trypsin, showing that proteinaceous components in both fractions are required for NE assembly activity. Both fractions were also inactivated by washing in extraction buffer that had been modified to contain Triton X-100, CHAPS, or octylglucoside at a micelle concentration of 1.5 mM (see Helenius and Simons, 1975), but neither fraction was affected by any of the detergents at half this concentration (data not shown). These results indicate that the components of both NEP-A and NEP-B are sensitive to detergents and are possibly vesicles. In addition, the sensitivity of both fractions to 0.1 M sodium carbonate, a treatment that has been shown to lyse the vesicles of the ER and remove all peripheral membrane proteins (Fujiki et al., 1982), provides further evidence that vesicle integrity and/or peripheral membrane proteins are required for NE assembly.

High-salt treatment completely inactivated NEP-B, but did not affect NEP-A. In contrast, *N*-ethylmaleimide treatment inactivated NEP-A but not NEP-B. These results show that the two particulate fractions required for NE assembly can be distinguished by their sensitivities to high salt and *N*-ethylmaleimide. For all these procedures, control experiments (described in Materials and Methods) were performed to ensure that the effects seen on NE assembly were not due to residual reagents carried over into the incubation mixtures used to test for NE assembly.

The activity of α-glucosidase, an enzyme marker for the ER (Burns and Touster, 1982; Wilson and Newport, 1988), was assayed in both NEP-A and NEP-B to determine whether ER was distributed equally in both fractions. As shown in Fig. 5, the specific activity of α-glucosidase was ~10 times greater in NEP-A than in NEP-B, indicating that much of the ER is in NEP-A. On the other hand, NEP-B appeared to contain relatively little ER. We do not know whether the ER in NEP-B plays a role in NE assembly. However, since NEP-A by itself cannot support NE assembly, these results strongly suggest that the ER alone is not sufficient, in agreement with the results of Wilson and Newport (1988).

### ATP Requirement for NE Assembly

Sucrose density centrifugation removed most of the mitochondria from the NEP-A fraction (Fig. 3, *A* and *B*) and very few remained in the $S_{200}$ fraction. It was therefore possible to reconstitute an extract containing few mitochondria in order to study the requirements for ATP in NE assembly. An ATP-regenerating or -depleting system was added to samples of $S_{200}$ or $S_{200}$ and sucrose-purified NEP-A and incubated at 18°C for 30 min before the addition of sperm. In $S_{200}$ alone, vesicles bound to the sperm chromatin but failed to fuse with each other, regardless of whether an ATP-regenerating or -depleting system was present (Fig. 6, *A* and *C*). However, in $S_{200}$ and sucrose-purified NEP-A a normal NE formed around each sperm in the presence of the ATP-regenerating system (Fig. 6 *D*), whereas vesicles bound to the sperm but did not fuse together in the presence of the ATP-depleting system (Fig. 6 *C*). These results provide strong evidence that ATP is not required for the binding of vesicles to chromatin, but is essential for fusion between vesicles.

### Roles of NEP-A and NEP-B in NE Assembly

The possible roles of NEP-A and NEP-B in NE assembly were investigated by preparing reconstituted extracts in which the amount of either fraction was reduced below the usual concentration. Preliminary experiments showed that complete NE assembly could occur when the concentration of either NEP-A or NEP-B was decreased to as little as one-fifth of its original concentration. Therefore, NE assembly was examined in reconstituted extracts where the ratio of NEP-B to NEP-A was the same as that found in the initial extract, or where one of the fractions was present at one-fifth of this concentration while the other remained the same. Representative micrographs of the NEs formed under these conditions are shown in Fig. 7. It was clear that the number

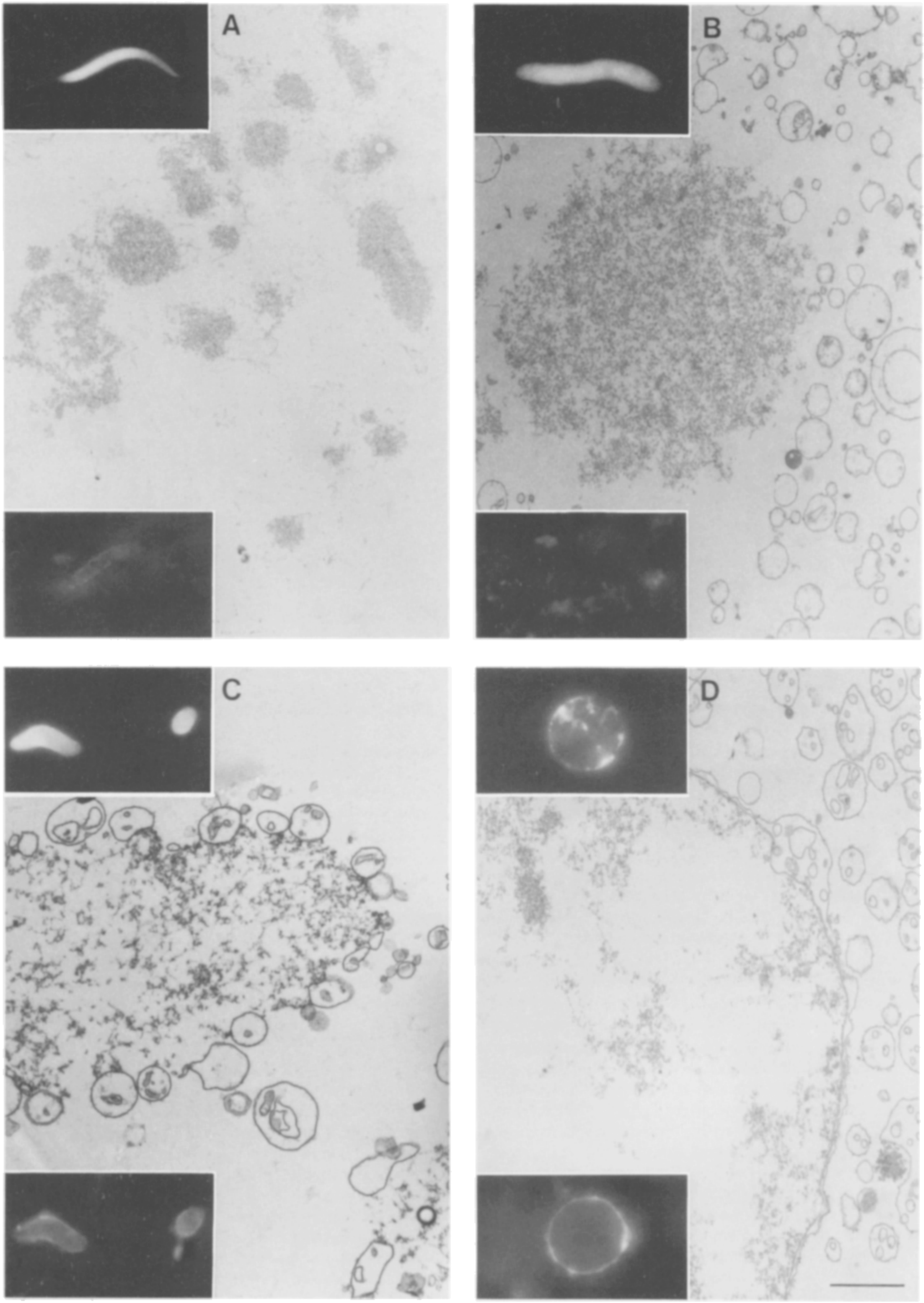

A
B
C
D

*Table I. NE Assembly after Treatment of NEP-A or NEP-B*

| Treatment | NEP-A | NEP-B |
|---|---|---|
| Extraction buffer | + | + |
| 15 μg/ml trypsin | – | – |
| Detergent | – | – |
| 0.1 M $Na_2CO3$ | – | – |
| 0.9 M KCl | + | – |
| 1 mM *N*-ethylmaleimide | – | + |

NEP-A and NEP-B were treated with extraction buffer modified to contain the reagents listed in the left-hand column, as described in Materials and Methods. The treated fractions were then assayed for their ability to participate in NE assembly.
+, Retains activity. –, Loses activity.

of pores that formed in the NE was much greater when NEP-A was depleted (Fig. 7 *A*) than when NEP-B was depleted (Fig. 7 *C*). The pore density was intermediate when equal amounts of NEP-A and NEP-B were used to reconstitute the NE (Fig. 7 *B*). As shown in Fig. 8, over a 25-fold increase in the ratio of NEP-B to NEP-A the density of pores increased approximately fourfold. This finding suggests that NEP-B may have a role in nuclear pore formation.

## Discussion

The ability of cell-free extracts of frog eggs to assemble a NE around sperm chromatin, metaphase chromosomes, or naked DNA has been demonstrated by many laboratories (Lohka and Masui, 1983; 1984; Newmeyer et al., 1986*a*,*b*; Newport, 1987; Sheehan et al., 1988; Wilson and Newport, 1988). In this paper we have shown that, in addition to soluble factors, NE assembly requires at least two particulate fractions, which we have called NEP-A and NEP-B. We have separated NEP-A and NEP-B from the low-speed extract by differential centrifugation and have devised protocols to recover the fractions largely free of soluble components. NEP-A has been further enriched by centrifugation on a discontinuous sucrose density gradient, but we have been unable to fractionate NEP-B further. One reason for this may be that NEP-B is more labile than NEP-A. Our only assay for NEP-B activity tests for the assembly of complete NEs in the presence of NEP-A and cytosol, and if the level of NEP-B falls to <20% of the original concentration, no NEs are formed (unpublished observations). Since the recovery of NEP-B on centrifugation (step E) may be low, it becomes difficult to assay for the presence of NEP-B in dilute solutions.

What are the roles of NEP-A and NEP-B in NE assembly? Ultrastructural studies show that when demembranated sperm are incubated in cytosol with NEP-A, the sperm chromatin decondenses but does not acquire a NE, and that the vesicles in NEP-A fail to bind to the chromatin. A NE also does not form around sperm incubated in cytosol with NEP-B. However, in this case vesicles attach to the chromatin surface, although they do not fuse together. A normal NE forms only in the presence of cytosol, NEP-A, and NEP-B. From these data we conclude that NEP-B is involved in the targeting of membranes to the surface of the chromatin, while NEP-A provides a pool of vesicles that contributes many of the NE membranes. Interestingly, sperm incubated in cytosol without NEP-B expand to a greater extent and are more fragile than those incubated in cytosol with NEP-B (Fig. 4, *A* and *B*). This may indicate that during NE assembly the vesicles in NEP-B limit nuclear expansion by cross-linking chromatin fibers, while the vesicles in NEP-A may allow for expansion of the nuclear envelope without the formation of additional attachments to the chromatin.

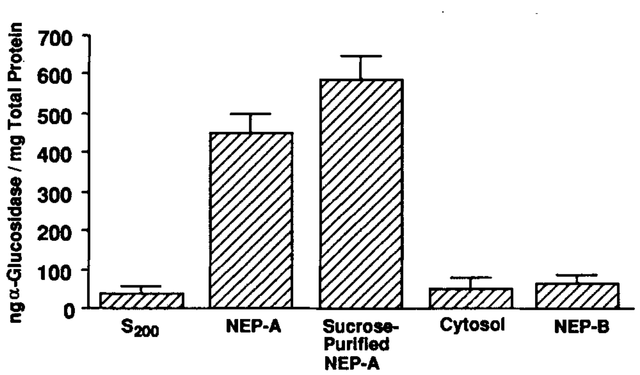
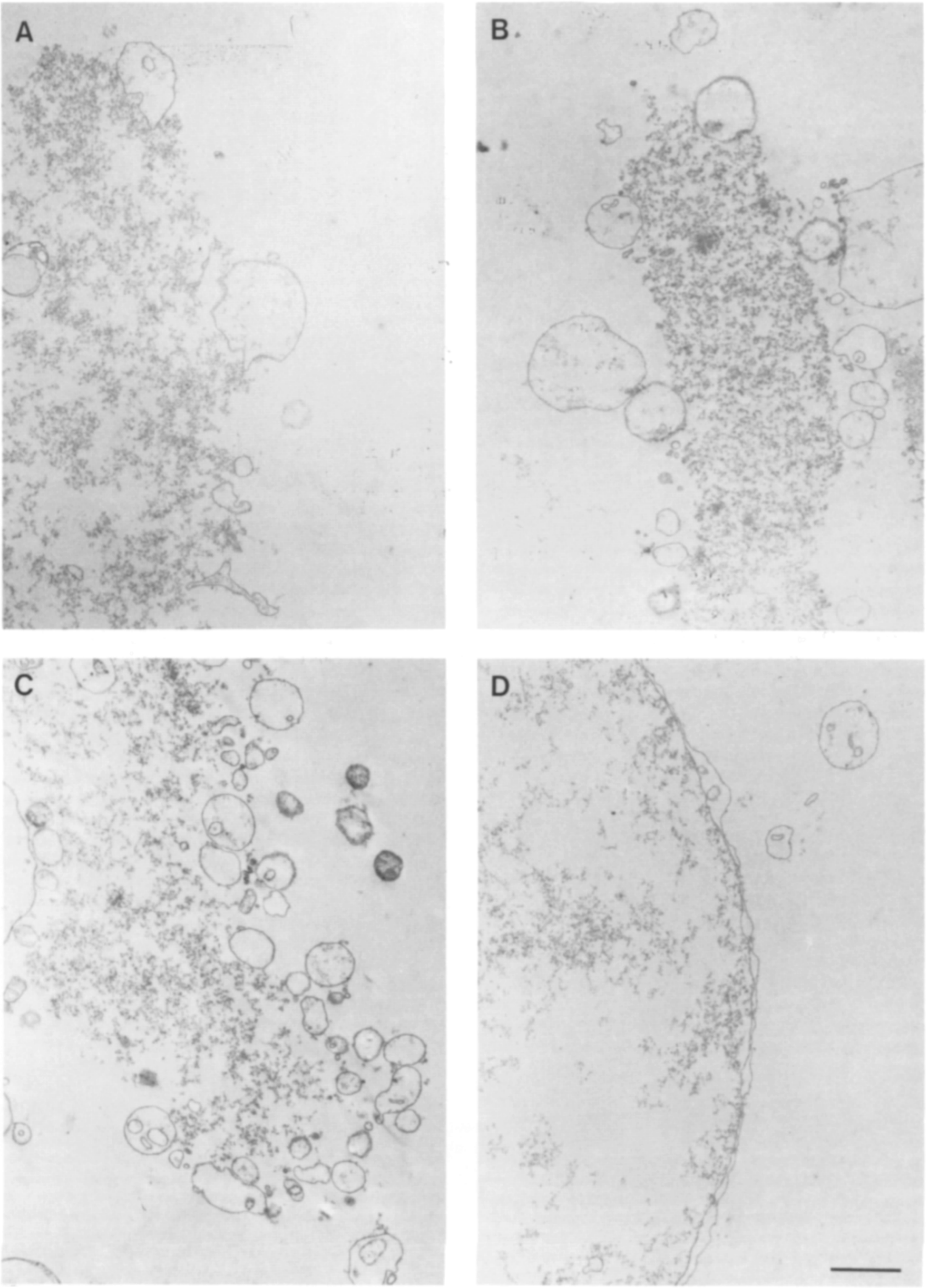


*Figure 5.* Levels of α-glucosidase in $S_{200}$, NEP-A, cytosol, and NEP-B. The various fractions were assayed for α-glucosidase activity as described in Materials and Methods and the levels of α-glucosidase were normalized against total protein in each fraction. NEP-A and sucrose-purified NEP-A were found to have levels of α-glucosidase activity ~10 times greater than those of the other fractions.

The different properties of NEP-A and NEP-B described above could be due either to the existence of two distinct populations of vesicles in NEP-A and NEP-B, or to protein complexes in NEP-B that sediment independently of vesicles. We believe the first of these possibilities to be correct since: (*a*) ultrastructural studies show that vesicles are present in NEP-B as well as in NEP-A; (*b*) the vesicles present in $S_{200}$ are removed by the same conditions that sediment NEP-B; and (*c*) NEP-B is sensitive to three detergents at the lowest concentrations that inactivated NEP-A. Although non-membrane-associated proteins could also be disrupted by detergents, it is less likely that they would show the same sensitivities to three different detergents as the vesicles in NEP-A. These lines of evidence indicate that vesicles are an essential component of NEP-B. The vesicles in NEP-A and

*Figure 4.* Light and electron micrographs of sperm incubated in cytosol (*A*), cytosol plus NEP-A (*B*), cytosol plus NEP-B (*C*), and cytosol plus NEP-A and NEP-B (*D*). For each panel, the upper inset shows chromatin visualized by 4′,6-diamidine-2-phenylindole dihydrochloride staining, while the lower inset shows the same nuclei stained with 3,3′-dihexyloxacarbocyanine to reveal membranes. In cytosol alone (*A*) or in cytosol plus NEP-A (*B*) the sperm enlarge considerably as the chromatin decondenses, but no NE forms and the vesicles from NEP-A do not attach to the surface of the chromatin (*B*). In cytosol plus NEP-B (*C*) no NE assembles around the sperm, though the sperm appear more compact than those incubated in cytosol alone and vesicles attach to the chromatin surface as they do in $S_{200}$ (Fig. 2 *A*). When cytosol, NEP-A, and NEP-B are all present (*D*) NE assembly is complete. All LM insets were photographed at the same magnification as those in Fig. 2. Bar, 1 μm.


A
B
C
D

NEP-B are distinguished from each other by their different sedimentation rates, sensitivities to high-salt and *N*-ethylmaleimide, and levels of $\alpha$-glucosidase. Furthermore, when vesicles in NEP-B bind chromatin they are often close enough to each other to fuse into a continuous membrane, but fail to do so in the absence of NEP-A (e.g., Figs. 2 *A*, 4 *C*, and 6 *B*). If the vesicles in NEP-A and NEP-B were the same, one might expect a similar capacity for vesicle fusion in both fractions. These points lead us to conclude that NEP-A and NEP-B contain populations of vesicles that are distinct from each other.

The binding of vesicles to chromatin and fusion between vesicles are probably mediated by peripheral and/or integral membrane proteins. At least some of the proteins in NEP-B are probably peripheral membrane proteins that can be removed by high salt, and both NEP-A and NEP-B are sensitive to trypsin. Wilson and Newport (1988) have also shown that vesicles involved in NE assembly fail to bind sperm chromatin after trypsin treatment. In their experiments, vesicles from both NEP-A and NEP-B may have been in the vesicle fraction, since our results show that only vesicles in NEP-B can bind chromatin. Thus, trypsin treatment may have destroyed the ability both of NEP-B to bind chromatin and of NEP-A to fuse with other vesicles. Wilson and Newport (1988) also reported that the vesicle fraction was not sensitive to high salt, unlike NEP-B. Perhaps in their experiments sufficient NEP-B remained in the supernatant to permit NE assembly when their supernatant and vesicle fractions were combined.

NEP-A, which is required for the fusion of vesicles into the continuous double membrane of the NE, was inactivated by mild treatment with the sulfhydryl-blocking agent *N*-ethylmaleimide. Vesicle fusion between the ER and the Golgi complex, between the cisternae of the Golgi stack, and between early endosomes all require a cytosolic protein, called NSF, which is sensitive to *N*-ethylmaleimide under similarly mild conditions (Malhotra et al., 1988; Beckers et al., 1989; Diaz et al., 1989); but the relationship between NSF and the *N*-ethylmaleimide–sensitive protein required for NE assembly is not known. Newmeyer and Forbes (1990) have recently shown that the repair of isolation-induced damage to rat liver nuclei in *Xenopus* cell-free extracts is sensitive to *N*-ethylmaleimide. Since the fusion of vesicles to the NE is thought to play a role in the repair process, one would predict that NEP-A rather than NEP-B would be involved.

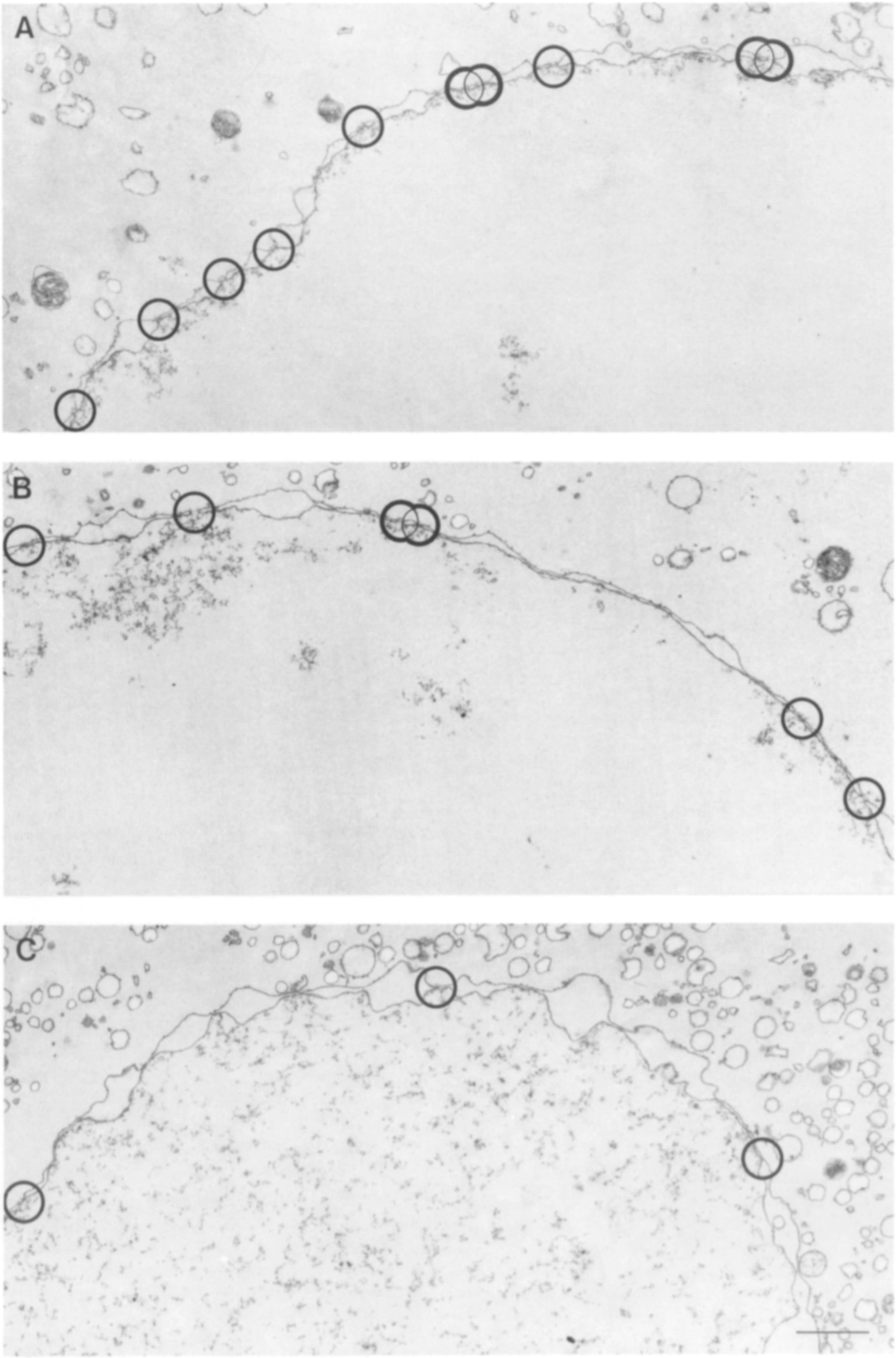
The assembly of nuclear pores may require components in NEP-B, since varying its relative concentration alters the density of pores seen in the NE. This suggests that one or more precursor components of the nuclear pore complex are enriched in NEP-B. While it is probable that these components reside within the vesicles that bind to chromatin, we cannot exclude the possibility that other components that copurify with vesicles are responsible. We also cannot say why the changes in pore density did not more closely parallel the changes in the relative amount of NEP-B, but we suspect that NE assembly may be highly cooperative, thus limiting the range of pore densities that can be attained in a complete NE by varying precursor concentrations. In their recent work, Sheehan et al. (1988) observed "prepore" structures that were associated with chromatin during the assembly of NEs in *Xenopus* egg extracts, especially when the soluble components were in excess of the vesicular components. We did not observe similar structures, possibly because we only used precursor ratios that gave complete NEs. Their soluble fraction may have contained vesicles that fractionated as NEP-B in our experiments, thus favoring the formation of pores at a high density.

Recently, Finlay and Forbes (1990) demonstrated that morphologically normal, yet functionally inactive, nuclear pores form when a soluble fraction of *Xenopus* egg extracts was passed over a wheat germ agglutinin affinity column to remove the *N*-acetylglucosamine–conjugated components of the nuclear pore complex. Since in all of our experiments the presence of pores was scored morphologically, it is probable that the determinants of nuclear pore assembly that persist in wheat germ agglutinin–depleted extracts are found in NEP-B, whereas those removed by the affinity column are in the cytosol. We have attempted to locate a known component of the nuclear pore complex by using polyclonal antibodies (gift of L. Gerace, Research Institute of Scripps Clinic) against gp210, an integral membrane glycoprotein of the rat liver pore complex (Greber et al., 1990). Unfortunately, these antibodies failed to cross-react with any of the *Xenopus* fractions described here (data not shown).

From our results we propose the model for NE assembly shown in Fig. 9. Our model is similar to that of Wilson and Newport (1988), but differs in that we propose that there are two distinct vesicle populations in *Xenopus* eggs required to form a complete NE. One population in NEP-B is probably specific to the NE and can bind to sperm chromatin by an ATP-independent process. We do not know what components of the sperm chromatin are recognized, nor which proteins mediate the binding. Furthermore, it seems likely that the NEP-B is in some way involved in the assembly of nuclear pores, as discussed above. The vesicles in NEP-A, which contain a marker for the ER, are unable to bind chromatin by themselves, but can fuse with chromatin-bound vesicles by an ATP-dependent process to form the double membrane of the NE. Hence, in our model vesicles in NEP-B specifically target NE precursors to the chromatin surface during the early steps of NE assembly, and other vesicles, perhaps from the ER, then fuse with them to provide much of the membrane of the NE.

Our model is consistent both with the observations presented here and with the findings of others. In previous studies, Burke and Gerace (1986) observed that cell-free extracts from CHO cells required ATP for complete NE assembly around mitotic chromosomes, while only binding of

*Figure 6.* Effects of depletion of ATP on vesicle binding and NE assembly. Sperm were incubated in $S_{200}$ (*A* and *B*) or $S_{200}$ plus sucrose-purified NEP-A (*C* and *D*) for 60 min at 18°C in the presence of an ATP-depleting system (Schlossman et al., 1984) (*A* and *C*) or an ATP-regenerating system (*B* and *D*). Vesicles attach to the chromatin surface in an ATP-independent manner (*A*–*C*) whether NEP-A is present or not (*A* and *C*). Vesicles only fuse together to form a complete NE in the presence of both ATP and NEP-A. Bar, 1 $\mu$m.


A
B
C

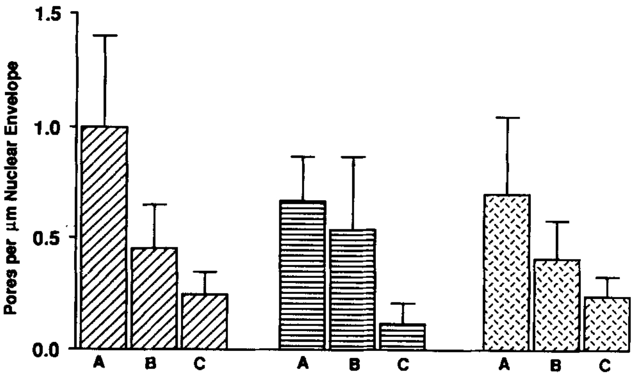


*Figure 8.* Nuclear pore densities for NEs assembled with different concentrations of NEP-A and NEP-B. NEs such as those shown in Fig. 7 were scored blind for the numbers of nuclear pores and the length of NE membrane in the micrographs. The results of three experiments are shown. (*A*) NEP-A at one-fifth of normal concentration and NEP-B at normal concentration. (*B*) NEP-A and NEP-B both at normal concentrations. (*C*) NEP-A at the normal concentration and NEP-B at one-fifth normal concentration. Similar results were obtained in five of six experiments, in which the pore densities in a total of 50–70 nuclei were examined for each experimental condition. The means and standard deviations of nuclear pore densities for these five experiments were 0.74 ± 0.15 (*A*), 0.50 ± 0.15 (*B*), and 0.22 ± 0.06 (*C*) per µm NE.

vesicles, without fusion, occurred in the presence of the nonhydrolyzable ATP analogue, ATP-γ-S. Wilson and Newport (1988) have also shown that ER vesicles alone are not sufficient for NE assembly in egg extracts. On the other hand, the NE membrane shares biochemical markers with the ER and is often continuous with it (Franke, 1974). Since lamin B also remains associated with the ER during mitosis in chick tissue culture cells (Stick et al., 1988), one would expect NE membrane proteins to copurify with ER proteins. However, we find relatively little α-glucosidase activity in NEP-B, suggesting that at least some membrane proteins of the NE do not segregate with the ER vesicles. These differences may be due to the cell types studied. The NE of a *Xenopus* oocyte, which matures into an unfertilized egg, has an extremely high density of nuclear pores (Franke and Scheer, 1970). Therefore, when the NE is disassembled during meiotic maturation, vesicles that form may contain mostly nuclear pore precursors and few, if any, ER proteins. In contrast, the density of nuclear pores is less in cultured cells and there may be a higher level of ER proteins in the membrane of the NE. Thus, when the NE is disassembled the vesicles that form will contain both ER and NE membrane proteins. *Xenopus* eggs may therefore exhibit an extreme case of the partitioning of NE proteins into separate populations of vesicles during NE disassembly. Nevertheless, the vesicle-associated proteins involved in binding to chromatin, vesicle–vesicle fusion, and nuclear pore assembly are likely to be conserved in eukaryotes, and the two NE precursor fractions that we have isolated from *Xenopus* eggs may facilitate the identification and analysis of these proteins.

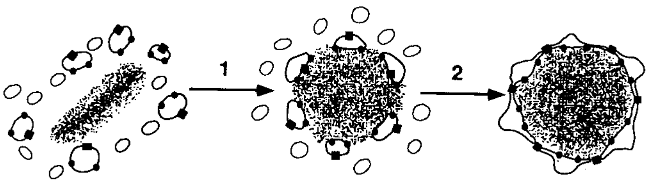


*Figure 9.* A model for NE assembly. Based on the data presented in this paper we propose that two distinct populations of vesicles are required for NE assembly. The vesicles present in the NEP-B fraction bind to the sperm chromatin in an ATP-independent manner, via proteins designated by the solid circles. This binding could be directly to the chromatin or could be mediated by some other components of the cytosol that have already bound to the chromatin. Vesicles in the NEP-A fraction fuse to the chromatin-bound vesicles in a process that requires ATP. The NEP-B vesicles also appear to be involved in targeting nuclear pores to the NE (via proteins designated by *solid squares*), though we have little information on how this process occurs.

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*Figure 7.* NE assembly in the presence of different concentrations of NEP-A and NEP-B. (*A*) NEP-A at one-fifth of normal concentration and NEP-B at normal concentration. (*B*) NEP-A and NEP-B both at normal concentrations. (*C*) NEP-A at normal concentration and NEP-B at one-fifth concentration. The level of NEP-A was reduced (*A*) by mixing 100 µl $S_{200}$ with one one-fifth of the NEP-A pellet normally used. The level of the NEP-B was reduced (*C*) by adding 20 µl $S_{200}$, which contains NEP-B, to 80 µl cytosol, which does not. NEP-A was then resuspended in the diluted $S_{200}$. Structures identified as nuclear pores are circled. The density of nuclear pores was highest when the ratio of NEP-A to NEP-B was 1:5 and lowest when the ration was 5:1 (also see Fig. 8). Bar, 1 µm.

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